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Inhibition of Tumor-induced Angiogenesis by Sulfated Chitin Derivatives[1]

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ABSTRACT

The effect of antimetastatic sulfated chitin derivatives (SCM-chitin III) on angiogenesis induced by B16-BL6 cells was examined in syngeneic mice. SCM-chitin III caused a marked decrease of the number of vessels toward tumor mass (angiogenic response) without affecting the tumor cell growth when coinjected with tumor cells (on day 0), or injected into tumor site on day 1 or 3 after tumor inoculation. In contrast, carboxymethyl chitin as well as heparin had no effect. Invasion of endothelial cells through reconstituted basement membrane (Matrigel) toward tumor-conditioned media was significantly inhibited by SCM-chitin III in a Transwell chamber assay. SCM-chitin III also inhibited the haptotactic migration of endothelial cells to fibronectin-substrate, but did not inhibit the chemotactic activity in tumor conditioned media *in vitro*. SCM-chitin III did not directly affect the viability and the growth of tumor cells and endothelial cells *in vitro*. These results suggest that inhibition of lung tumor metastasis by SCM-chitin III may in part be due to the inhibition of tumor-associated angiogenesis.

INTRODUCTION

The development of new capillary blood vessels (angiogenesis) occurs in normal and pathological conditions, including embryonic development, wound healing, inflammation, and neoplasia (1–3). In tumor-associated angiogenesis, angiogenic factors from tumors stimulate endothelial cells within a venule to degrade the vascular basement membrane and to migrate into the surrounding tissues toward the tumor mass, and to promote the proliferation of endothelial cells in a capillary sprout (4). There are many evidences that angiogenesis is important for the progressive growth of solid tumors (5). Angiogenesis also permits the shedding of metastatic tumors from the primary site (6). Consequently, inhibition of angiogenesis may lead to control of tumor growth and metastasis.

Several angiogenesis inhibitors have been identified from normal tissues or cells such as protamine (7), interferons (8), platelet factor 4 (9), angiostatic steroids (10), and M_r 140,000 protein regulated by a cancer suppressor gene (11). However, some of these inhibitors have problems concerning their therapeutic applications because of their excessive toxicity and limited efficacy.

Chitin is a homogeneous polysaccharide composed of *N*-acetylglucosamine residues, while heparin is a structurally heterogeneous sulfated polysaccharide composed of repeating units of glucosamine and uronic acid (glucuronic acid or iduronic acid). We have recently reported that chemically modified chitin derivatives, into which 6-*O*-sulfate and 6-*O*-carboxymethyl groups were introduced, significantly inhibited the lung tumor colonization of B16-BL6 melanoma in experimental and spontaneous lung metastasis model (12). In addition, SCM-chitin[3] III suppressed tumor cell invasion of basement membrane and the migration to laminin-substrate, but had no toxicity against tumor cells and endothelial cells *in vitro* (13).

In this study, we described the effect of SCM-chitin III on neovascularization initiated by B16-BL6 cells in syngeneic mice and on the invasion of endothelial cells through the basement membrane toward tumor-conditioned media *in vitro*.

MATERIALS AND METHODS

Animals. Inbred 7- to 10-week-old female C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The mice were maintained in the Laboratory for Animal Experiments, the Institute of Immunological Science, Hokkaido University, under laminar air flow conditions.

Cells and Cell Culture. Highly metastatic subline of murine B16 melanoma, B16-BL6, was kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, TX. Melanoma cells were maintained as monolayer cultures in Eagle's MEM supplemented with 7.5% FBS, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine. Rat lung endothelial cells were kindly provided by Dr. M. Nakajima, M. D. Anderson Cancer Center, Houston, TX. RLE cells were maintained in 1.0% gelatin-coated plastic tissue culture plates containing a 1:1 ratio of DMEM:F12 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FBS.

Chitin Derivatives and Other Reagents. Chitin was prepared from queen crab shells by the method of Hackman (14) and powdered to 45–60 mesh before use. A 6-*O*-carboxymethyl chitin was prepared from chitin according to the method described previously (15) and the degree of substitution used was 0.80. The sulfation of CM-chitin was carried out by the general method of Horton and Just (16). Briefly, CM-chitin was treated with distilled pyridine to remove water and resuspended in pyridine. Chlorosulfonic acid-pyridine mixture was added to the chitin suspension and the mixture was boiled under reflux for 90 min with stirring. The supernatant liquid was decanted and the residue, dissolved in ice water, was adjusted to pH 9 with 2 M NaOH. The precipitate formed by the addition of ethanol was redissolved in water, dialyzed against deionized water to remove free salt, and subsequently lyophilized. The degree of sulfation was estimated by the quantitative analyses for sulfur in the products (17). The molecular weights of chitin derivatives were estimated from viscosity measurements by using Ubbelohde-type viscometer by applying the equation proposed for heparin (18). Average molecular weights of chitin derivatives used in this study were 24,000 for SCM-chitin III and 63,000 for CM-chitin. Chemical analyses of the products were previously described in detail (12). A partially *N*-desulfated SCM-chitin III was prepared as follows; 200 mg of SCM-chitin III were suspended in 5% methanol-dimethyl sulfoxide and stirred at 50°C for 90 min. The residue was neutralized by 1 M NaOH,

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[3] The abbreviations used are: SCM-chitin III, sulfated chitin derivatives; MEM, Eagle's minimal essential medium; CM-chitin, carboxymethyl chitin; DMEM:F12, Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 medium; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; FBS, fetal bovine serum; TCM, tumor-conditioned medium; FGF, fibroblast growth factor; EGF, epidermal growth factor; i.d., intradermal(ly); i.t., intratumoral(ly); RLE, rat lung endothelial.

subsequently precipitated by acetone, and air dried. Heparin-Sepharose CL-6B was purchased from Pharmacia AB Laboratory, Uppsala, Sweden. SCM-chitin III-Sepharose 4B was prepared by coupling reactions as follows. Briefly, Sepharose 4B was swelled with 1 mM HCl, and washed 5 times with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) on a glass filter (G3) for 15 min. Partially *N*-desulfated SCM-chitin III dissolved in coupling buffer was added to the swelling gel under heterogeneous conditions, and incubated at 0°C overnight. The mixture was then incubated with 0.2 M glycine at 25°C for 2 h to block the unreacted-active groups. The products were repeatedly washed with coupling buffer to remove excess glycine, and resuspended in coupling buffer. The coupling of SCM-chitin III to Sepharose 4B was confirmed by the IR spectrum as a reference of Sepharose 4B. Heparin-Sepharose CL-6B and SCM-chitin III-Sepharose 4B were designated as heparin beads and SCM-chitin III beads, respectively. The beads were washed twice with PBS and resuspended in serum-free MEM before use. Heparin sodium salt (Lot TLP3856; specific activity, 197.1 units/mg) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Clupeine (protamine from herring testis) was purchased from Sigma Chemical Co., St. Louis, MO. Bovine acidic FGF and recombinant human EGF were purchased from Biomedical Technologies, Inc., Stoughton, MA. Tumor-conditioned medium was prepared from the culture supernatant of B16-BL6 cells. Confluent monolayers of tumor cells grown in 75-cm^2 Falcon tissue culture flasks, were rinsed twice with PBS, and incubated at 37°C for 24 h in 5 ml of serum-free DMEM:F12. The TCM was passed through a 0.2 μm Millipore filter and stored at −20°C before use. All the reagents and media in this study were endotoxin free (approximately <1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co. Ltd., Tokyo, Japan).

Assay of Tumor-induced Angiogenesis. The assay of tumor angiogenesis in syngeneic mice was carried out according to the method described by Kreisle and Ershler (19) with some modifications (20). C57BL/6 mice were inoculated i.d. with B16-BL6 melanoma cells (5×10^5) at two sites on the back. The i.t. injection of chitin derivatives was performed on various days after tumor inoculation. Three days after the injection of chitin derivatives, mice were killed immediately after i.v. injection (0.2 ml) of 1% Evans blue, and the skin was separated from the underlying tissues. Each of the inoculation sites was located with a dissecting microscope, and angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass. The tumor size was approximated by averaging the diameters of short and long axis of the remnant of injected cells. All counts were made by a single observer in a blinded manner.

Cell Migration Assay. Endothelial cell migration along a gradient of substratum-bound fibronectin (haptotactic migration) was assessed in Transwell cell culture chambers (Costar 3422, Cambridge, MA) according to the methods as reported by McCarthy *et al.* (21) with some modifications (22). Polyvinylpyrrolidone-free polycarbonate filters with 8.0-μm pore size (Nucleopore, Pleasanton, CA) were precoated with 5 μg of fibronectin in a volume of 50 μl on the lower surface, and dried overnight at room temperature. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of RLE cells were harvested by a short trypsinization (0.25% trypsin and 0.02% EDTA at 37°C for 1 min), washed 3 times with serum-free MEM, and resuspended to a final concentration of 2×10^6/ml in DMEM:F12 containing 0.1% bovine serum albumin. Cell suspensions (100 μl) were added to the upper compartment of the chamber, chitin derivatives were added to the lower compartment and incubated at 37°C in a 5% CO_2 atmosphere. After a 4-h incubation, the filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at ×400, and each assay was performed in triplicate. The chemotactic migration of RLE cells was measured by using Transwell cell culture chambers as described above. The lower surface of the filters was precoated with 5 μg of gelatin. RLE cell suspensions (2×10^5/100 μl) were added to the upper compartment of the chamber, chemoattractants were added to the lower compartment and incubated at 37°C for 18 h. In the inhibition assay, the chemoattractants were preincubated with 5 mg heparin beads or SCM-chitin III beads at 0°C for 2 h with stirring. The following procedures were the same as those of the haptotactic migration assay.

Cell Invasion Assay. Endothelial cell invasion through reconstituted basement membrane (Matrigel) was assayed according to the method of Albini *et al.* (23) with some modifications (22). In Transwell cell culture chambers, the lower surface of the filters was precoated with 5 μg of fibronectin, and then 10 μg of Matrigel (Collaborative Research, Inc., Bedford, MA) were applied to the upper surface of the filters. The filters thus prepared were designated Matrigel/fibronectin-coated filters. RLE cells (2×10^5/100 μl) were added to the upper compartment, chemoattractants were added to the lower compartment in the presence or absence of chitin derivatives in both compartments, and incubated at 37°C for 18 h. The following procedures were the same as those of the migration assay.

Cell Proliferation Assay. RLE cells suspended in DMEM:F12 containing 10% FBS were seeded at 2×10^3 cells/well into 1% gelatin-coated 96-well tissue culture plate (Falcon) and were allowed to attach overnight. The media were aspirated and replaced with DMEM:F12 containing 0.1% bovine serum albumin. The cultures were incubated at 37°C for 48 h in the presence or absence of chitin derivatives, and pulsed with 0.5 μCi/well of [^{3}H]thymidine (specific activity, 23 Ci/mmol; Amersham International, Buckinghamshire, United Kingdom) for the last 4 h before termination. The cells were then trypsinized and harvested by an automatic cell harvester. The radioactivity was measured in a liquid scintillation counter and expressed as mean cpm ± SD in triplicate culture.

Statistical Analysis. The statistical significance of differences between groups was calculated by applying the Student's 2-tailed *t* test.

RESULTS

Effect of SCM-Chitin III on Tumor-induced Angiogenesis in Mice. Tumor-associated angiogenesis contributes to tumor metastasis as well as growth of solid tumors. A decreased rate of angiogenesis is closely associated with a low incidence of hematogenous metastasis (24). We first examined the effect of antimetastatic SCM-chitin III on tumor-induced angiogenesis *in vivo*. B16-BL6 cells (5×10^5) were injected i.d. on the back of syngeneic mice and the angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass. Fig. 1 shows that angiogenic response (number of vessels) at the tumor injection site increased in a time-dependent fashion and was proportional to the tumor growth (tumor size) within 5 days after tumor inoculation. Thereafter, increased tumor growth, but little or no change in the number of vessels, was

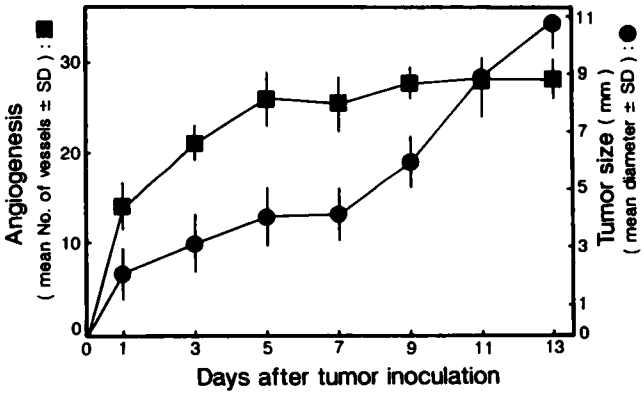

Fig. 1. Time course of angiogenesis and tumor growth at tumor injection sites. Three C57BL/6 mice per group were inoculated i.d. with B16-BL6 cells (5×10^5) at two sites on the back. At various days after tumor inoculation, mice were killed and the skin was separated from underlying tissues. Angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass.

Table 1 *Inhibition of tumor-induced angiogenesis by SCM-chitin III*

Three C57BL/6 mice per group were given injections i.t. of 100 μg of SCM-chitin III on day 0, 1, 3, or 7 after i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Three days after the injection of chitin derivatives, mice were killed and the skin was separated from underlying tissues. Angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass.

Administered i.t. with:	Dose (μg/mouse)	Angiogenesis (mean no. of vessels ± SD)	Tumor size (mm)
Day 0			
PBS		21 ± 3	3 ± 1
SCM-chitin III	100	13 ± 2[a]	4 ± 1
CM-chitin	100	22 ± 3	4 ± 1
Heparin	100	24 ± 2	4 ± 1
Clupeine	100	14 ± 2[a]	3 ± 1
Day 1			
PBS		24 ± 2	3 ± 1
SCM-chitin III	100	15 ± 3[a]	3 ± 1
CM-chitin	100	23 ± 2	3 ± 1
Heparin	100	25 ± 1	4 ± 1
Day 3			
PBS		27 ± 1	4 ± 1
SCM-chitin III	100	17 ± 4[a]	4 ± 1
CM-chitin	100	26 ± 2	4 ± 2
Heparin	100	25 ± 2	4 ± 1
Day 7			
PBS		31 ± 4	6 ± 2
SCM-chitin III	100	31 ± 2	6 ± 1
CM-chitin	100	32 ± 4	7 ± 1
Heparin	100	33 ± 4	7 ± 1

[a] $P < 0.001$.

Table 2 *Dose response of SCM-chitin III on tumor-induced angiogenesis*

Three C57BL/6 mice per group were given injections i.t. of various doses of SCM-chitin III on day 1 after i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Three days after the injection of chitin derivatives, mice were killed and the skin was separated from underlying tissues. Angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass.

Administered i.t. with:	Dose (μg/mouse)	Angiogenesis (mean no. of vessels ± SD)	Tumor size (mm)
Day 1			
PBS		20 ± 2	3 ± 1
SCM-chitin III	20	14 ± 3[a]	3 ± 1
	100	9 ± 2[b]	3 ± 1
	500	8 ± 1[b]	3 ± 1
Clupeine	100	8 ± 1[b]	3 ± 1

[a] $P < 0.01$.
[b] $P < 0.001$.

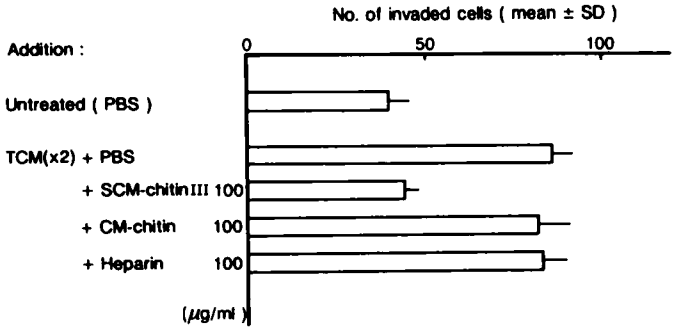

Fig. 2. Inhibition of RLE cell invasion through Matrigel/fibronectin-coated filters by SCM-chitin III. Filters were precoated with Matrigel (10 μg) on the upper surface and with fibronectin (5 μg) on the lower surface. RLE cells (2×10^5) were seeded into the upper compartment in Transwell chambers and TCM (×2) was added into the lower compartment, in the presence or absence of SCM-chitin III (100 μg/ml) in both compartments. After an 18-h incubation, the invaded cells on the lower surface were visually counted.

Table 3 *Inhibition of haptotactic migration of RLE cells on fibronectin-coated filters by SCM-chitin III*

RLE cells (2×10^5) were seeded on the filters precoated on the lower surface with 5 μg fibronectin. After a 4-h incubation, the migrated cells on the lower surface were visually counted.

Treatment	No. of migrated cells (Mean ± SD)	p^a
Addition in lower compartment[b]		
PBS	41 ± 6	
SCM-chitin III		
20 μg/ml	36 ± 5	
100 μg/ml	21 ± 3	<0.001
500 μg/ml	11 ± 3	<0.001
CM-chitin, 100 μg/ml	42 ± 7	
Heparin, 100 μg/ml	38 ± 2	
Treatment of fibronectin substrate with:[c]		
SCM-chitin III, 100 μg	19 ± 4	<0.001
CM-chitin, 100 μg	42 ± 5	
Heparin, 100 μg	33 ± 8	
Treatment of RLE cells with:[d]		
SCM-chitin III, 100 μg	39 ± 3	
CM-chitin, 100 μg	42 ± 3	
Heparin, 100 μg	42 ± 8	

[a] Compared with untreated control (PBS) by Student's 2-tailed *t* test.
[b] SCM-chitin III was added into lower compartment of Transwell chambers.
[c] Fibronectin-coated filters were preincubated with SCM-chitin III for 1 h at room temperature.
[d] RLE cells were preincubated with SCM-chitin III for 1 h on ice.

observed. The background vascularity of surrounding tissues was observed around 3–5 vessels by projecting toward randomized points (data not shown). We next examined the effect of SCM-chitin III on tumor angiogenesis at various days after tumor inoculation. SCM-chitin III (100 μg) was injected i.d. with tumor cells (on day 0) or i.t. on day 1, 3, or 7 after the implantation, and angiogenesis was assessed 3 days after the injection. Table 1 shows that SCM-chitin III caused a marked decrease of neovascularization when injected on day 0, 1, or 3 but not on day 7. This inhibition was dose dependent, ranging from 20 to 500 μg/mouse on day 1 following tumor inoculation (Table 2). The use of SCM-chitin III did not affect the tumor growth (size) in comparison with untreated control (PBS). Herring protamine, clupeine, also inhibited the tumor angiogenesis, except when injected on day 7. In contrast, both CM-chitin and heparin, at the dose of 100 μg, had no effect.

Effect of SCM-Chitin III on Invasion of Endothelial Cells into Matrigel. An invasion of capillary endothelial cells through the basement membrane from existing blood vessels toward tumor mass is one of the essential events of tumor neovascularization. Therefore, we tested the effect of SCM-chitin III on endothelial cell invasion through reconstituted basement membrane (Matrigel) in a Transwell chamber assay. Fig. 2 shows that rat lung endothelial cells in the upper compartment were able to invade through Matrigel to the fibronectin-coated lower surface of the filters. The addition of TCM from B16-BL6 cells into the lower compartment promoted the invasion of RLE cells approximately 2-fold. The enhanced invasion was significantly inhibited to the control level (PBS) by the addition of 100 μg/ml SCM-chitin III into both compartments. Neither CM-chitin nor heparin, at the concentration of 100 μg/ml, inhibited RLE cell invasion through Matrigel/fibronectin-coated filters.

Effect of SCM-Chitin III on Migration of Endothelial Cells. The endothelial cell invasion in this system can be subdivided into three sequential steps; degradation of basement membrane (Matrigel) components, haptotaxis on extracellular matrix proteins, and chemotaxis to conditioned media from tumors. To further investigate the influence of SCM-chitin III on tumor neovascularization, we examined the effect of SCM-chitin III

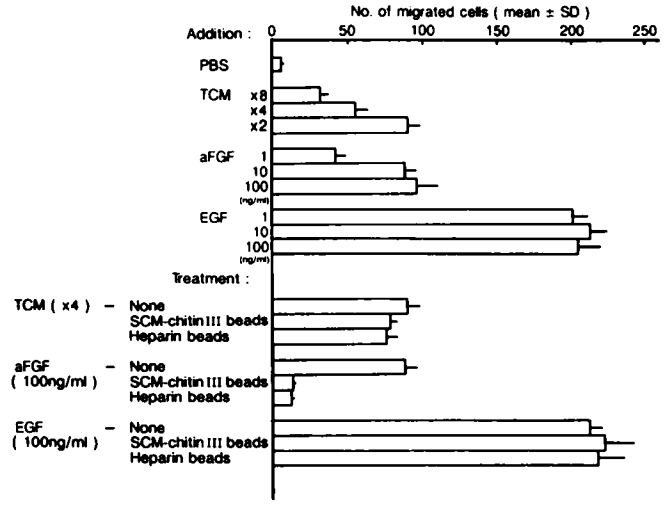

Fig. 3. Effect of SCM-chitin III on TCM-induced RLE cell chemotaxis. RLE cells (2×10^5) were seeded onto the filters which were precoated with gelatin (5 μg) on the lower surface, and chemoattractants, which had been treated or untreated with SCM-chitin III beads, were added into the lower compartment in Transwell chambers. After an 18-h incubation, the migrated cells on the lower surface were visually counted.

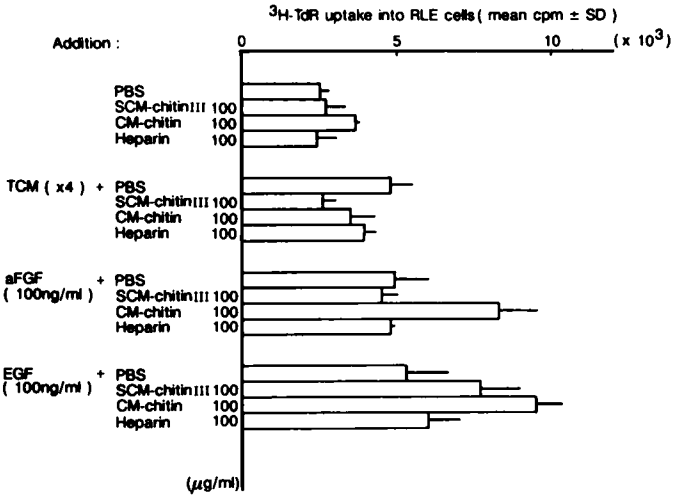

Fig. 4. Effect of SCM-chitin III on growth of RLE cells. RLE cells (2×10^3) were seeded with or without SCM-chitin III (100 μg/ml) into gelatin-coated wells in the presence or absence of angiogenic factors. After a 48-h incubation, the cells were pulsed with [^{3}H]thymidine for the last 4 h before termination, and the radioactivity was counted.

on haptotaxis of RLE cells *in vitro*. Table 3 shows that SCM-chitin III inhibited the haptotactic migration of RLE cells on fibronectin-coated filters in a concentration-dependent manner ($P < 0.001$). CM-chitin, however, did not affect the migration, nor did heparin. Pretreatment of fibronectin-substrate with SCM-chitin III caused a marked decrease of RLE cell migration, whereas CM-chitin and heparin did not. On the other hand, preincubation of RLE cells with SCM-chitin III did not affect their migration. These results suggest that SCM-chitin III was able to inhibit the haptotactic migration of RLE cells through interaction between fibronectin-substrate and SCM-chitin III. Furthermore, we examined the effect of SCM-chitin III on chemotaxis of RLE cells to TCM in Transwell chambers. The lower surface of the filters were coated with gelatin and RLE cells were incubated in the upper compartment with or without TCM in the lower compartment. Fig. 3 shows that the migration of RLE cells to gelatin-coated filters was not observed without TCM after an 18-h incubation (5 ± 2). When TCM was added in the lower compartment, the migration of RLE cells was chemotactically stimulated in a concentration-dependent manner of TCM. Other chemoattractants, acidic FGF or EGF, also stimulated cell migration at various concentrations used. Preincubation of TCM with SCM-chitin III beads did not affect the chemotactic response of RLE cells or that of heparin beads. On the other hand, chemotaxis of RLE cells to aFGF was significantly inhibited by the pretreatment with both SCM-chitin III beads and heparin beads, while the chemotaxis to EGF was not inhibited by any treatment. These results indicate that SCM-chitin III did not affect the chemotactic migration of endothelial cells to TCM.

Effect of SCM-Chitin III on Growth of Endothelial Cells. Endothelial cell proliferation which occurred within a capillary sprout is an important step in capillary formation as well as in their locomotion. We tested the influence of SCM-chitin III on the growth of endothelial cells *in vitro*. Figure 4 shows that 100 μg/ml SCM-chitin III, CM-chitin, and heparin did not directly affect RLE cell growth after a 48-h incubation period. The incubation of B16-BL6 cells with 100 μg/ml SCM-chitin III for 3 days did not affect their viability and growth *in vitro* (data not shown). TCM promoted the growth of RLE cells by about 2 times as compared with the control. When 100 μg/ml SCM-chitin III was added to RLE cells in the presence of TCM, the mitogenic effect of TCM was inhibited approximately 40% over that of the control. In contrast, an inhibitory effect on the enhanced RLE cell growth by aFGF or EGF was not observed when SCM-chitin III was added to the culture. A similar result was observed by counting the cell number under the same conditions (data not shown).

DISCUSSION

We have demonstrated here that sulfated chitin derivatives inhibited tumor-associated angiogenesis in syngeneic mice. The angiogenic response (number of vessels) to B16-BL6 cells logarithmically increased within 5 days after tumor inoculation, and formed a plateau over 5 days (Fig. 1). We also observed that the vessels are likely to become dilated, depending on the growth of tumor mass and increased blood supply. However, no leakage of dye (Evans blue) into the surrounding region was observed by the i.v. injection. A single administration of 100 μg of SCM-chitin III at the tumor site inhibited the tumor angiogenesis without affecting tumor cell growth on day 0, 1, or 3 after the implantation, but not on day 7 (Table 1). SCM-chitin III (100 μg/ml) also did not affect the growth and the viability of tumor cells and RLE cells *in vitro*. (Fig. 4; data not shown). These results suggest that SCM-chitin III may prevent the formation of new capillary vessels at the early phase of tumor angiogenesis without affecting the tumor cell growth.

A neovascularization is composed of a characteristic series of steps. The new capillary formation is initiated by local degradation of vascular basement membrane in response to angiogenic factors (25, 26). Endothelial cells have been shown to produce degrading enzymes of basement membrane components such as type IV collagenase (27, 28). We have recently found that SCM-chitin III inhibited the activity of type IV collagenase and heparanase more potently than that of heparin (13). This implies that SCM-chitin III may act as an inhibitor of type IV collagenase from RLE cells, which results in the prevention of degradation of basement membrane. After degradation of basement membrane, endothelial cells migrate into the surrounding tissue which was constructed with extracellular

matrix components, toward tumor angiogenic stimuli (26). We showed that RLE cells were able to chemotactically respond to the TCM (Fig. 3). However, both SCM-chitin III beads and heparin beads were unable to absorb the chemotactic activity in TCM, indicating that SCM-chitin III did not interact with chemotactic factors from tumors. Chemotactic factors in TCM are not characterized yet, but at least they may not be FGF families which preferentially bind to heparin. Heparin has been reported to promote locomotion and growth of endothelial cells induced by acidic FGF *in vitro*, which resulted in the augmentation of angiogenesis (29). The i.t. administration of heparin (100 μg) failed to augment the angiogenic response at the tumor site (Table 1). This failure may be due to the lack of heparin-binding angiogenic factors from B16-BL6 cells. RLE cell migration along a gradient of substratum-bound fibronectin (haptotaxis) was significantly inhibited by the addition of SCM-chitin III into the culture or the pretreatment of fibronectin-substrate with SCM-chitin III (Table 3). In addition, SCM-chitin III bound to heparin-binding domain of fibronectin with high affinity.[4] This indicates that SCM-chitin III could inhibit migration of endothelial cells on fibronectin substrate probably by its binding to the heparin-binding domain in this molecule. These results suggest that SCM-chitin III-mediated inhibition of tumor angiogenesis in mice may mainly be due to the inhibition of endothelial cell migration to extracellular matrix components, including fibronectin.

We have recently reported that multiple i.v. or i.t. administration of SCM-chitin III caused a marked decrease of lung tumor colonization of B16-BL6 cells before or after the amputation of primary tumors in spontaneous lung metastasis model (12). The mechanism responsible for the inhibition of lung metastasis by SCM-chitin III was partially characterized. SCM-chitin III interfered with tumor cell arrest in lungs, probably through the inhibition of tumor cell adhesion to subendothelial matrix (30). It also may not be due to the inhibition of platelet aggregation and blood coagulation induced by tumor cells because SCM-chitin III has much lower levels of anticoagulant activity and anti-platelet aggregation activity than that of heparin (30). SCM-chitin III also inhibited the tumor cell invasion of Matrigel, the tumor cell adhesion and migration to laminin, and the proteolytic degradation of basement membrane components *in vitro* (13).

In conclusion, we demonstrated that SCM-chitin III inhibited angiogenesis induced by B16-BL6 cells in mice, and prevented the invasion of endothelial cells through Matrigel and the migration to fibronectin-substrate *in vitro*. SCM-chitin III may interfere with neovascularization at an early growth phase not only in primary tumor sites but also in metastatic foci. This would partly contribute to the inhibition of lung tumor metastasis by SCM-chitin III in spontaneous lung metastasis model.

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