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Inhibition of Tumor Cell Arrest in Lungs by Antimetastatic Chitin Heparinoid

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We have investigated the effect of sulfated chitin derivatives on the intravascular events in the metastatic cascade. 6-O-Sulfated carboxymethyl chitin (SCM-chitin III), as well as heparin, significantly inhibited the arrest of B16-BL6 cells in lungs after co-injection with radiolabeled tumor cells, but carboxymethylated chitin (CM-chitin) had no effect. Heparin showed a potent inhibitory effect on tumor cell-elicited platelet aggregation and on blood coagulation, which can subsequently enhance the survival, arrest and invasiveness of tumor cells, whereas SCM-chitin III showed much weaker properties. In contrast, SCM-chitin III was found to inhibit the adhesion of tumor cells to subendothelial matrix, while heparin did not. SCM-chitin III was still active in inhibiting experimental lung metastasis even in mice which had been pretreated with anti-asialo GM1 serum or carrageenan to eliminate NK cells or macrophages. Thus, these results suggest that SCM-chitin-mediated inhibition of tumor metastases is distinct from that by heparin and may be due to interference with tumor cell arrest in the capillaries and consequently to the inhibition of tumor cell adhesion to subendothelial matrix.

Key words: Chitin heparinoid — Metastasis — Tumor arrest

A complex series of steps is required to permit the successful establishment of tumor metastasis.[1-3] Arrest and extravasation of circulating tumor cells in the capillaries are important steps in the metastatic cascade. Tumor cell arrest is initiated by tumor cell-endothelial cell contact[4,5] and stabilized by tumor cell-associated platelet thrombus which may facilitate the subsequent penetration of tumor cells through endothelial cells[6,7] and their underlying subendothelial matrix.[8,9] Although the formation of tumor emboli arises from the aggregation of platelets and the activation of the coagulation cascade induced by some tumor cells, the correlation between metastatic potential and the ability of tumor cells to induce platelet aggregation remains undefined.[10-15]

Several attempts have been made to inhibit tumor metastasis experimentally by using anticoagulants.[11,16-19] Suemasu and Ishikawa[20] have found that heparin and dextran sulfate successfully inhibited the experimental pulmonary metastasis of Sato lung carcinoma cells, and this inhibition may be partially due to its effect in changing the ionic properties of tumor cell surfaces. Tsubura *et al.*[21] have shown that sulfated polysaccharides were able to reduce the blood-borne pulmonary metastasis in rats by interfering with a step in the coagulation pathway such as the formation of tumor emboli caused by platelet aggregation at the early stage of tumor lodgement. Agostino[22] reported that experimental pulmonary metastasis was enhanced by intravenous injection of ellagic acid, which activated the blood coagulation factor. On the other hand, Irimura *et al.*[23] have demonstrated that chemically modified heparin without anticoagulant activity inhibited both lung metastasis of mouse melanoma cells and degradative enzyme (heparanase) activity of the melanoma cells.

Chitin is a homogeneous polysaccharide composed of N-acetylglucosamine residues, while heparin is a heterogeneous sulfated polysaccharide composed of repeating units of glucosamine and uronic acid (glucuronic acid or iduronic acid). 6-O-Carboxymethylation of chitin leads to elimination of undesirable properties of chitin, such as the adsorption of serum proteins[24] and antigenicity.[25] We recently reported that sulfated CM-chitin with a high degree of sulfation (SCM-chitin III) caused a marked decrease of lung tumor colonization of B16-BL6 melanoma cells in the experimental and spontaneous lung metastasis models, although SCM-chitin III has a much lower level of anticoagulant activity than that of heparin.[26]

In the present study, we focus on the intravascular events in the metastatic cascade, and investigate the effect of SCM-chitin III on the process of tumor cell arrest to clarify the mechanism of inhibition of tumor metastasis by SCM-chitin III.

MATERIALS AND METHODS

Animals Inbred 7- to 10-week-old female C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. The mice were maintained in the Laboratory for Animal Experiments, the Institute of

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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 I. J. Fidler, M.D. Anderson Cancer Center, Houston, Texas. Melanoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. Rat lung endothelial cells (RLE cells) were kindly provided by M. Nakajima, M.D. Anderson Cancer Center, Houston, Texas. RLE cells were maintained in 1.0% gelatin-coated plastic tissue culture plates containing a 1:1 ratio of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 media (DMEM: F12; GIBCO Laboratories, Grand Island, NY) supplemented with 10% FBS.

Chitin heparinoid and heparin Chitin was prepared from Queen Crab shells by the method of Hackman[27] and powdered to 45–60 mesh before use. 6-O-Carboxymethyl chitin (CM-chitin) was prepared from chitin according to the method described previously[28]; the degrees of substitution used were 0.40, 0.56 and 0.80. The sulfation of chitin and CM-chitin was carried out by the general method of Horton and Just.[29] Briefly, chitin or 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 off and the residue, suspended 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 means of quantitative analyses for sulfur in the products.[30] The molecular weights of chitin heparinoids were estimated from viscosity measurements using an Ubbelohde-type viscometer by applying the equation proposed for heparin.[31] Average molecular weights of chitin derivatives used in this study were 24000 for SCM-chitin III and 63000 for CM-chitin. Chemical analyses of the products were previously described in detail.[26] Heparin sodium salt (Lot TLP3856, specific activity: 197.1 units/mg) was purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Experimental metastasis assay Experimental pulmonary metastasis was assessed by means of tumor cell injection into the lateral tail vein of mice. C57BL/6 mice were injected intravenously (iv) with 20 μl of rabbit anti-asialo GM1 antisera (Wako Pure Chemical Industries, Ltd.) or intraperitoneally (ip) with 1.2 mg of carrageenan to abolish the activity of NK cells or macrophages, respectively. Twenty-four hours later, B16-BL6 melanoma cells (5×10^4) were admixed with or without chitin heparinoid in PBS and inoculated into mice. The mice were killed 14 days after tumor inoculation. The lungs were fixed in Bouin's solution and the lung tumor colonies were counted under a dissection microscope.

Lung retention of radiolabeled tumor cells B16-BL6 melanoma cells in the exponential growth phase were labeled with [^{125}I]iododeoxyuridine (^{125}I-IUdR) (specific activity: 200 mCi/mmol, New England Nuclear, Boston, MA). ^{125}I-IUdR-labeled tumor cells (3×10^4) in a volume of 0.2 ml were injected iv with or without chitin heparinoid into the lateral tail vein of the C57BL/6 mice. The mice were exsanguinated at times ranging from 30 min to 8 h after the injection. The lungs, liver, spleen, kidneys and blood were collected from each mouse, and rinsed in 70% ethanol. The radioactivity in each organ was measured in a gamma counter.

Platelet aggregation Plastic tubes and pipets were used for all procedures. Blood was obtained from C57BL/6 mice by heart puncturing, using heparin (final concentration, 10 units/ml) as an anticoagulant. Platelet-rich plasma (PRP) was prepared from the blood by centrifugation at 230 g for 7 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 1500*g* for 10 min at room temperature. The platelet number in PRP was adjusted to $3\times10^5/\mu$l with PPP. Platelet aggregation was measured photometrically by using a dual-channel aggregometer Model 440 (Chromo-Log, USA) at 37°C under stirring at 1000 rpm. Tumor cell suspension in PBS (5×10^5 cells/27.5 μl) was added to 250 μl of PRP in the presence or absence of 5 μl of chitin heparinoid. Aggregation was recorded as an increase in light transmission, with that of PPP representing 100% transmission.

Chromogenic assay for coagulation The anticoagulant activity of chitin heparinoids was measured by the method using a chromogenic substrate with some modification (Testzym AT-III 2 kit, Daiichi Kagaku Yakuhin Co., Ltd., Tokyo). Thrombin (6 mg/ml) dissolved in dilution buffer (100 m*M* 2-amino-2-hydroxymethyl-1,3-propanediol (pH 7.4), containing monomethylamine hydrochloride) was treated with various concentrations of chitin heparinoid at 37°C for 5 min in the presence of antithrombin III (AT-III). Substrate solution (S-2238; H-D-phenylalanyl-L-pipecolyl-L-arginyl-*p*-nitroanilide·2 hydrochloride salt) at the concentration of 1.4 mg/ml was added, and the mixture was incubated at 37°C for 5 min. The enzyme reaction was stopped by addition of sodium citrate and the absorbance of the mixture at 405 nm was measured.

Preparation of subendothelial matrix RLE cells (2×10^3/well) were seeded onto 96-well microtiter plates precoated with 1.0% gelatin and grown in DMEM:F12 containing 10% FBS. Every 48 h the culture medium was

replaced with freshly prepared medium. After incubation for 7 days, RLE cell monolayers were washed twice with hypotonic buffer (5 m*M* Tris-HCl pH 7.5, 0.5 mg/ml BSA, 0.1 m*M* $CaCl_2$) and then incubated with the buffer for 30 min at 37°C. RLE cells were lysed with 0.5% Nonidet P-40 in hypotonic buffer for 60 min at 37°C. The subendothelial matrix was then washed three times with PBS containing 10% FBS and used immediately for adhesion assay. The subendothelial matrix characteristic of RLE cells contains type IV collagen, laminin and heparan sulfate proteoglycan, but no type I collagen and little chondroitin sulfate proteoglycan.[32]

Cell adhesion assay Cell adhesion assay was based on the method previously described.[33] B16-BL6 cells in their exponential growth phase were labeled with 0.3 μCi/ml of ^{125}I-IUdR in culture medium (MEM containing 5% FBS). After incubation at 37°C for 24 h, cells were washed with warm PBS to remove unbound radiolabel, harvested by a short trypsinization (0.25% trypsin and 0.02% EDTA at 37°C for 1 min) and resuspended in cold serum-free MEM to form a single cell suspension. Radiolabeled tumor cells (2×10^4/well) were added to confluent monolayers of RLE cells or the cell matrix described above. Experiments were terminated by removing the nonadherent cells through vacuum aspiration. The cultures were washed three times with PBS to assure the removal of remaining nonadherent cells and the adherent cells were lysed with 50 μl of 0.1 *N* NaOH. The lysate was absorbed on cotton swabs and the radioactivity was measured by gamma counting. The binding capacity (number of cell bound/substrate) was given by: $A/B\times C$ where A is cpm of substrate-bound tumor cells, B is cpm of total tumor cells added in culture plates and C is the number of tumor cells added in culture plates. Time course studies were performed initially to determine the optimal time for tumor cell adhesion at 37°C and on the basis of the results, the assay time was standardized at 5 min.

Statistical analysis The statistical significance of differences between the groups was determined by applying Student's two-tailed *t* test.

RESULTS

Effect of SCM-chitin III on experimental lung metastasis in NK cell- or macrophage-deficient mice We have previously demonstrated that SCM-chitin III significantly inhibited the lung tumor colonization of B16-BL6 melanoma cells in experimental and spontaneous lung metastasis models, but CM-chitin did not.[26] To clarify the mechanisms responsible for the reduction of tumor metastasis by SCM-chitin III, we first examined the effect of SCM-chitin III on the function of NK cells or macrophages *in vivo*. SCM-chitin III was co-injected iv with B16-BL6 cells into untreated mice or mice pretreated 24 h earlier with anti-asialo GM1 antiserum or carrageenan and the lung tumor colonies were counted 14 days after tumor inoculation. As shown in Table I, a

Table I. Effect of Chitin Heparinoid on the Experimental Lung Metastases of B16-BL6 Melanoma in Anti-asialo GM1- and Carrageenan-treated Mice

Treatment of mice		Dose (μg/mouse)	No. of lung metastases on day 14 mean±SD (range)	$P^{a)}$
Untreated	PBS	—	76±8 (67–84)	
	SCM-chitin III	250	5±3 (2–9)	<0.001
	Heparin-Na	250	17±4 (13–22)	<0.001
	CM-chitin	250	75±8 (66–84)	
Anti-asialo GM1 (20 μl)	PBS	—	358±14 (344–376)	
	SCM-chitin III	250	117±12 (101–134)	<0.001
	Heparin-Na	250	153±16 (127–167)	<0.001
	CM-chitin	250	339±39 (287–391)	
Carrageenan (1.2 mg)	PBS	—	223±10 (215–239)	
	SCM-chitin III	250	26±6 (22–35)	<0.001
	Heparin-Na	250	18±6 (13–25)	<0.001
	CM-chitin	250	224±15 (208–244)	

B16-BL6 melanoma cells (2×10^4) were injected iv with or without chitin heparinoid into groups of untreated C57BL/6 mice or mice pretreated 24 h earlier with the indicated agent or antiserum. Lung tumor colonies were determined 14 days after tumor inoculation.
a) Compared with its respective untreated control (PBS) by Student's two-tailed *t* test.

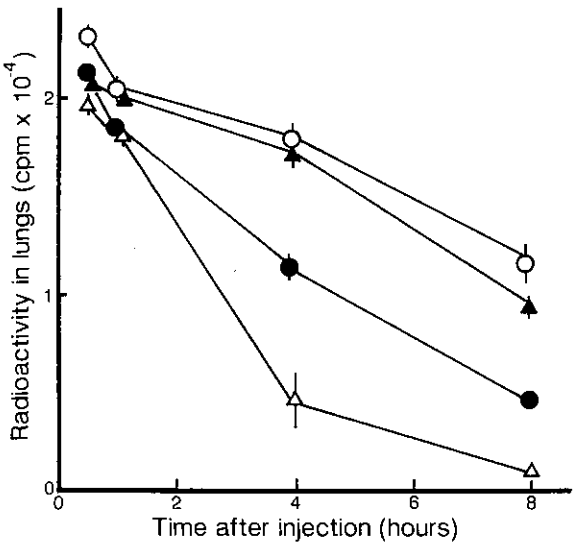

Fig. 1. Lung retention of ^{125}I-IUdR-labeled tumor cells co-injected with chitin heparinoid into mice. ^{125}I-IUdR-labeled B16-BL6 cells (3×10^4) were inoculated iv with PBS (○), 250 μg of SCM-chitin III (●), CM-chitin (▲), or heparin (△) into C57BL/6 mice. The radioactivity in the lungs was measured at various times after the injection.

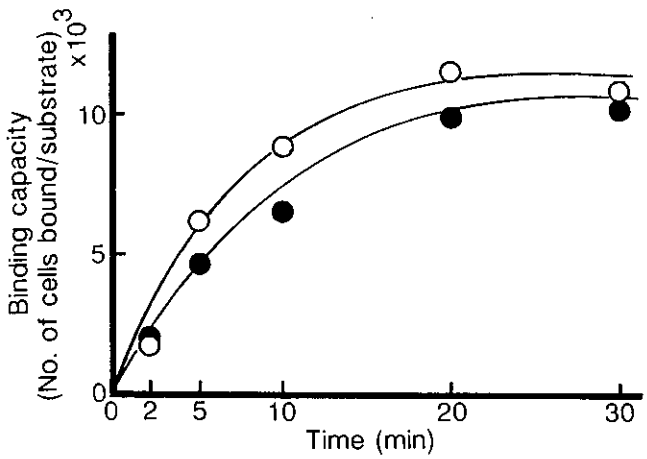

Fig. 2. Adhesion of tumor cells to RLE cell monolayer and the cell matrix. ^{125}I-IUdR-labeled B16-BL6 cells (2×10^4) were added to RLE cell monolayer (○), or the cell matrix (●). At various incubation times, non-adherent cells were washed away and the radioactivity of the remaining adhering cells was counted.

significant enhancement of lung tumor colonization was observed in antiasialo GM_1 antisera- or carrageenan-treated mice in comparison with that of untreated mice. The co-injection of 250 μg of SCM-chitin III with tumor cells as well as heparin caused a marked decrease of the number of lung tumor colonies in both untreated and pretreated mice.

We also observed that 500 μg/ml SCM-chitin III failed to activate peritoneal macrophages and alveolar macrophages *in vitro* and *in vivo* (data not shown).

Inhibition of tumor cell arrest in the lungs by SCM-chitin III We next studied the effect of SCM-chitin III on the arrest of tumor cells in the lungs. ^{125}I-IUdR-labeled B16-BL6 cells were injected with or without 250 μg of SCM-chitin III and the radioactivity in the lungs was monitored with a gamma counter at various times after tumor inoculation. The radioactivity of the lungs in SCM-chitin III-injected mice decreased more rapidly than that of untreated mice for 4–8 h after tumor inoculation (Fig. 1). A significant reduction of tumor cell arrest in the lungs was observed in heparin-injected mice. However, no difference was detected between untreated and CM-chitin-injected mice. There were no discernible differences between untreated and SCM-chitin III-treated mice in the arrest and retention of labeled tumor cells in liver, spleen, kidneys and blood after tumor injection (data not shown).

Effect of SCM-chitin III on tumor cell adhesion to endothelial cells and the subendothelial matrix Initial arrest of the circulating tumor cells in the target organ was characterized by endothelial cell-tumor cell contact.[4,5] To investigate the mechanism of the reduction of tumor cell arrest in the lungs by the co-injection of SCM-chitin III, we examined the effect of SCM-chitin III on the adhesion of tumor cells to confluent monolayers of endothelial cells *in vitro*. ^{125}I-IUdR-labeled B16-BL6 cells were added to lung endothelial (RLE) cell monolayers and incubated at 37°C for appropriate times. The adhesion of tumor cells to RLE cell monolayers was time-dependent and the maximal cell adhesion was noted after more than 10 min (Fig. 2). Table II shows that 500 μg/ml heparin was partially able to inhibit the tumor cell adhesion to RLE cell monolayers (15% of the control binding). However, neither SCM-chitin III nor CM-chitin showed any effect.

The adhesion of tumor cells to the subendothelial matrix following endothelial cell retraction may be an important step in stable arrest of tumor cells in target tissue.[8,9] We next studied the effect of SCM-chitin III on tumor cell adhesion to the subendothelial matrix. ^{125}I-IUdR-labeled tumor cells were added to the subendothelial matrix of RLE cells and the adherent tumor cells were counted. As shown in Fig. 2, suboptimal adhesion of tumor cells to the subendothelial matrix was observed on incubation for 5–10 min. Table II shows that 500 μg/ml SCM-chitin III inhibited the tumor cell adhesion to the subendothelial matrix (33% of the control

Table II. Inhibition of Tumor Cell Adhesion to RLE Cell Monolayer and the Cell Matrix

Treatment	Concentration (μg/ml)	Binding capacity (No. of cell bound ± SD/substrate)	
		cell monolayer	cell matrix
Untreated (PBS)	—	6577 ± 532	4562 ± 598
SCM-chitin III	500	6678 ± 142	3039 ± 129 (33%)[a]
CM-chitin	500	6394 ± 580	3960 ± 138
Heparin	500	5570 ± 181 (15%)	4309 ± 475

^{125}I-IUdR-labeled B16-BL6 melanoma cells (2×10^4) were added to confluent RLE cell monolayer or its cell matrix in the presence or absence of chitin heparinoid or heparin. After a 5-min incubation, non-adherent cells were washed away and attached cells were counted. The values in parentheses represent percent inhibition.
a) $P<0.02$.

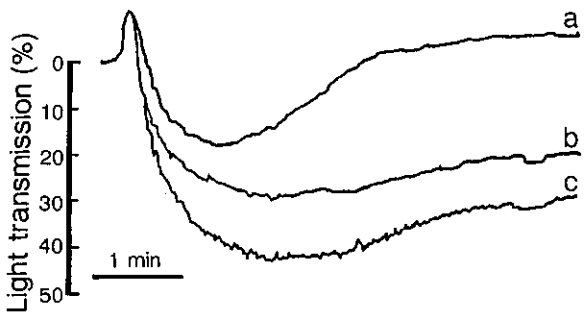

Fig. 3. Effect of chitin heparinoid on B16-BL6-induced platelet aggregation. Mouse PRP was pretreated with PBS (c), 100 μg/ml heparin (a), or 100 μg/ml SCM-chitin III (b) at 37°C for 1 min. Tumor cell suspension (5×10^5) was added to PRP and the percentage light transmission was monitored with a dual-channel aggregometer.

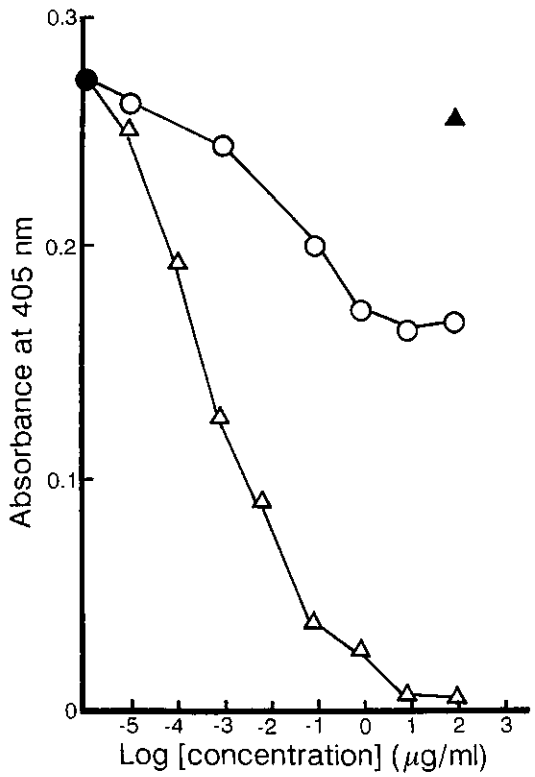

Fig. 4. Anticoagulant activity of chitin heparinoid in the presence of AT-III. Thrombin (6 mg/ml) was treated with various concentrations of SCM-chitin III (○), CM-chitin (▲), heparin (△), or PBS (●) at 37°C for 5 min in the presence of AT-III. Substrate solution was added, and the mixture was incubated at 37°C for 5 min. The enzyme reaction was stopped with sodium citrate and the absorbance of the mixture at 405 nm was measured.

binding). However, neither heparin nor CM-chitin showed any effect.

Effect of SCM-chitin III on the interaction of tumor cells with platelets Tumor cell adhesion to endothelial cells or subendothelial matrix is thought to be a weak association.[34] The development of platelet thrombus may result in the protection of tumor cells from host immune responses and mechanical forces which would cause the dislodgment of tumor cells, and consequently lead to stabilization of the tumor cell-endothelial cell contact.[35] To assess whether or not SCM-chitin III is able to inhibit platelet thrombus formation, we first tested the effect of SCM-chitin III on tumor cell-induced platelet aggregation *in vitro* (Fig. 3). An addition of B16-BL6 cells to heparinized platelet-rich plasma (PRP) elicited platelet aggregation (Fig. 3, c). A pretreatment of PRP with 100 μg/ml heparin caused a reduction in the magnitude of aggregation in response to B16-BL6 cells (Fig. 3, a). However, 100 μg/ml SCM-chitin III showed a slightly inhibitory effect on tumor cell-induced platelet aggregation (Fig. 3, b). We also observed that 100 μg/ml heparin inhibited the thrombin-induced platelet aggregation of washed platelet suspension (WPS), but SCM-chitin III did not (data not shown).

We further investigated the effect of SCM-chitin III on the blood coagulation reaction coincident with platelet aggregation. The anticoagulant activity was measured by using a chromogenic substrate kit. Thrombin was pretreated with SCM-chitin III in the presence of antithrombin III (AT-III). The mixture was then incubated with a substrate at 37°C for 5 min and the absorbance at 405 nm was measured. Figure 4 shows that the anticoagulant activity of heparin was dose-dependent and the maximal inhibition was noted at more than 10 μg/ml. On the other hand, the inhibition of thrombin activity by SCM-chitin III was only 30% of that by heparin at the highest concentration used in this study. CM-chitin showed no detectable anticoagulant activity.

DISCUSSION

Tumor cell arrest and extravasation are achieved by interaction of tumor cells with blood cells and/or vascular endothelial cells, and are characterized by the following steps[36]: (1) initial arrest with tumor cell-endothelial contact; (2) formation of tumor cell-associated platelet thrombus; (3) separation of endothelial cells and adhesion to subendothelial matrix; (4) penetration of subendothelial matrix. The development of platelet thrombus in the early stages of tumor cell arrest has been extensively studied. However, the functional role of platelet thrombus in tumor cell arrest and extravasation in the metastatic cascade remains unsettled.

We recently reported that sulfated chitin derivatives (SCM-chitin III) dramatically inhibited the lung metastasis of B16-BL6 cells in experimental and spontaneous lung metastasis models but CM-chitin had no effect.[26] We have demonstrated here that tumor cell arrest in lungs was significantly inhibited by iv coinjection of SCM-chitin III. However, SCM-chitin III showed little or no inhibition of tumor cell-induced platelet aggregation at concentrations ranging from 1 to 1000 μg/ml (Fig. 3 and data not shown), and exhibited low levels of antithrombogenic activity *in vitro* (Fig. 4). These results suggest that inhibition of tumor cell arrest in lungs by SCM-chitin III may not be due to interference with platelet thrombus formation, which is caused by tumor cell-induced platelet aggregation and activation of the coagulation cascade. The mechanism of the slight inhibition of platelet aggregation by SCM-chitin III is still unknown. In contrast, the anticoagulant heparin potently inhibited both tumor cell- and thrombin-induced platelet aggregation. B16 melanoma cells have been shown to activate procoagulant factor X and consequently to lead to the generation of thrombin.[37] Therefore, the reduction of lung tumor colonization and tumor cell arrest by heparin may be in part due to the prevention of platelet thrombus formation.

Tumor cell invasion of the subendothelial matrix following endothelial cell retraction may be important for the stabilization of tumor cell arrest at a secondary site. Three sequential steps of tumor cell invasion of the subendothelial matrix were proposed[38]: (a) adhesion of tumor cells via cell surface receptors to matrix components such as fibronectin and laminin, (b) local degradation of the matrix by a tumor cell-derived proteolytic enzyme, (c) tumor cell migration into the region of the matrix modified by proteolysis. Moreover, Liotta[38] suggested that interaction of laminin with laminin receptors on the tumor cell surface is important for successful lung colonization by tumor cells. In this study, we showed that SCM-chitin III (500 μg/ml) inhibited tumor cell adhesion to the subendothelial matrix (including laminin), although it was unable to inhibit the adhesion of tumor cell to endothelial cell monolayers. SCM-chitin III did not affect tumor cell adhesion to the subendothelial matrix at concentrations of less than 100 μg/ml (data not shown). Recently, we found that SCM-chitin III significantly inhibited tumor cell adhesion, migration to laminin, proteolytic degradation of basement membrane components and invasion of reconstituted basement membrane Matrigel *in vitro* in a dose-dependent manner.[39] These results suggest that SCM-chitin III may inhibit tumor cell arrest in the lungs through the prevention of some steps in the invasive process including tumor cell adhesion to subendothelial matrix following endothelial cell retraction.

SCM-chitin III had no direct cytotoxic effect on B16-BL6 cells or endothelial cells *in vitro*, nor did it affect their cell growth at concentrations ranging from 0.1 to 500 μg/ml (data not shown). It seems likely that SCM-chitin III is not concerned with NK cell- or macrophage-mediated inhibition of tumor metastasis (Table I). Other biological properties of SCM-chitin III in relation to the metastatic cascade are now under investigation.

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