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SELECTIVE SULFATION OF CHITIN DERIVATIVES FOR BIOMEDICAL FUNCTIONS

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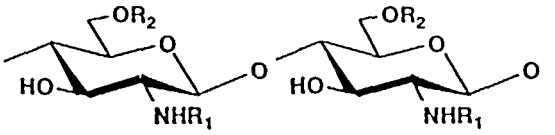
ABSTRACT

The highest antithrombogenic activity was achieved by the *N*- and *O*-sulfation of partially *N*-deacetylated (70%) 6-*O*-carboxymethyl (0.60)-chitin (SCM-DAC-70) among various modified chitin derivatives. But a *O*-sulfated 6-*O*-carobxymethyl-chitin (SCM-chitin) was a quite inert heparinoid for the inhibition of blood clotting. It was also suggested that the distribution of *N*-sulfate and *N*-acetyl groups on the C-2 position

of glucosamine residue might be essential to the selective adsorption of SCM-DAC-70 to antithrombin-III (AT-III) to inhibit thrombin activity. Kinetic evaluations demonstrated the noncompetitive inhibition of thrombin activity by SCM-DAC-70 and competitive inhibition of thrombin by SCM-DAC-70-AT-III complex. We were unable to find such a specific inhibition by the SCM-DAC-70 complex with blood clotting factors other than AT-III. SCM-chitin was found to inhibit the metastasis of cancer cells at about 80–85% without any side effect different from those of heparin. It was suggested that the inhibition of metastasis of B16-BL6 melanoma cells was achieved by bifunctional inhibitions for heparanase and type IV collagenase without growth inhibition of cancer cells.

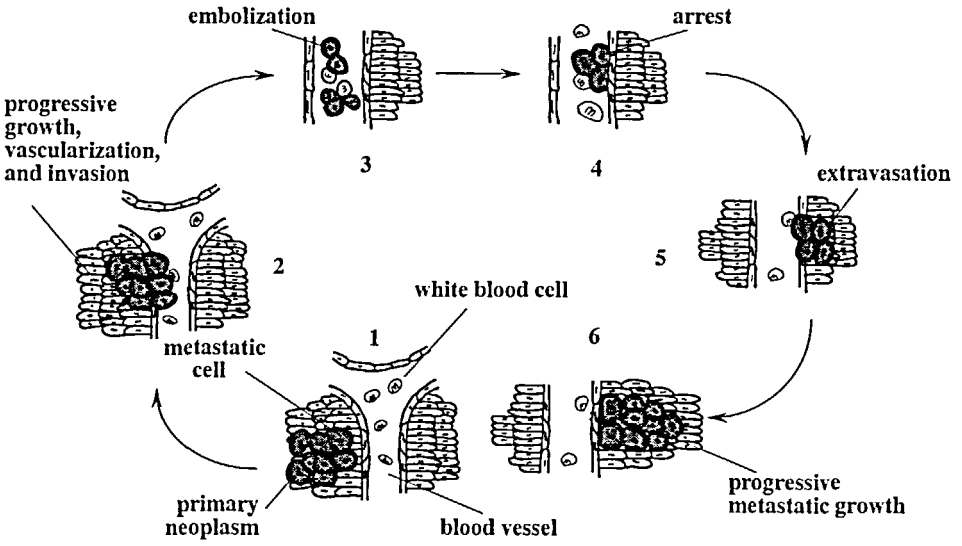
INTRODUCTION

Chitin, a natural abundant mucopolysaccharide, is known to be the β-1,4-glycan of *N*-acetyl-*D*-glucosamine (GlcNAc). Chitin is also known to be a biodegradable [1] and nontoxic [2] polysaccharide. Chitosan is supplied as a natural product or chemical derivative of chitin in *N*-deacetylated form, as shown in Fig. 1. As the fundamental chemical structure of chitin is close to that of heparin, there have been several studies on the preparation of chitin heparinoids [3–8]. Muzzarelli et al. reported the preparation of chitin heparinoid by the sulfation of *N*-(carboxymethyl)chitosan as a new type of heparinoid derived from partially deacetylated chitin (degree of deacetylation, 58%). The advantage of their compound was reported to be the lack of any adverse effect on cellular structure [9] rather than their antithrombogenic actions compared with sulfated chitosan. However, the toxic behaviors of chitin heparinoids, such as hypertension, have not been established. The introduction of the *O*-carboxymethyl group to sulfated dextran is a useful



$R_1 = COCH_3$	$R_2 = H$	Chitin
$= COCH_3$	$= H, CH_2COOH$	CM-chitin
$= COCH_3$	$= H, SO_3H$	S-chitin
$= COCH_3 : 0.3$ $H, SO_3H : 0.7$	$= CH_2COOH, SO_3H$	SCM-chitin
$= COCH_3 : 0.3$	$= H, SO_3H$	S-chitosan
$H, SO_3H : 0.7$	$= CH_2COOH, SO_3H$	SCM-DAC-70

FIG. 1. Chemical structures of chitin derivatives.



SCHEME 1. General scheme of metastasis of cancer cells.

method to cancel hypertension following intravenous injection [10]. Though SCM-chitin was observed to interact with thrombin rather than with antithrombin III (AT-III) [11], a partial *N*-deacetylation reaction (70%) and, then, *N*- and *O*-sulfations were used to prepare the chitin heparinoid with advanced properties including low toxicity [12]. The degree of interaction became higher with AT-III than with thrombin by *N*- *O*-sulfations of 6-*O*-CM-chitin (SCM-DAC-70) and it was enhanced again by further sulfation of the C-3 hydroxyl group [13]. An equivalent mole of SCM-DAC-70 was shown by gel permeation chromatography (GPC) to be enough to inhibit thrombin activity in the presence of AT-III, although three equivalent moles of SCM-DAC-70 were found to be adsorbed to AT-III both by GPC assay and fluorescence titration. As the full range of inhibition of blood clotting by SCM-DAC-70 was almost similar level as that of heparin in the presence of AT-III, the inhibitory activity of SCM-DAC-70 molecule seemed to be one-third that of heparin, generally.

Since SCM-chitin, the *N*-acetylated form of SCM-DAC-70, as shown in Fig. 1, is almost inactive in suppressing blood clotting and platelet coagulation (1/1,000–1/10,000 that of heparin), including a poor degree of mouse peritoneal macrophage activation, SCM-chitin was expected to reflect another side of heparin functions, such as antimetastasis of carcinomas by the inhibition of hydrolytic enzyme activity in cancer cells [14–18]. The more sulfated CM-chitin was found to be a better inhibitor of heparanase and type IV collagenase which were secreted from cancer cells on metastasis, although heparin inhibited only heparanase activity and showed side effects such as vein bleeding after repeated administrations. As the denaturation of lactoferrin was observed only by the adsorption of C-3 sulfated chitin, the denaturation of enzymes was suggested to be the main factor in the inhibition of enzymatic activities on the antimetastasis of carcinoma as shown in Scheme 1.

EXPERIMENTAL

Materials. Chitin was prepared from Queen Crab shells according to the method of Hackman [19] and powdered to 45–60 mesh before use. Reagents of reagent grade were obtained from Wako Pure Chemical Industries Ltd. and used without further purification. Bovine fibrinogen was purchased from Seikagaku Kogyo Co. Ltd. as a 95% clottable one (Lot 100). Heparin sodium salt of 159 units/mg (Lot M9R8164) was purchased from Nakarai Chemical Industries Ltd. Bovine thrombin was supplied from Mochida Pharmaceutical Co. Ltd. as a 1000 NIH units per vial (Lot 3B428). Bovine Plasma AT-III was obtained from Boehringer Mannheim GmbH (Lot 1443301). The protein concentrations were determined by their absorbance at 280 nm using absorption coefficients of 6.0 for AT-III [20], 17.9 for thrombin [21], and 1.506 for fibrinogen [22]. The molecular weights of proteins used in this study were 56,000 for AT-III and 37,000 for thrombin. The synthetic chromogenic substrate from thrombin, Boc-Val-Pro-Arg-MCA [23], was purchased from Peptide Institute Inc. Ltd. Sephadex G-100(Fine) and the gel filtration calibration kit for molecular weight determination were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

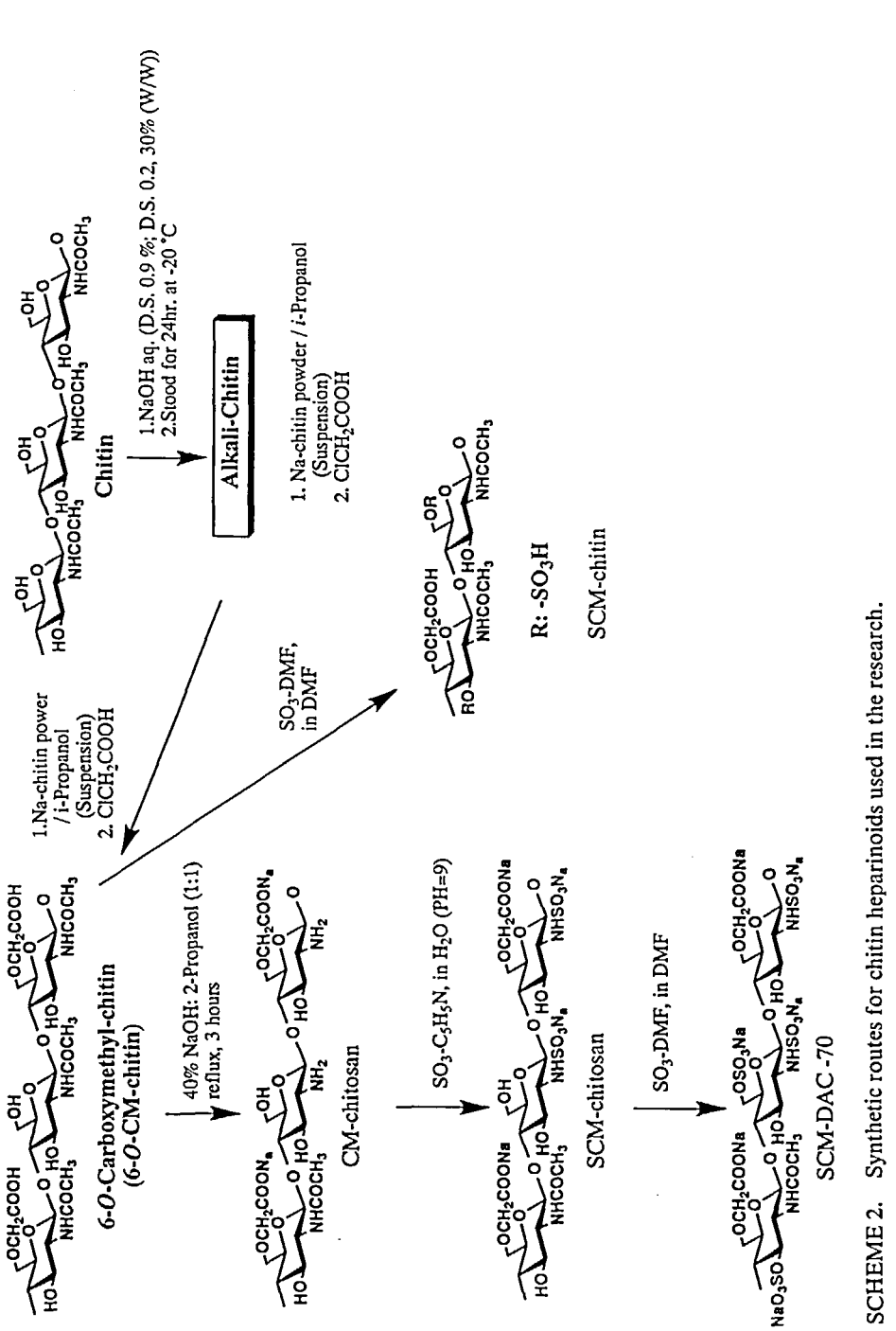
Carboxymethyl(CM)-chitin. 6-O-CM-chitin was prepared according to the method described previously (Scheme 2), and the degree of substitution at the C-6 hydroxyl group was 0.60 based on elemental analysis, potentiometric titration, and the IR spectrum [21]. The molecular weight was assumed from viscosity to be 63,000, using the viscosity equation proposed by Kaneko et al. [24].

Partial N-Deacetylation of CM-chitin. The *N*-deacetylation of CM-chitin was carried out in a 40% NaOH/isopropanol system at the reflux temperature for 5 hours to prepare CM-chitosan (70% of *N*-deacetylation).

Sulfation of CM-chitosan. Selective *N*-sulfation of CM-chitosan was carried out by the method of Whistler and Kosic [8]. In brief, 7.0 g CM-chitosan (degree of carboxymethylation, 0.60; degree of deacetylation, ~70%) was dissolved in water and the sulfating reagent (sulfur trioxide/pyridine, 3 equivalent mol/NH_2) was added dropwise for 3 hours at pH 9–10 (adjusted by 4M NaOH aqueous solution). The reaction mixture was dialyzed against deionized water to remove impurities and then lyophilized. Yield: 8.1 g. Little free amino group was observed by the ninhydrin test. The *O*-sulfations of CM-chitin and *N*-sulfated CM-chitosan were successively followed by the use of the SO_3/DMF system [9]. Chemical and physicochemical properties of heparinoids are listed in Table 1 together with those of *N*-sulfated chitosan (S-DAC).

Estimation of the Degree of Sulfation. The degree of sulfation was estimated by the quantitative analysis of sulfur content in the products according to a method reported previously [11].

Molecular Weight. The molecular weights of chitin heparinoids were estimated from viscosity measurements using a Ubbelohde-type viscometer by applying the proposed viscosity equation for heparin [25].



SCHEME 2. Synthetic routes for chitin heparinoids used in the research.

TABLE 1. Analytical Data for Chitin Heparinoids

Sample	S, %[a]	$-SO_3^-$[b]	$-COO^-$[b]	MW × 10^4[c]
S-chitin II	8.54	0.73		1.3
III	10.22	0.87		2.4
Sulfated chitin				
SCM-chitin I	1.43	0.12	0.8	1.8
II	6.70	0.57	0.4	1.0
III	7.66	0.65	0.56	2.4
Sulfated deacetyl chitin		NHAc[b]		
S-chitosan	11.89	1.32 (0.30)	0.6	4.2
SCM-chitosan	5.90	0.68 (0.30)	0.6	4.7
CM-chitin [*O*-(carboxymethyl) chitin]	0	0	0.8	6.3

[a]Degree (%) of sulfation.
[b]Number of functional group/GlcNAc residue.
[c]Average molecular weights were determined by viscosity equations for heparin.

Clotting Assay. The anticoagulant activity of heparinoids was measured with the use of bovine fibrinogen in the presence of an equivalent amount of AT-III and thrombin [11].

Amidolytic Methods to Estimate Anticoagulant Activity

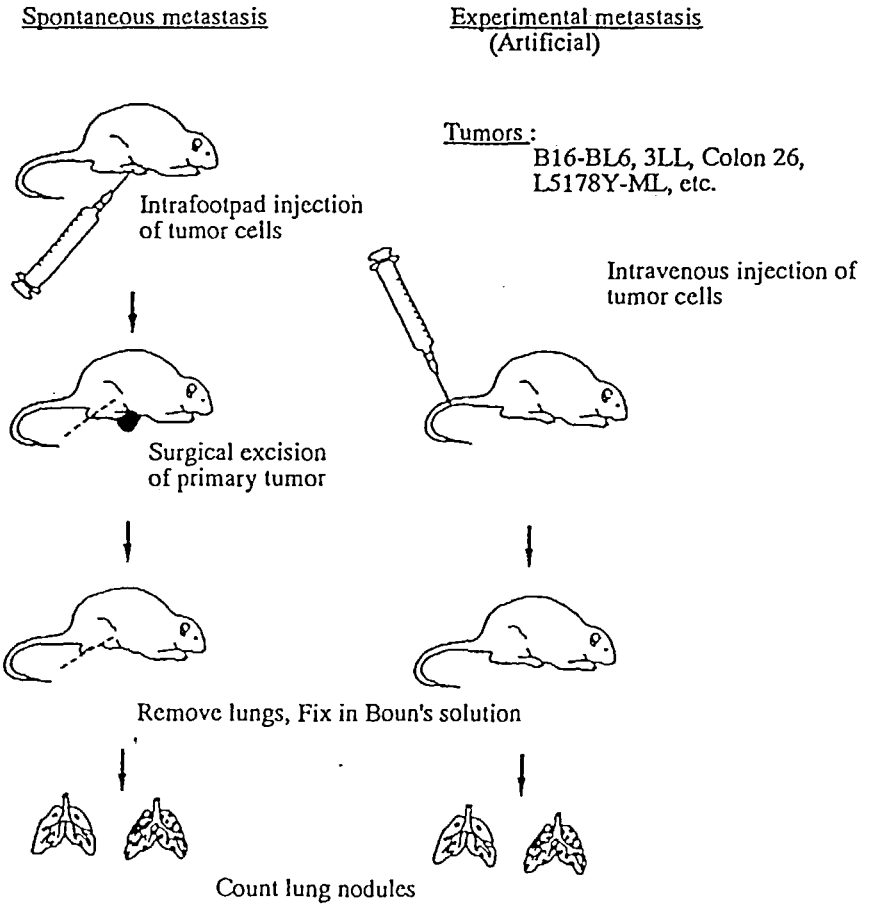
Screening Assay. The assay was performed substantially as described by Uchiyama and Nagasawa [26]. 100 μL heparin or heparinoids solutions (0–10 μg/mL Tris-HCl buffer, pH 7.4) was incubated for 1 minute at 30°C. 50 μL thrombin (1.0 NIH unit/mL Tris-HCl buffer, pH 7.4) was added. 250 μL substrate solution (Boc-Val-Pro-Arg-MCA, 43 μM) was added after a minute of incubation, and the fluorescence intensity was measured under excitation at 380 nm using a Hitachi Spectrofluorophotometer Model MPF-2. Kinetic analyses were also made under the condition mentioned above and calculated by their Lineweaver–Burk plots.

Toxicity Assay for Animal. Inbred C57BL/6 female mice, 7 to 10 weeks of age, were injected intravenously with 0.1 mL polysaccharide saline solution (10 mg/mL), and the weights of spleen, lung, and liver were measured after 3 days.

Gel Filtration Chromatography. The affinity of heparinoids with AT-III or thrombin was investigated by gel chromatography using a Sephadex G-100 (Fine) column (1.0 × 18.0 cm). The mixture was allowed to stand for 1 minute at 25°C before loading, and it was eluted with a 0.1 M NaCl aqueous solution at a rate of 0.3 mL/min while monitoring by optical density at 220 nm. The apparent molecular weights of the complexes were estimated by means of a calibration curve using ribonucrease A (13.7K), chymotrypsinogen A (25K), ovalbumin (43K), and bovine serum albumin (67K) [12].

Tumor Metastasis Assay (14). Experimental pulmonary metastasis was assessed by means of tumor cell injection into the lateral tail veins of mice. C57BL/6 mice were injected intravenously (i.v.) with B16-BL6 melanoma (5×10^4) admixed with or without chitin derivatives. The mice were killed 14 days after tumor inoculation, and the number of lung tumor colonies was counted. In spontaneous pulmonary metastasis assay, mice were inoculated subcutaneously with B16-BL6 cells (5×10^5) in the right hind footpad. The primary tumors were surgically removed on Day 21 after tumor inoculation. The injection of chitin derivatives was performed on various days before or after the amputation. Mice were killed 14 days after the amputation, and the number of lung tumor colonies was counted. An outline of experiment is shown in Scheme 3.

Tumor Invasion and Migration Assay [17]. The invasive activity of tumor cells was investigated in a Transwell cells culture chamber. The upper surface of the



SCHEME 3. Outline of antimetastatic assays.

filters (8 μm pore size) in the culture chambers was precoated with basement membrane Matrigel (5 μg) and the lower surface was coated with 5 μg fibronectin or laminin. The filters thus prepared were designated Matrigel/fibronectin- and Matrigel/laminin-coated filters, respectively. Cell suspensions of B16-BL6 (2×10^5/100 mL) were added to the upper compartment in the presence or absence of chitin derivatives and incubated at 37°C for 6 hours. The cells that had migrated through the filter to the lower surface were manually counted. Tumor cell migration along a gradient of substratum-bound fibronectin or laminin was assayed by using filters precoated with fibronectin or laminin on the lower surface. The following procedures were the same as those of the invasion assay.

Heparanase Inhibition and Type IV Collagenolysis Assay. Heparanase and type IV collagenase activities were assessed by a method described previously [17]. Briefly, ^{3}H-labeled Heparin sulfate (HS) was incubated at 37°C with partially purified heparanase in the presence or absence of chitin derivatives. [^{3}H] HS degradation products in the supernatant were analyzed by size-exclusion chromatography. Heparanase activity was determined by measuring the radioactivity of each fraction. ^{3}H-labeled type IV collagen was incubated at 37°C for 48 hours in the presence or absence of chitin derivatives. Type IV collagenolytic activity was calculated from the radioactivity in the supernatant.

RESULTS AND DISCUSSION

Preparation of heparinoids: *N*-Sulfation of chitosan is achieved smoothly by the use of SO_3/pyridine under mild condition. The sulfation of the remaining C-6 hydroxyl group of 6-O-CM-chitin (degree of substitution = 0.60) is not as easy because of the loose crystalline structure, although that of chitin is much easier because of its rigid crystalline structure as shown in the case of carboxymethylation (Scheme 1). But 3,6-*O*-sulfations were carried out smoothly by applying the SO_3/DMF or SO_3/DMSO system. The remaining C-6 hydroxyl group of 6-O-CM-chitin is required to tritylate for the specific sulfation of the C-3 hydroxyl group. The molecular weight of heparinoids is also a serious problem because of the rather drastic condition required to modify the C-3 hydroxyl group compared to the C-6 hydroxyl group.

Heparinoid Activity. When the inhibitor actions were analyzed by the transformation of fibrinogen to fibrin in the presence of AT-III, the anticoagulant activity by SCM-DAC-70 was the highest among the chitin heparinoids and almost showed a similar degree of inhibition, as shown in Fig. 2. The anticoagulant activity by SCM-chitosan (only the amino group of 6-O-CM-DAC-70 was sulfated) was far away from that by SCM-DAC-70, suggesting the serious contribution of the *O*-sulfated group to full anticoagulant activity, as shown in Fig. 3. Kinetic analyses of the inhibitory action using a synthetic substrate demonstrated noncompetitive inhibition by SCM-DAC-70 on direct interaction with thrombin ($K_i = 9.26 \times 10^{-8}$ M) and competitive inhibition with AT-III ($K_i = 3.33 \times 10^{-8}$ M) as well as that with heparin (as shown in Figs. 4a and 4b), suggesting an inhibition mechanism similar to that of heparin.

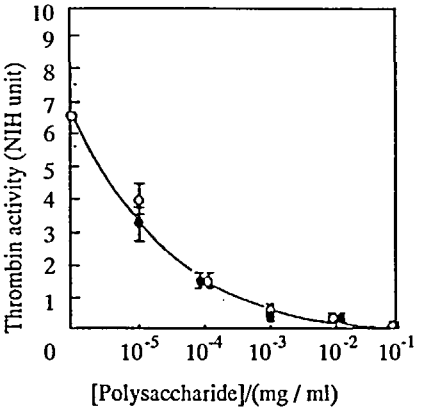


FIG. 2. Anticoagulant activity of SCM-DAC-70 (○) and heparin (●) using bovine fibrinogen as a substrate in the presence of antithrombin-III (AT-III).

Size of Complexes between SCM-DAC-70 and AT-III or Thrombin. Sephadex G-100 gel chromatography was applied to investigate the affinity of SCM-DAC-70 to AT-III or thrombin as shown in Figs. 5a and 5b. Because the molecular weight of thrombin is about 40K, that of AT-III is 60K, and that of SCM-DAC is 14K, the smaller affinity of SCM-DAC-70 to thrombin than to AT-III was clearly shown. The maximum heparinoid adsorption was estimated to be 5 moles for thrombin and 3 for AT-III. However, an equivalent mole of SCM-DAC-70 to AT-III was found to be enough to reach a maximum degree of inhibition [27]. Another two equivalent moles of SCM-DAC-70 were suggested to be nonspecific adsorption to AT-III. As an almost similar degree of inhibition to heparin was

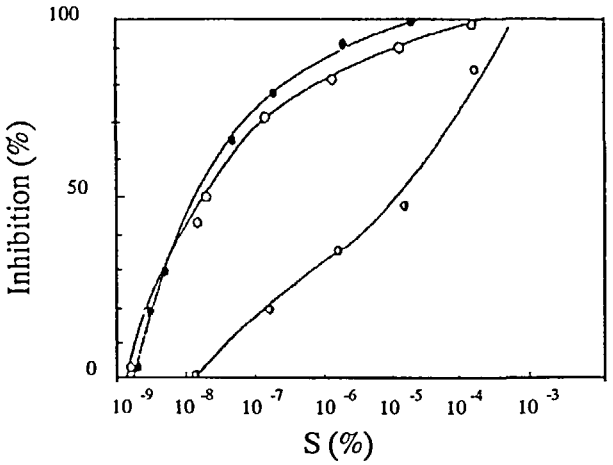
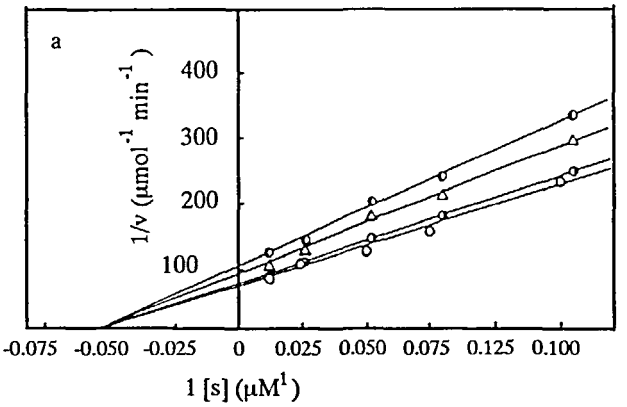


FIG. 3. Antithrombogenic activity of SCM-chitosan (◑), SCM-DAC-70 (○), and heparin (●) in the presence of AT-III.



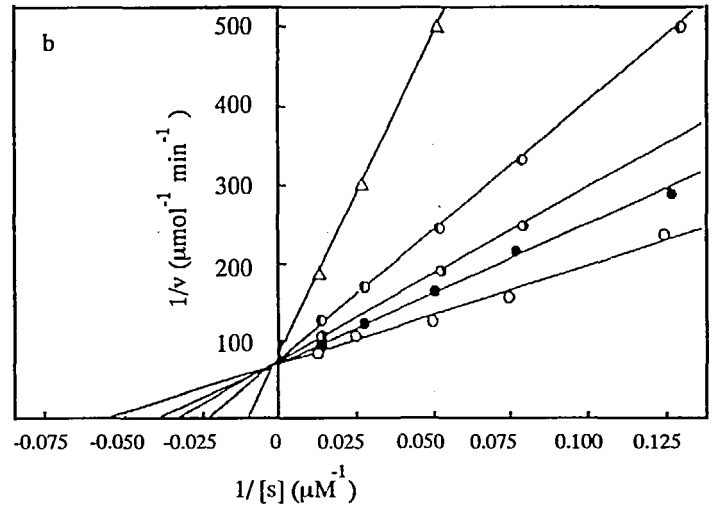


FIG. 4a. Effect of polysaccharides on the hydrolytic action of thrombin ([Thrombin] = 0.025 NIH U/mL, Boc-Val-Pro-Arg-MCA as a substrate, pH 7.4 and 30°C). (○) Without inhibitor, (◑) SCM-chitosan, (◐) SCM-DAC-70, and (△) heparin.

FIG. 4b. Effect of AT-III and heparinoides complexes on the hydrolytic action of thrombin (same conditions as in Fig. 4a). (○) without inhibitor, (●) only AT-III, (◑ AT-III and SCM-chitosan, (◐) AT-III and SCM-DAC-70, and (△) AT-III and heparin.

shown by SCM-DAC-70 through the inhibitions of fibrinogen transformation and the hydrolytic activity of the synthetic substrate, the unit activity of SCM-DAC-70 would be 1/3 that of heparin for the inhibition of thrombin activity by AT-III complex, as seen in Fig. 6. The maximum adsorption number of SCM-DAC-70 to AT-III was also confirmed by fluorescence titration [27]. The specificity of SCM-DAC-70 was investigated by applying other coagulation factors and demonstrated to be of the highest affinity for AT-III.

Hypertension Following the Intravenous Injection of Heparinoids. The weights of mice organs were measured after 3 days of intravenous injection of chitin heparinoids. Little increase of organ weight was observed for the administration of carboxymethylated chitin heparinoid, including body weight. There was little increase by *N*-sulfonated DAC-70 (S-chitosan). The biodegradability was almost suppressed by the sulfonation of chitin derivatives using lysozyme as a temporal marker of biodegradability.

Effect of Chitin Derivatives on Experimental and Spontaneous Lung Metastasis of B16-BL6 Melanoma. In the experimental lung metastasis assay, the number of lung tumor colonies was significantly reduced by co-injection of B16-BL6 with 250 μg of 6-O-sulfated chitin (S-chitin) with a high degree of sulfation, as was also the case with heparin (Experiment I of Table 2). *N*-Deacetylated chitin derivatives,

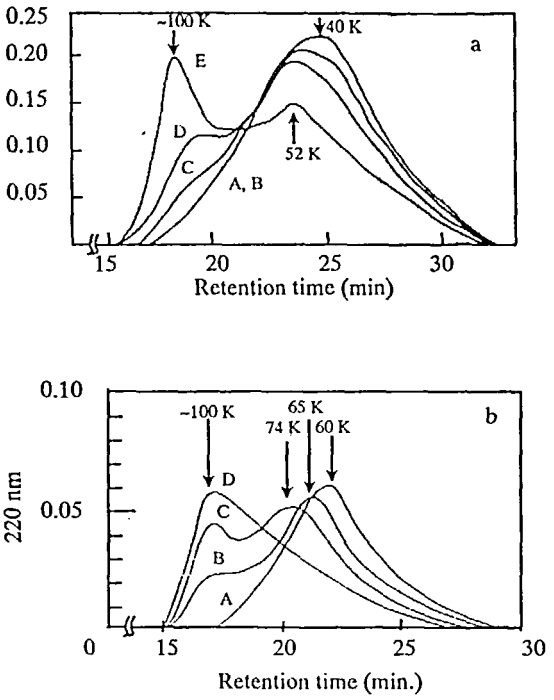


FIG. 5a. Sephadex (G-100) gel filtration chromatography of thrombin and SCM-DAC-70. 150 μL of protein solutions was loaded on the column and eluted by 0.1 M NaCl solution with 0.30 mL/min. The apparent molecular weights were determined by means of calibration curves using ribonulease A (13.7 K), chymotrypsinogen A (25 K), ovalbumin (43 K), and bovine serum albumin (67 K). The ratios indicated were (A) 0, (B) 0.63, (C) 6.3, (D) 63.0, and (E) 630.

FIG. 5b. Sephadex (G-100) gel filtration chromatography of AT-III and SCM-DAC-70. The method of Fig. 5a was followed. The ratios indicated were (A) 0, (B) 0.58, (C) 5.8, and (D) 58.0.

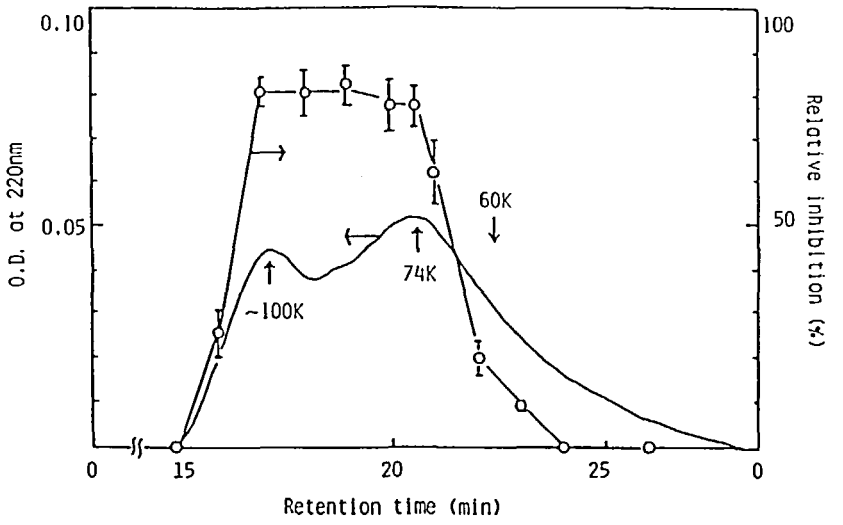


FIG. 6. Effective ratio of AT-III and SCM-DAC-70 to inhibit thrombin activity (Sephadex G-100 gel chromatography). (O) Relative inhibition of thrombin activity by the complex.

TABLE 2. Effect of Chitin Heparinoids on Experimental Lung Metastasis by Intravenous Injection of B16-BL6 Melanoma Cells[a]

Administered i.v. with	Dose, μg/mouse	Number of lung metastasis on Day 14		P^b
		Mean ± SD	Range	
Experiment I:				
Untreated (PBS)	–	124 ± 34	92–165	
Heparin-Na	250	8 ± 4	4–12	<.001
S-chitin II (8.54)[c]	250	52 ± 7	47–65	<.005
III (10.22)	250	15 ± 7	7–24	<.001
S-chitosan (11.89)	250	103 ± 9	89–111	
SCM-chitosan (5.90)	250	134 ± 23	96–156	
Experiment II:				
Untreated (PBS)	–	62 ± 14	44–78	
SCM-chitin I (1.43)	250	58 ± 18	40–90	
II (6.70)	250	30 ± 10	20–42	<.005
III (7.66)	250	8 ± 2	4–12	<.001

[a]Five C57BL/6 mice per group were infected with B16-BL6 (5×10^4) with or without chitin heparinoids. Mice were killed 2 weeks after tumor inoculation, and lung colonies were measured.

[b]Compared with untreated control by Student's two-tailed *t*-test.

[c]The degree (%) of sulfation.

S- and SCM-chitosan, however, were not effective for the inhibition of experimental lung metastasis, although they were fully 6-*O*- and *N*-sulfated derivatives. The antimetastastic activity of SCM-chitin carrying various amounts of sulfate and carboxymethyl groups at the C-6 position was also examined (Experiment II of Table 2). A higher degree of sulfation resulted in a smaller number of tumor colonizations. The highest degree of inhibition was observed when 250 μg SCM-chitin III (highest degree of sulfation in this study) was co-injected with tumor cells, although it had a lower degree of sulfation than S-chitin III. The therapeutic effect of S-chitin III was investigated for spontaneous lung metastasis in mice. Experiment I of Table 3 shows that seven intermittent i.v. administrations of SCM-chitin III before amputation of primary tumors caused a remarkable decrease of lung tumor colonies in a dose-dependent manner. In particular, intermittent administrations of 200 μg SCM-chitin III showed about a fivefold greater effect than those of heparin in the inhibition of lung metastasis. S-Chitin II (smaller sulfur content than S-chitin III) was also more effective than heparin, whereas CM-chitin did not show any inhibitory effect. SCM-

TABLE 3. Effect of Chitin Heparinoids on Spontaneous Lung Metastasis by Intrafootpad Injection of B16-BL6 Melanoma Cells

Administered i.v.	Dose, μg/mouse	Number of lung metastasis on Day 14		P^b
		Mean ± SD	Range	
Experiment I				
Untreated (PBS)	—	66 ± 22	35–84	
SCM-chitin III on Days				
7,9,11,3,15,17,19	200	4 ± 3	0–8	<.001
	100	11 ± 8	4–22	<.005
	50	36 ± 26	16–71	
S-chitin II	200	11 ± 8	6–23	<.005
CM-chitin	200	41 ± 18	25–72	
Heparin-Na	200	19 ± 16	8–43	<.01
Experiment II				
Untreated (PBS)	—	52 ± 10	39–65	
SCM-chitin I on Days				
22,24,26,28,30,32,34	200	62 ± 18	41–80	
II	200	14 ± 7	7–24	<.001
III	200	9 ± 6	1–17	<.001
S-chitin	200	14 ± 8	7–26	<.001
CM-chitin	200	54 ± 22	34–88	
SCM-chitosan	200	74 ± 7	68–83	
Heparin-Na	200	24 ± 7	17–34	<.001

[a]Five C57BL/6 mice per group were administered i.v. with chitin heparinoids at the indicated times after tumor inoculation. Primary tumors were surgically removed on Day 21, and mice were killed 2 weeks after tumor excision.

[b]Compared with untreated control by Student's two-tailed *t*-test.

chitin III was also effective when it was administrated seven times after the amputation of primary tumors. These results suggest that the 3- or 6-O-sulfate group of GlcNAc residue is essential for the inhibition of tumor metastasis.

Inhibition of Tumor Cell Arrest in Lungs by SCM-chitin III. The effect of SCM-chitin III on tumor cell arrest was investigated to analyze the mechanism of tumor antimetastasis. Lung retention of ^{125}I-labeled B16-BL6 cells was reduced more rapidly for 4–8 hours after tumor inoculation by co-injection of SCM-chitin III than that of untreated mice, as shown in Fig. 7. A significant reduction of tumor cell arrest in lung was observed in heparin-injected mice. However, little difference was detected between untreated mice and SCM-chitin-injected mice.

Inhibition of Tumor Cell Invasion by SCM-chitin III. As SCM-chitin III showed a weak influence on tumor cell-induced platelet aggregation and on blood coagulation, these properties might stimulate the early phase of tumor cell arrest in blood vessels. Also, inhibition of tumor cell invasion to basement membrane following endothelial cell retraction might be significant in the stabilization of tumor cell arrest at the secondary site. Thus, the effect of SCM-chitin III on tumor cell invasion of basement membrane was investigated by Transwell chamber assay as listed in Table 4. SCM-chitin III could potentially inhibit tumor cell invasion through Matrigel/fibronectin- and Matrigel/laminin-coated filters and of heparin, whereas little inhibition was observed in the presence of CM-chitin or SCM-chitosan.

To clarify the mechanism of inhibition of tumor cell invasion by SCM-chitin III, the influence of heparinoid on tumor cell migration through laminin and on the matrix degrading enzymes (heparanase and type IV collagenase) were investigated from tumor cells in vitro. The inhibition of haptotactic migration of B16-BL6 cells through laminin-coated filters is shown in Fig. 8 in a SCM-chitin III concentration-dependent manner. CM-chitin doesn't show any inhibitory effect at a concentration of 500 μg/mL. SCM-chitin III inhibits HS degradation by partially purified hepar-

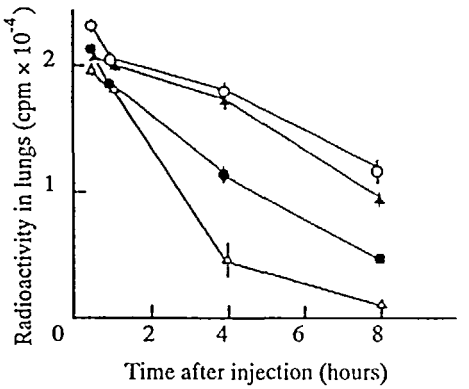


FIG. 7. Lung retention of ^{125}I-IUdR-labeled tumor cells co-injected with chitin heparinoid into mice. ^{125}I-IUdR-labeled B16-BL6 cells (3×10^4) were inoculated IV with PBS (○), 250 μg SCM-chitin III (●), CM-chitin (▲), and heparin (△) into C57BL/6 mice. The radioactivity in the lungs was measured at various times after the injection.

TABLE 4. Inhibition of Tumor Cell Invasion on Matrigel/Fibronection-or Matrigel/Laminin-Coated Filters by Chitin Derivatives[a]

Treatment	Number of invaded cells	
	Matrigel/ fibronectin	Matrigel/ laminin
Untreated	115 ± 11[b]	73 ± 5
SCM-chitin:		
I (1.43)[c]	116 ± 11	47 ± 7
II (6.70)	58 ± 10[d]	9 ± 5[d]
III (7.66)	46 ± 8[d]	7 ± 2[d]
CM-chitin	111 ± 9	75 ± 9
SCM-chitosan (5.90)	107 ± 19	68 ± 9
Heparin	52 ± 5[d]	45 ± 6[d]

[a]B16-BL6 cells (2×10^5) were seeded with or without chitin derivatives into the upper compartment of Transwell chambers. Filters were precoated with either fibronectin (5 μg) or laminin (5 *mg*) on the upper surface. After 8 hours of incubation, the invading cells on the lower surface were visually counted.

[b]Mean ± SD.

[c]Numbers in parentheses are the degree (percentage) of sulfation.

[d]$P < .001$ by Student's two-tailed *t*-test.

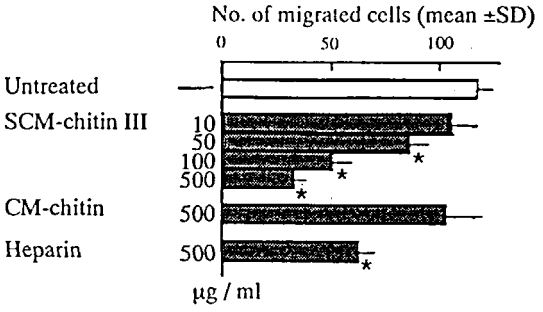


FIG. 8. Dose-response of SCM-chitin III on haptotactic migration of tumor cells. B16-BL6 cells (2×10^5) were seeded on the filters precoated on the lower surface with 5 μg laminin. Various concentrations of SCM-chitin III were added into the lower compartment of Transwell chambers. After a 6-hour incubation, the migrant cells on the lower surface were visually counted. *$P > 0.001$ compared with the control by Student's two-tailed *t* test.

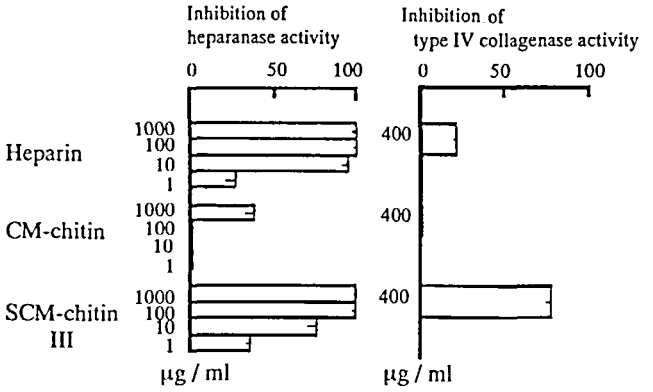


FIG. 9. Effect of sulfated chitin derivatives on heparanase and type IV collagenase activities by B16-BL6 melanoma cells. *N*-[^{3}H]-Acetylated HS (5 μg) was incubated at 37°C for 3 hours with 26.7 μg partially purified heparanase in 50 μL 0.2 M sodium acetate buffer, pH 5.6, in the presence or absence of chitin derivatives. ^{3}H-Labeled type IV collagen film was treated at 37°C for 3 hours with or without chitin derivatives and incubated with B16-BL6 cells (5×10^4) suspended in 400 μL of a medium containing 0.5% BAS for 48 hours.

TABLE 5. Biological Activities of SCM-Chitin III and Heparin[a]

	SCM-chitin III	Heparin
Carbohydrate composition	*N*-Acetylglucosamine	Glucosamine + uronic acid
Tumor metastasis in mice:		
Experimental	↓	↓
Spontaneous	↓	↓
Tumor cell arrest in lungs	↓	↓
Tumor invasion	↓	↓
Tumor migration (haptotaxis)	↓	↓
Tumor cell adhesion:		
To endothelium	−	±
To extracellular matrix	↓	↓
Enzymatic degradation of extracellular matrix:		
Heparanase	↓	↓
Type IV collagenase	↓	−
Tumor angiogenesis	↓	−
Coagulation/platelet aggregation	±	↓
Tumor cell growth	−	−

[a]↓ = inhibition; ± = partial inhibition; − = no effect.

anase from B16-BL6 cell as does heparin in a concentration-dependent manner as shown in Fig. 9, whereas little influence of CM-chitin was observed at the indicated concentrations except for 1000 μg/mL. On the other hand, degradation of ^{3}H-labeled type IV collagen was inhibited over 24 hours by more than 75% after treatment with 400 μg/mL SCM-chitin III and by approximately 20% inhibition after treatment with heparin. Little inhibition was shown by CM-chitin. A summary of the biological functions of SCM-chitin III and heparin are listed in Table 5.

CONCLUSION

The highest anticoagulant activity was shown by *N*-, 6-*O*-. and 3-*O*-sulfated 6-O-CM-chitin (SCM-DAC-70) in the presence of AT-III among several 6-O-CM-chitin derivatives including SCM-chitosan, in which only amino groups were sulfated at 70%, whereas only a faint anticoagulant activity was shown by SCM-chitin in which 3-*O*- and 6-*O*-hydroxyl groups were sulfated. The introduction of a carboxymethyl group might suppress hypertension. The one-third antithrombogenic activity of SCM-DAC-70 compared to that of heparin indicates 3 moles of SCM-DAC-70 adsorption to AT-III, which was confirmed by fluorescence titration.

Antimetastasis of B16-BL6 melanoma cells was shown by SCM-chitin III which was deeply sulfonated at the hydroxyl groups of the 6-O-CM-GlcNAc residue. The antimetastasis of SCM-chitin III was suggested to be a specific inhibition toward enzymes, such as heparanese and type IV collagenase, which were secreted from melanoma cells and invaded vein wall. Specific sulfation at the C-3 hydroxyl group seems to be a main factor for controlling both anticoagulant and antimetastatic activities. As the degree of antimetastatic activity is around 80–85% thrombogenic and antimetastatic activities can be enhanced by applying site-specific sulfation of CM-chitin.

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