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Abrogation of the interaction between osteopontin and αvβ3 integrin reduces tumor growth of human lung cancer cells in mice

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Summary Osteopontin (OPN) is a multifunctional cytokine involved in cell signaling by interacting with αvβ3 integrins. Recent clinical studies have indicated that OPN expression is associated with tumor progression and poor prognosis among patients with lung cancer. However, the biological role of OPN in human lung cancer has not yet been well-defined. The purpose of this study is to investigate and provide evidence for the causal role of OPN regarding tumor growth and angiogenesis in human lung cancer. In this study, we developed a stable OPN transfectant from human lung cancer cell line SBC-3 which does not express the intrinsic OPN mRNA. To reveal the in vivo effect of OPN on tumor growth of human lung cancer, we subcutaneously injected OPN-overexpressing SBC-3 cells (SBC-3/OPN) and control cells (SBC-3/NEO) into the nude mice. Transfection with the OPN gene significantly increased in vivo tumor growth and neovascularization of SBC-3 cells in mice. These in vivo effects of OPN were markedly suppressed with administration of anti-αvβ3 integrin monoclonal antibody or anti-angiogenic agent, TNP-470. Furthermore, recombinant OPN protein enhanced human umbilical vein endothelial cell (HUVEC) proliferation in vitro, and this enhancement was significantly inhibited with the

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addition of anti-αvβ3 integrin antibody. Taken together, these results suggest that OPN plays a crucial role for tumor growth and angiogenesis of human lung cancer cells in vivo by interacting with αvβ3 integrin. Targeting the interaction between OPN and αvβ3 integrin could be effective for future development of anti-angiogenic therapeutic agents for patients with lung cancer.
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1. Introduction

Lung cancer is one of the most frequently diagnosed solid tumors in the world, and is the leading cause of cancer-related deaths in Japan [1]. Despite advancement and improvements in surgical and medical treatments, the prognosis of lung cancer patients remains extremely poor [2]. These facts indicate how important it is to identify novel target molecules for the development of new anticancer therapies for human lung cancer.

Tumor growth and metastasis depend on blood supply and vessel formation. Therefore, anti-angiogenic therapy appears to be an attractive and rational approach for the treatment of solid tumors including lung cancer [3,4]. One approach to anti-angiogenic therapy is to inhibit the adhesive interactions required for tumor angiogenesis. The migration and proliferation of vascular endothelial cells is dependent on their adhesiveness to extracellular matrix (ECM) proteins through a variety of cell adhesion receptor including αvβ3 integrin [5,6]. Thus, the interaction between ECM and αvβ3 integrin may be a therapeutic target for lung cancer patients.

Osteopontin (OPN) is a multifunctional phosphoprotein that binds to αv integrin at the arginine-glycine-asparatic acid (RGD) motif of the central portion and exerts cell-adhesion and migration activity [7,8]. OPN is one of the ECM proteins produced by cancer cells, and is revealed to be overexpressed in various human tumors including the lung, breast, colon, ovary, and gastric cancers [9—14]. Previous studies suggested that OPN may be involved in the angiogenesis of cancer cells. For example, Senger et al. reported that OPN promotes vascular endothelial cell migration via αv integrin in cooperation with vascular endothelial growth factor (VEGF), suggesting that OPN may be involved in angiogenesis [15]. Shijubo et al. demonstrated that coexpression of OPN and VEGF is closely associated with angiogenesis and poor prognosis in stage I lung adenocarcinoma [16]. Thus, OPN is postulated to be related with tumor progression and angiogenesis in various cancers.

Recently, much interest has been focused on OPN expression in human lung cancer. Donati et al. investigated on the correlation between OPN expression in tumor tissues and survival of 136 patients with stage I non-small cell lung cancer (NSCLC), and indicated that OPN expression is a significant unfavorable prognostic factor for survival among patients with stage I NSCLC [17]. Hu et al. also reported that OPN expression was associated with tumor growth, tumor staging, and lymph node invasion of patients with NSCLC. They further analyzed OPN levels in plasma, and suggest that plasma OPN levels may serve as a biomarker for diagnosing or monitoring patients with NSCLC [18]. These findings from these clinical studies imply that OPN may be a therapeutic target and useful biomarker for human lung cancer. However, the biological and functional role of OPN in lung cancer animal model and therapeutic trials targeting OPN and its receptor, αvβ3 integrin, have not yet been reported.

In this study, we first developed stable transfectants from human small cell lung cancer (SCLC) cell line SBC-3 that constitutively secrete mouse OPN. We demonstrated that OPN transfected SBC-3 cells significantly increased in vivo tumorigenecity and neovascularization in comparison with the control cells in mice. In addition, we evaluated the therapeutic efficacy of anti-mouse αvβ3 integrin antibody (RMV-7) against OPN-overexpressing SBC-3 cells inoculated mice. The biological significance of OPN in tumor growth and angiogenesis of lung cancer and potential treatment using RMV-7 antibody are also discussed.

2. Materials and methods

2.1. Cell lines and reagents

Human small cell lung cancer cell line, SBC-3 cells, was kindly provided by Dr. I. Kimura (Okayama University, Okayama), and cultured in RPMI1640 (Koujin Bio, Saitama, Japan) medium containing 10% (v/v) fetal calf serum. HUVEC were purchased from Clonetics (San Diego, CA) and maintained with EGM-2 medium (Clonetics) on collagen-coated plastic flasks. The anti-mouse αvβ3 antibody (RMV-7) was kindly provided by Prof. Okumura (Department of Immunology, Juntendo University), and has been proven to interfere with OPN-mediated cell migration, adhesion, and proliferation [19,20]. Anti-human αvβ3 monoclonal antibody (LM609) was purchased from Chemicon International (Australia). The monoclonal antibody against murine CD31 was purchased from Pharmingen (San Diego, CA). The monoclonal antibody against murine OPN was purchased from Immuno-Biological Laboratories (Gunma, Japan). The polyclonal rabbit anti-single stranded DNA (ssDNA) was purchased from Dakocytomation (Tokyo, Japan). TNP-470 (6-*O*-(*N*-chloroacethyl-carbamoyl)-fumagillol), a semisynthetic analog of fumagillin derived from *Aspergillus fumigatus*, was kindly provided by Takeda Chemical Industries (Osaka, Japan).

2.2. Transfection

5×10^5 SBC-3 cells were transfected with Lipofectamine Reagent (Invitrogen) using 8 μg of purified murine OPN cDNA cloned into the eukaryotic cDNA expression vector BMGneo as previously described [21]. This plasmid was designated as BMGneo-mOPN. Two days later, the cells were placed in G418 sulfate (Geneticin; Invitrogen) at 1 mg/ml for selection. Four weeks after transfection, G418-resistant colonies were expanded and isolated with limiting dilution. The

resulting selected and isolated SBC-3 cells transfected with BMGneo-mOPN and BMGneo were designated as SBC-3/OPN and SBC-3/NEO, respectively.

2.3. Detection of OPN and VEGF transcription by RT-PCR

Expression of OPN and VEGF mRNA were assessed by RT-PCR. Total RNAs were extracted from cultured cell lines with TRIzol reagent (Invitrogen). The primers for the RT-PCR were: OPN sense primer (5′-AGTCGACATGAGATTGGCAGTGATTTGC-3′), OPN anti-sense primer (5′-ACTCGAGGCCTCTTCTTTAGTTGACCTC-3′), VEGF sense primer (5′-TGCACCCATGGCAGAAGGAGG-3′), and VEGF anti-sense primer (5′-TCACCGCCTCGGCTTGTCACA-3′). RT-PCR was conducted using a Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, NJ) according to the manufacture's instructions.

2.4. Determination of OPN protein secretion by ELISA

5×10^5 SBC-3/OPN transfectants were cultured in 6-well plates with 2% FCS in RPMI 1640 medium overnight, followed by incubation in 3 ml serum free medium for an additional 24 h. Secreted murine OPN protein level in culture supernatant was measured with the commercial ELISA kit (Immuno-Biological Laboratories, Gunma, Japan) according to the manufacturer's instruction.

2.5. Western blot analysis

Conditioned medium from SBC-3/OPN and SBC-3/NEO cells were subjected to western blot analysis. Samples were separated on 10% acrylamide gels and transferred to a nitrocellulose filter with electroblotting at 4 °C. The filters were blocked in phosphate-buffered saline (PBS) containing 10% dry milk, washed in PBS containing 1% dry milk and 0.5% Tween-20, and then incubated with polyclonal rabbit anti-mouse OPN antibody (Immuno-Biological Laboratories, Gunma, Japan) at room temperature for 1 h. Filters were again washed and then incubated with horseradish-peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech) for 1 h. Filters were then washed with TBST (150 mM NaCl, 10 mM Tris, pH 8.0, 0.05% Tween-20), and specific proteins were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

2.6. In vitro cell growth rates

SBC-3/NEO and SBC-3/OPN were placed onto 96-well plates at 2×10^3 cells/well in triplicate. At designated time points, the number of cells were quantified using a colormetric MTT assay as described previously [22].

2.7. In vitro cell migration assay

SBC-3/OPN and SBC-3/NEO were transfered to 6-well culture plates at 5×10^5 cells/well and incubated with 2% FCS in RPMI 1640 medium overnight. The cells were washed in PBS, and 3 ml of serum free medium were added to each well. After 24 h, 3 ml of conditioned serum-free medium were collected and subjected to in vitro cell migration assay. In vitro cell migration was performed using a cell culture insert with 8 μm micropore membrane (Falcon; Becton Dickinson, Franklin Lake, NJ) as previously described [21]. Briefly, the suspension of HUVEC (5×10^4 cells/200 μl in RPMI 1640 containing 0.1% BSA) was added to the upper chamber and the collected medium was added to the lower chamber. In order to confirm cell migration mediated by OPN, we conducted additional experiments by treating the cells with GRGDS peptide (Sigma) at the concentration of 100 μM or anti-human αvβ3 antibody at the concentration of 10 μg/ml. After incubation at 37 °C for 8 h, the filters were fixed with 10% formalin, and stained with crystal violet. The cells on the upper surface of the filters were removed by swabbing with a cotton swab, and the cells that had migrated to the lower surface were counted under a microscope at the magnification of 200×. All assays were performed in triplicate and at least three independent experiments were performed.

2.8. Soft agar colony formation assay

Six-well culture plates were covered with a layer of 0.5% agar in RPMI 1640 medium containing 20% (v/v) fetal calf serum to prevent the attachment of the cells to plastic substratum. Cell suspensions (5×10^3 cells/well) of the SBC-3/OPN or SBC-3/NEO cells were prepared with 0.3% agar and poured into 6-well plates. After 2 weeks of incubation at 37 °C, the colonies containing at least 50 cells were counted. All assays were performed in triplicate.

2.9. Mice

Female athymic BALB/c nude mice, 6—7 weeks old, were purchased from Charlsriver Co., Ltd. (Tokyo, Japan) and maintained in our animal facilities under specific pathogen-free conditions. All animal experiments were performed according to the Guidelines on Animal Experimentation as established by Juntendo University, School of Medicine.

2.10. In vivo tumorigenicity

SBC-3/OPN and SBC-3/NEO cells were harvested from the culture flask with 0.05% Trypsin-EDTA (Invitrogen), washed three times, resuspended in PBS. Cell viability was determined by trypan blue dye exclusion test and cells were inoculated subcutaneously (s.c.) into the left flank of nude mice (1×10^7 cells/mouse). To investigate whether tumor growth is mediated by the interaction between OPN and its receptor, the RMV-7 antibody was administered to SBC-3/OPN or SBC-3/NEO inoculated mice. Briefly, RMV-7 (200 μg/mouse) and control isotype-matched IgG (200 μg/mouse) were administered intraperitoneally from day 3 after inoculation three times a week for 3 weeks. TNP-470 (30 mg/kg) was also administered subcutaneously from day 7 twice a week for 3 weeks to reveal the involvement of angiogenesis in in vivo tumor growth. Tumor growth was measured with a digital caliper in two perpendicular diameters every week. Tumor volumes were calculated from the length (a) and width (b) by using the following formula:

volume (mm^3) = $ab^2/2$. Each group consisted of 10 mice. All experiments were performed twice.

2.11. Immunohistochemical staining

Histological sections were obtained from SBC-3/OPN and SBC-3/NEO tumor tissues resected from mice. After resection, tumor tissues were immediately embedded and frozen in Tissue-Tek OCD compound (Miles Laboratories, Elkhart, TN), and sections were cut at 4 μm thickness. Immunohistochemical staining for murine OPN and CD31 was performed as previously described [23]. To quantify apoptotic cell number in the tumor, we performed immunohistochemical staining for ssDNA. Briefly, the sections were fixed with 4% paraformaldehyde (PFA) and then incubated at 4 °C overnight with rabbit anti-ssDNA antibody diluted to 1:400. Specific binding was detected through avidin-biotin peroxidase complex formation with biotin conjugated goat anti-rabbit IgG (Vectastain ABC kit, Vector, Burlingame, CA) and diaminobendizine (DAB) (Sigma, St. Louis, MI) as the substrate. Staining was absent when isotype-matched immunoglobulin was used as the control.

2.12. HUVEC proliferation assay

A 96-well flat bottom plastic assay plate (Corning, NY) was coated with recombinant mouse OPN (RD systems, Inc., CA; 10 μg/ml), polylysine (100 μg/ml) or BSA (10 mg/ml) in PBS and incubated overnight at 4 °C. The plate was washed with PBS and non-specific adhesion sites were blocked with 1% BSA in PBS for 1 h at 37 °C. After washing the wells with PBS, 5×10^3 cells in 100 μl of EGM-2 medium diluted with OPTI-MEM (Invitrogen) to 1/5 were seeded to each well. For some experiments, the HUVEC suspensions were pretreated with GRGDS peptide at the concentration of 100 μM or anti-human αvβ3 antibody at the concentration of 10 μg/ml for 1 h at 37 °C. Then after 48 h incubation, 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8, Dojindo, Kumamoto, Japan) was added to each well. The plate was further incubated at 37 °C for 6 h for color development. Absorbance was measured at 450 nm on a microplate reader with microplate manager (Bio-Rad, Richmond, CA). All experiments were performed in triplicate.

2.13. Statistics

Statistical analysis was performed with analysis of variance (ANOVA). All data are presented as mean ± standard deviation. Differences between means were considered statistically significant at $P < 0.05$.

3. Results

3.1. Generation of stable transfectants that secretes murine OPN

BMGneo-mOPN or BMGneo were transfected into SBC-3 cells. Two OPN transfected SBC-3 clones (SBC-3/OPN#5 and SBC-3/OPN#6) and two control clones (SBC-3/NEO#1 and SBC-3/NEO#3) were constructed. To verify the expression of OPN and VEGF mRNA in transfectants, we conducted RT-PCR for OPN and VEGF, respectively. As shown in Fig. 1A, high levels of OPN mRNA expression were detected in the SBC-3/OPN cells, while there were no detectable expression levels observed in the SBC-3/NEO cells. For VEGF mRNA, there was no difference in the level of expression between SBC-3/OPN and SBC-3/NEO cells. Thus, transfection with OPN gene into SBC-3 cells does not affect the expression of other angiogenic inducers like VEGF mRNA. Secreted OPN protein from transfectants was confirmed with both western blot analysis and ELISA kit (Fig. 1B and C). OPN-transfected clones secreted significant amounts of OPN, while control clones did not. The clone SBC-3/OPN#5 secreted the high-

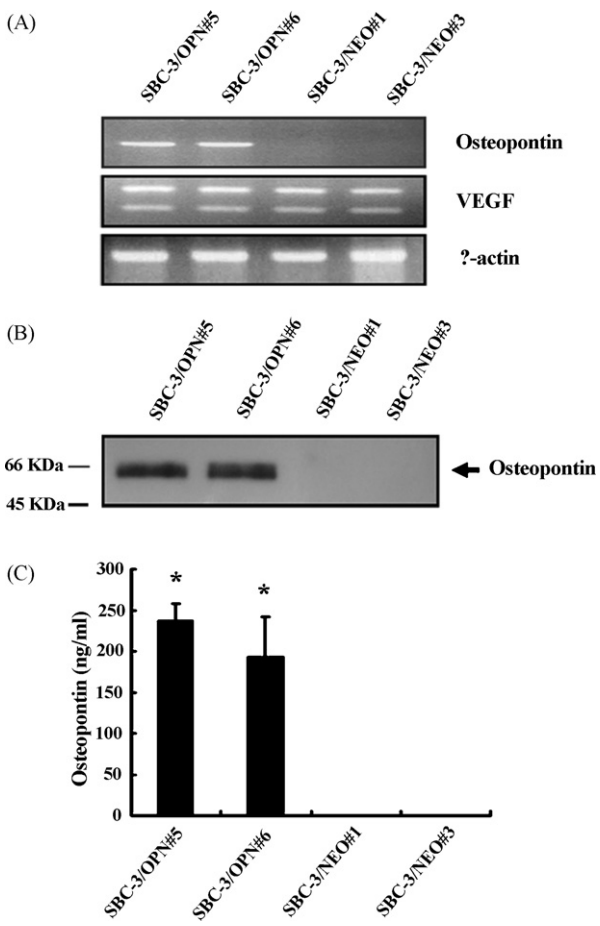

Fig. 1 (A) Expression of OPN and VEGF mRNA determined with RT-PCR analysis. Total RNAs were extracted from each clone and 1 μg of RNAs were subjected to RT-PCR analysis for OPN (top panel), VEGF (middle panel) and β-actin (bottom panel) expression. (B) Western blot analysis of secreted mouse OPN protein. Conditioned mediums from SBC-3/OPN and SBC-3/NEO clones were subjected to western blot analysis using polyclonal antibody against OPN. The arrow indicates the expression of OPN, and molecular standards are shown on the left in KD. (C) Secretion of OPN protein from SBC-3/OPN and SBC-3/NEO cells. Conditioned medium from each clones were collected and subjected to ELISA analysis. Note that the clone SBC-3/OPN#5 secreted the highest level of OPN protein into the culture medium. $^*P < 0.05$ vs. SBC-3/NEO#1 and SBC-3/NEO#3.

est level of OPN protein into the culture medium. Therefore, we utilized this clone in the subsequential experiments.

3.2. In vitro cell growth rate of stable OPN-transfectant

Cells were seeded onto 96-well plates and the number of cells was quantified at specific time intervals with MTT assay. Cultured SBC-3/OPN and SBC-3/NEO cells displayed similar in vitro growth rates (data not shown).

3.3. Biological activity of OPN protein secreted from the transfectant

Since endothelial cell migration is essential for tumor angiogenesis, we conducted migration assay using HUVEC. Conditioned medium from SBC-3/OPN cells significantly stimulated HUVEC migration as compared with conditioned medium from SBC-3/NEO cells. Moreover, HUVEC migration toward the culture medium of SBC-3/OPN cells was almost completely suppressed with the addition of GRGDS peptide and anti-human $\alpha v\beta 3$ antibody (Fig. 2). These results suggest that OPN secreted from SBC-3/OPN is actually biological active and stimulates HUVEC migration by interacting with $\alpha v\beta 3$ integrin.

3.4. Effect of OPN transfection on colony formation

We evaluated whether transfection with OPN gene affects colony formation of SBC-3 cells in vitro with soft agar colony formation assay. As shown in Fig. 2B, there was no significant difference in the number of colonies between SBC-3/OPN and SBC-3/NEO cells. Thus, colony formation of SBC-3 cells in vitro was not affected by transfection with the OPN gene.

3.5. In vivo tumorigenecity of OPN transfectant

To investigate whether OPN has any role in tumor growth in vivo, SBC-3/OPN#5 clone and SBC-3/NEO#1 clone were injected subcutaneously into the left flank of the nude mice. As shown in Fig. 3 A and B, in contrast to the absence of any significant changes in in vitro cell growth, the in vivo growth rate of SBC-3/OPN#5 was significantly faster than that of the SBC-3/NEO#1 cells. We also tested in vivo tumor growth of the other SBC-3/OPN clone, SBC-3/OPN#6, to confirm its enhanced in vivo tumorigenecity. As expected, SBC-3/OPN#6 demonstrated enhanced in vivo tumor growth compared to SBC-3/NEO#1 (data not shown).

3.6. Expression of OPN protein in SBC-3/OPN and SBC-3/NEO tumors

To investigate whether enhanced tumor growth of SBC-3/OPN clones in vivo was mediated by secreted OPN, immunohistochemical staining for OPN was conducted. The OPN-positive cell number was significantly greater in the SBC-3/OPN induced tumor in comparison with that of the SBC-3/NEO tumor (Fig. 3C). These results suggest that

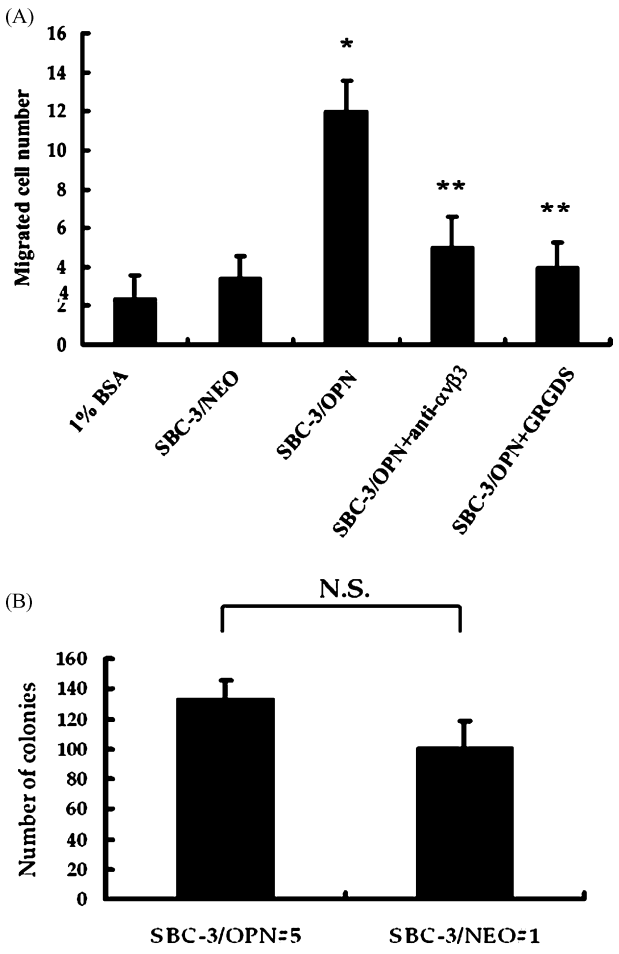

Fig. 2 (A) Migration of HUVEC toward conditioned medium from OPN-transfected cells. Cells were placed in the upper chamber and culture medium from SBC-3/NEO and SBC-3/OPN were added to the lower chamber. After 8 h incubation, cells that migrated through the porous filter were counted at $\times 200$ magnification. Enhanced migration of HUVEC toward the culture medium from SBC-3/OPN was abrogated with the addition of either GRGDS peptide (100 μM) or anti-human $\alpha v\beta 3$ antibody (10 μg/ml) to the upper chambers. Data are presented as mean $\pm$ S.D. $^{*}P < 0.0001$ vs. 1% BSA and SBC-3/NEO; $^{**}P < 0.001$ vs. SBC-3/OPN. (B) Soft-agar colony formation by SBC-3/OPN and SBC-3/NEO cells. Cells were seeded at an initial density of 5×10^3 cells into 6-well culture plates in triplicate in 0.3% agar. Colonies containing at least 50 cells were scored after 2 weeks of growth. Total colony per well were counted and presented as the mean $\pm$ S.D.

secreted OPN from SBC-3/OPN transfectants enhanced in vivo tumorigenesis.

3.7. Effect of OPN transfection on tumor angiogenesis

To investigate whether transfection with OPN gene results in increased tumor angiogenesis in vivo, we performed immunohistochemistry for CD31 and counted the microvessels in the SBC-3/OPN#5 and SBC-3/NEO#1 induced tumors of the nude mice. As shown in Fig. 4A, the number of CD31-

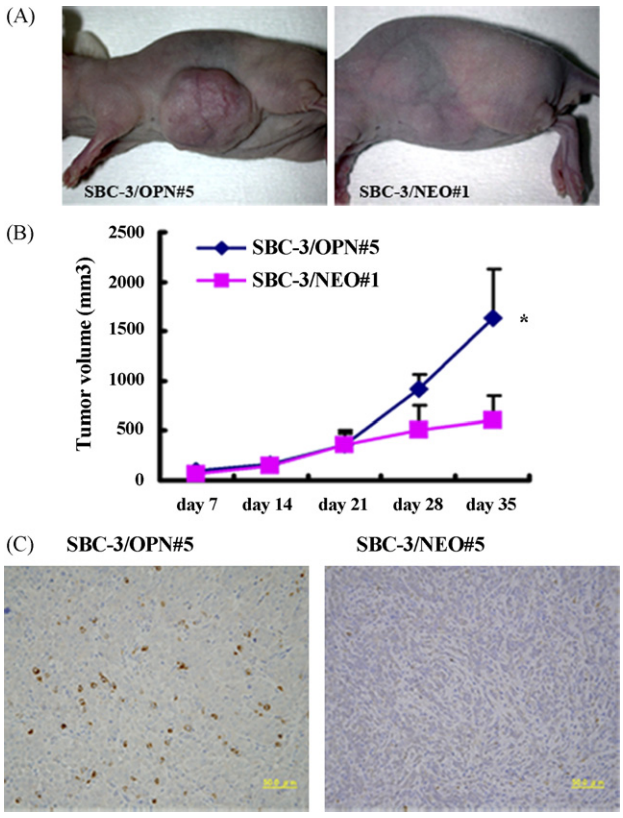

Fig. 3 Effect of OPN gene transfer into SBC-3 cells on tumor growth in mice. The SBC-3/OPN#5 and SBC-3/NEO#1 cells were inoculated s.c. into the left flanks of nude mice. (A) Representative photographs of the tumors at day 35 after inoculation with either the SBC-3/OPN#5 cells or the SBC-3/NEO#1 cells. (B) Tumors were measured with a digital caliper in two perpendicular diameters every week. The tumor volumes were calculated as described in Section 2. Each group consisted of 10 mice. $^{*}P < 0.05$ vs. SBC-3/NEO#1. (C) Representative sections of OPN expression in tumors derived from SBC-3/OPN and SBC-3/NEO. Cryostat sections of tumors developing in nude mice were stained with anti-mouse OPN monoclonal antibody (original magnification $\times 400$).

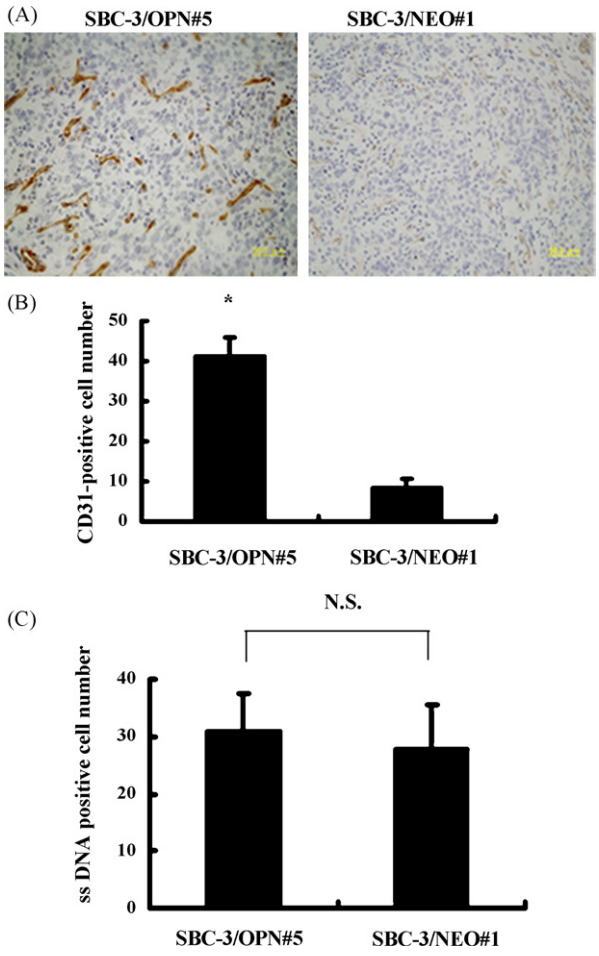

Fig. 4 (A and B) Vascularization of tumors derived from SBC-3/OPN#5 and SBC-3/NEO#1 cells. Cryostat sections of tumors developing in nude mice were stained with anti-CD31 monoclonal antibody. (A) Representative sections were depicted ($\times 200$). (B) Quantification of microvessel density in tumors. The number of CD31-positive microvessels in five fields of tumors that demonstrated the highest vascularity was counted at $\times 200$ and presented as mean $\pm$ S.D. $^{*}P < 0.001$ vs. SBC-3/NEO#1. (C) Quantification of ss DNA staining in SBC-3/OPN and SBC-3/NEO cells developed in nude mice. The number of ss DNA positive cells in SBC-3/OPN#5 tumor was not significantly different from that of SBC-3/NEO#1 tumor.

positive vascular endothelial cells was markedly increased in the SBC-3/OPN#5 induced tumor compared to that of the SBC-3/NEO#1 induced tumor. As shown in Fig. 4B, greater than tenfold the number of microvessels was identified in the SBC-3/OPN#5 induced tumor compared with the SBC-3/NEO#1 induced tumor. These results strongly imply that OPN upregulates tumor angiogenesis of SBC-3 cells in mice.

3.8. Effect of OPN transfection on tumor cell apoptosis

We evaluated whether transfection with OPN gene affects tumor cell apoptosis of SBC-3 cells in vivo with immunohistochemical staining for ssDNA. As shown in Fig. 4C, the number of apoptotic cells in the SBC-3/OPN induced tumor was not significantly different from that of the SBC-3/NEO induced tumor. These results suggest the apoptosis of SBC-3 cells in vivo was not affected by transfection with the OPN gene.

3.9. Effect of OPN on in vitro HUVEC proliferation

The endothelial cell proliferation is essential for tumor angiogenesis. Therefore, we performed HUVEC proliferation assay using recombinant mouse OPN protein. As shown in Fig. 5, immobilized OPN significantly stimulated HUVEC proliferation compared with immobilized polylysine and BSA. Interestingly, this enhanced HUVEC proliferation mediated by immobilized OPN was significantly inhibited with the addition of anti-human $\alpha v\beta 3$ antibody or GRGDS peptide. These results are consistent with our finding that migration of HUVEC to OPN was mediated by $\alpha v\beta 3$ integrin as shown in Fig. 2. Taken together, these findings imply the interaction between OPN and $\alpha v\beta 3$ integrins on vascular endothelial cells may play an important role in tumor angiogenesis.

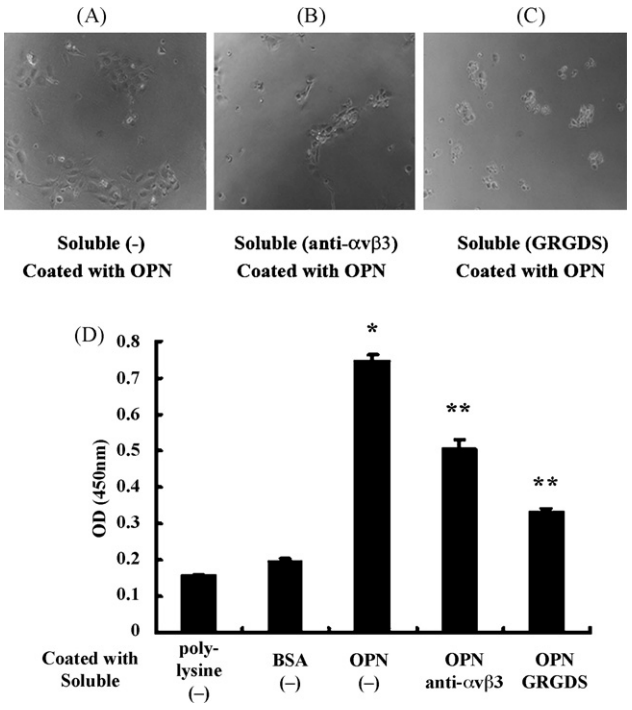

Fig. 5 Inhibitory effect of anti-αvβ3 antibody or RGD peptide on HUVEC proliferation mediated by OPN. (A–C) Representative microphotographs were depicted (×100). (D) Immobilized OPN significantly enhanced HUVEC proliferation and this enhancement was markedly suppressed by treatment with anti-αvβ3 antibody or RGD peptide. Data are presented as mean ± S.D. $^{*}P < 0.0001$ vs. coated with BSA, soluble (−); $^{**}P < 0.001$ vs. coated with OPN, soluble (−).

3.10. Effect of RMV-7 antibody or TNP-470 on growth of SBC-3/OPN tumor in vivo

Since the SBC-3/OPN#5 induced tumors revealed strong neovascularization and tumor growth, the SBC-3/OPN#5 induced tumors were treated with RMV-7 or anti-angiogenic agent, TNP-470, to investigate whether the accelerated SBC-3/OPN#5 tumor growth *in vivo* was directly associated with neovascularization mediated by the interaction between OPN and its receptor, αvβ3 integrin. As shown in Table 1, TNP-470 and RMV-7 administration significantly reduced *in vivo* tumor growth against SBC-3/OPN#5 cells with growth-inhibitory ratio (GIR) values (%) of 83.8% and 85.6%, respectively. In contrast to strong antitumor activity against SBC-3/OPN tumor, RMV-7 did not reveal any antitumor activity against the SBC-3/NEO tumor. These results suggest that the abrogation of the interaction between OPN and αvβ3 integrin could be an effective therapeutic modality in OPN-overexpressing lung cancer.

4. Discussion

OPN is a secreted multifunctional glycosylated phosphoprotein that is involved in tumor progression and metastasis through interaction with adhesion molecules such as integrins αvβ3, αvβ5, and αvβ1, and CD44 variants in a RGD sequence dependent or independent manner [24,25]. Angiogenesis plays a central role in the growth and metastasis of various cancers. The endothelial cell migration is dependent on their adhesive to extracellular matrix protein such as OPN through a variety of cell adhesion receptor including αvβ3 integrins [26]. It has been reported that overexpression of the αvβ3 integrin on tumor vasculature is associated with an aggressive phenotype of several solid tumor types [27,28]. Recent clinical studies also revealed that OPN, a ligand for αvβ3, overexpression is associated with tumor progression and poor survival of patients with lung cancer [17,18].

Table 1 Antitumor activity of RMV-7 or TNP-470 against SBC-3/OPN and SBC-3/NEO inoculated into nude mice

Cell line	Agent	Tumor volume (mm^3)	GIR (%)
SBC-3/OPN#5	TNP-470 (−)	506.9 ± 246.28	83.8
	TNP-470 (+)[b]	81.79 ± 34.4[*]	
	RMV-7 (−)	2272.45 ± 1126.73	85.6
	RMV-7 (+)[a]	326.35 ± 157.18[**]	
SBC-3/NEO#1	TNP-470 (−)	126.7 ± 27.98	27.1
	TNP-470 (+)[b]	92.36 ± 12.64	
	RMV-7 (−)	464.76 ± 167.49	3.6
	RMV-7 (+)[a]	448.17 ± 177.68	

Antitumor activity was evaluated in term of growth-inhibitory ratio (GIR, %), defined as [1 − (mean tumor volume of treated/mean tumor volume of control)] × 100 at day 32[a] after the first administration of RMV-7 or day 28[b] after the first administration of TNP-470. Data are presented as mean ± S.D.
[*] $P < 0.05$ vs. TNP-470 (−).
[**] $P < 0.05$ vs. RMV-7 (−).

In this study, we conducted *in vivo* tumorigenicity experiments using human lung cancer cell line, SBC-3 cells, to reveal whether interaction between OPN and its receptor αvβ3 plays a key role in tumor growth mediated by angiogenesis. The SBC-3 cell line was originally established from bone marrow aspirate of the 24-year-old male patient with small cell lung cancer [29]. Its subcutaneous implantability has been approved by Fukumoto et al. [30]. OPN-overexpressing SBC-3 cells significantly enhanced *in vivo* tumor growth compared to the control cells. Interestingly, *in vitro* cell growth rate and VEGF mRNA expression levels were similar among these cells. In contrast, transfection of SBC-3 cells with OPN gene significantly induced neovascularization *in vivo*. Apoptosis of SBC-3 cells *in vivo* and colony formation of SBC-3 cells *in vitro* were not affected by transfection with the OPN gene. These results imply that promotion of the tumor growth of SBC-3/OPN cells *in vivo* may be attributed to the hypervascularization induced by secreted OPN. In fact, recombinant human OPN protein enhanced HUVEC proliferation *in vitro*, and these effects of OPN were significantly suppressed with the addition of anti-αvβ3 integrin monoclonal antibody or RGD peptide. These results suggest that OPN is implicated in the process of angiogenesis by interacting with the αvβ3 integrin. In addition, we performed *in vivo* experiment to evaluate the metastatic effect of OPN. The cell suspensions of SBC-3/OPN or SBC-3/NEO cells were injected into a lateral tail vein of BALB/c nude mice. Unfortunately, we did not observe metastatic colonies in lungs. Although liver and kidney metastasis were observed, there

was no significant difference in the number of metastatic colonies in livers and kidneys between in SBC-3/OPN and SBC-3/NEO injected mice (data not shown).

The sustained growth of solid tumors is dependent on the vascular network, making tumor blood vessels a potential therapeutic target [3]. Since previous reports confirmed that OPN plays an important role in tumor progression and metastasis, various therapeutical trials targeting the interaction between OPN and its receptors have been proposed. Thalmann et al. reported that anti-OPN antibody inhibits the growth stimulatory effect of endogenous OPN for human prostate carcinoma cells [31]. In addition, a murine anti-human OPN antibody, which recognizes the RGD/thrombin cleavage region, inhibits the adhesion of MDA-MB-435 breast cancer cells to OPN [32]. Recent trials have used the siRNA technique to knock down OPN mRNA expression. Shevde et al. have demonstrated that suppression of OPN mRNA with siRNA reduced tumorigenecity of MDA-MB-435 breast cancer cells [33]. In addition, Wai et al. revealed that inhibition of OPN mRNA reduced metastatic potential in murine colon carcinoma cells [34]. Regarding anti-OPN receptor antibodies, Brooks et al. have reported that monoclonal antibody (LM609) against $\alpha v\beta 3$ integrin induces apoptosis of the proliferative angiogenic blood vessel cells and leads to tumor regression in breast cancer [35]. However, there are no studies with regard to the therapeutic trials targeting OPN and its receptor in lung cancer animal models.

In the present study, we evaluated therapeutic efficacy of anti-$\alpha v\beta 3$ integrin antibody (RMV-7) in OPN-overexpressing human lung cancer cells inoculated mice model. Treatment of mice with RMV-7 completely suppressed the *in vivo* tumor growth of SBC-3/OPN with GIR value of 85.6%, while growth rate of SBC-3/NEO *in vivo* was not attenuated by treatment with RMV-7. In the same way, anti-angiogenic agent, TNP-470, exhibited strong anti-tumor activity against SBC-3/OPN tumor with GIR value of 83.8%. These results suggest that interaction between OPN and $\alpha v\beta 3$ integrin plays a crucial role for tumor growth induced by up-regulated angiogenesis of human lung cancer cells in mice and anti-$\alpha v\beta 3$ antibody could be useful in anti-angiogenic treatment of human lung cancer.

Phase I study using vitaxin (humanized monoclonal anti-$\alpha v\beta 3$ integrin antibody) has demonstrated its safety and potential activity in some human cancers. This study revealed that one patient demonstrated partial response and seven patients exhibited stable disease course among the 14 patients evaluated [36]. Recently, McNeel et al. reported phase I trial of a monoclonal antibody specific for $\alpha v\beta 3$ integrin (MEDI-522) in patient with advanced multiple malignancies including lung cancer [37]. In their study, three patients with renal carcinoma demonstrated a prolonged and stable disease course among the 25 patients investigated. However, none of the patients with lung cancer revealed favorable therapeutic response. According to our previous report, OPN is predominantly expressed in NSCLC, but its expression level is variable [38]. In both phase I trials, they did not mention the issue of OPN expression in NSCLC. The reason why none of the patients with NSCLC revealed any response to treatment with anti-$\alpha v\beta 3$ antibody might have been due to the low expression of OPN in NSCLC cells in these patients. In fact, administration of RMV-7 antibody did not reduce *in vivo* tumor growth in SBC-3/NEO cells inoculated mice in our study. These results suggest that intratumoral OPN expression could be a surrogate marker in the prediction of therapeutic response for treatment with anti-$\alpha v\beta 3$ integrin antibody in lung cancer.

Conclusively, our study revealed that OPN is involved in tumor growth and angiogenesis of lung cancer by up-regulating vascular endothelial cell migration and proliferation via interacting with $\alpha v\beta 3$ integrin. OPN and its receptor could be effective target molecules in the future for anti-angiogenic therapy of patients with lung cancer.

Conflict of interest

None.

References

[1] Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, et al. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci USA 2001;98:13790—5.
[2] Chan DC, Earle KA, Zhao TL, Helfrich B, Zeng C, Baron A, et al. Exisulind in combination with docetaxel inhibits growth and metastasis of human lung cancer and prolongs survival in athymic nude rats with orthotopic lung tumors. Clin Cancer Res 2002;8:904—12.
[3] Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27—31.
[4] Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267: 10931—4.
[5] Eliceiri BP, Cheresh DA. The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 1999;103:1227—30.
[6] Varner JA. The role of vascular cell integrins alpha v beta 3 and alpha v beta 5 in angiogenesis. Exs 1997;79:361—90.
[7] Patarca R, Saavedra RA, Cantor H. Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. Crit Rev Immunol 1993;13:225—46.
[8] Takahashi K, Takahashi F, Tanabe KK, Takahashi H, Fukuchi Y. The carboxyl-terminal fragment of osteopontin suppresses arginine-glycine-asparatic acid-dependent cell adhesion. Biochem Mol Biol Int 1998;46:1081—92.
[9] Agrawal D, Chen T, Irby R, Quackenbush J, Chambers AF, Szabo M, et al. Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. J Natl Cancer Inst 2002;94:513—21.
[10] Chambers AF, Wilson SM, Kerkvliet N, O'Malley FP, Harris JF, Casson AG. Osteopontin expression in lung cancer. Lung Cancer 1996;15:311—23.
[11] Kim JH, Skates SJ, Uede T, Wong KK, Schorge JO, Feltmate CM, et al. Osteopontin as a potential diagnostic biomarker for ovarian cancer. JAMA 2002;287:1671—9.
[12] Tuck AB, Chambers AF. The role of osteopontin in breast cancer: clinical and experimental studies. J Mammary Gland Biol Neoplasia 2001;6:419—29.
[13] Tuck AB, O'Malley FP, Singhal H, Tonkin KS, Harris JF, Bautista D, et al. Osteopontin and p53 expression are associated with tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas. Arch Pathol Lab Med 1997;121:578—84.
[14] Ue T, Yokozaki H, Kitadai Y, Yamamoto S, Yasui W, Ishikawa T, et al. Co-expression of osteopontin and CD44v9 in gastric cancer. Int J Cancer 1998;79:127—32.
[15] Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. Stimulation of endothelial cell

migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol 1996;149:293—305.
[16] Shijubo N, Kojima H, Nagata M, Ohchi T, Suzuki A, Abe S, et al. Tumor angiogenesis of non-small cell lung cancer. Microsc Res Tech 2003;60:186—98.
[17] Donati V, Boldrini L, Dell'Omodarme M, Prati MC, Faviana P, Camacci T, et al. Osteopontin expression and prognostic significance in non-small cell lung cancer. Clin Cancer Res 2005;11:6459—65.
[18] Hu Z, Lin D, Yuan J, Xiao T, Zhang H, Sun W, et al. Overexpression of osteopontin is associated with more aggressive phenotypes in human non-small cell lung cancer. Clin Cancer Res 2005;11:4646—52.
[19] Takahashi F, Takahashi K, Okazaki T, Maeda K, Ienaga H, Maeda M, et al. Role of osteopontin in the pathogenesis of bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 2001;24:264—71.
[20] Takahashi K, Nakamura T, Koyanagi M, Kato K, Hashimoto Y, Yagita H, et al. A murine very late activation antigen-like extracellular matrix receptor involved in CD2- and lymphocyte function-associated antigen-1-independent killer-target cell interaction. J Immunol 1990;145:4371—9.
[21] Takahashi F, Akutagawa S, Fukumoto H, Tsukiyama S, Ohe Y, Takahashi K, et al. Osteopontin induces angiogenesis of murine neuroblastoma cells in mice. Int J Cancer 2002;98:707—12.
[22] Cui R, Takahashi K, Takahashi F, Tanabe KK, Fukuchi Y. Endostatin gene transfer in murine lung carcinoma cells induces vascular endothelial growth factor secretion resulting in up-regulation of in vivo tumorigenecity. Cancer Lett 2006;232:262—71.
[23] Hirama M, Takahashi F, Takahashi K, Akutagawa S, Shimizu K, Soma S, et al. Osteopontin overproduced by tumor cells acts as a potent angiogenic factor contributing to tumor growth. Cancer Lett 2003;198:107—17.
[24] Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EJ, Tognazzi K, Perruzzi CA, et al. Osteopontin expression and distribution in human carcinomas. Am J Pathol 1994;145: 610—23.
[25] Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 1996;271:509—12.
[26] Auerbach W, Auerbach R. Angiogenesis inhibition: a review. Pharmacol Ther 1994;63:265—311.
[27] Gasparini G, Brooks PC, Biganzoli E, Vermeulen PB, Bonoldi E, Dirix LY, et al. Vascular integrin alpha(v)beta3: a new prognostic indicator in breast cancer. Clin Cancer Res 1998;4:2625—34.
[28] Mitjans F, Sander D, Adan J, Sutter A, Martinez JM, Jaggle CS, et al. An anti-alpha v-integrin antibody that blocks integrin function inhibits the development of a human melanoma in nude mice. J Cell Sci 1995;108(Pt 8):2825—38.
[29] Miyamoto H. Establishment and characterization of an adriamycin-resistant subline of human small cell lung cancer cells. Acta Med Okayama 1986;40:65—73.
[30] Fukumoto H, Nishio K, Ohta S, Hanai N, Fukuoka K, Ohe Y, et al. Effect of a chimeric anti-ganglioside GM2 antibody on ganglioside GM2-expressing human solid tumors in vivo. Int J Cancer 1999;82:759—64.
[31] Thalmann GN, Sikes RA, Devoll RE, Kiefer JA, Markwalder R, Klima I, et al. Osteopontin: possible role in prostate cancer progression. Clin Cancer Res 1999;5:2271—7.
[32] Bautista DS, Xuan JW, Hota C, Chambers AF, Harris JF. Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. J Biol Chem 1994;269:23280—5.
[33] Shevde LA, Samant RS, Paik JC, Metge BJ, Chambers AF, Casey G, et al. Osteopontin Knockdown Suppresses Tumorigenicity of Human Metastatic Breast Carcinoma, MDA-MB-435. Clin Exp Metastasis 2006;23:123—33.
[34] Wai PY, Mi Z, Guo H, Sarraf-Yazdi S, Gao C, Wei J, et al. Osteopontin silencing by small interfering RNA suppresses in vitro and in vivo CT26 murine colon adenocarcinoma metastasis. Carcinogenesis 2005;26:741—51.
[35] Brooks PC, Stromblad S, Klemke R, Visscher D, Sarkar FH, Cheresh DA. Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. J Clin Invest 1995;96:1815—22.
[36] Gutheil JC, Campbell TN, Pierce PR, Watkins JD, Huse WD, Bodkin DJ, et al. Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin alphavbeta3. Clin Cancer Res 2000;6:3056—61.
[37] McNeel DG, Eickhoff J, Lee FT, King DM, Alberti D, Thomas JP, et al. Phase I trial of a monoclonal antibody specific for alphavbeta3 integrin (MEDI-522) in patients with advanced malignancies, including an assessment of effect on tumor perfusion. Clin Cancer Res 2005;11:7851—60.
[38] Zhang J, Takahashi K, Takahashi F, Shimizu K, Ohshita F, Kameda Y, et al. Differential osteopontin expression in lung cancer. Cancer Lett 2001;171:215—22.