Inhibition by Sulfated Chitin Derivatives of Invasion through Extracellular Matrix and Enzymatic Degradation by Metastatic Melanoma Cells

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ABSTRACT

We have investigated the effects of sulfated chitin derivatives and heparin on the invasion of B16-BL6 melanoma cells through reconstituted basement membrane Matrigel which contains laminin, type IV collagen, heparan sulfate proteoglycan, and entactin. 6-O-sulfated chitin (S-chitin) and 6-O-sulfated and carboxymethyl chitin (SCM-chitin) significantly inhibited the penetration of tumor cells through Matrigel in parallel with the increased degree of sulfation. However, 6-O- and N-deacetylated chitin derivative (SCM-chitosan) and CM-chitin had no effect. SCM-chitin with a high degree of sulfation (SCM-chitin III), which exhibited fairly low levels of anticoagulant activity, was more effective than intact heparin. SCM-chitin III and heparin were also shown to block the attachment and migration of tumor cells to laminin-coated substrates, which are considered to be involved in tumor invasion. The inhibition of cell attachment and migration by SCM-chitin III and heparin is likely to depend upon their specific binding to laminin molecules (possibly the heparin-binding domain). Degradation of heparan sulfate by heparanase was inhibited by SCM-chitin III and heparin in a dose-dependent manner. Surprisingly, SCM-chitin III could inhibit type IV collagenolytic activity of tumor cells more potently than heparin. Thus, nontoxic SCM-chitin III of low anticoagulant properties may provide a promising basis for the prevention of cancer metastasis.

INTRODUCTION

During the metastatic cascade, a tumor cell passes through several connective tissue barriers which consist of adhesive molecules such as fibronectin, laminin, and other glycoproteins and proteoglycans (1–7). Tumor invasion is a complex process involving cell adhesion, motility (migration), and the secretion of different classes of degradative enzymes. Therefore, understanding the mechanisms of invasion may help in the development of antitumor therapies.

Heparin is a structurally heterogenous sulfated glycosaminoglycan composed of repeating units of N-acetyl glucosamine and uronic acid (glucuronic acid or iduronic acid) and is known to exhibit a variety of biological properties such as inhibition of blood coagulation by binding to antithrombin III (8, 9), potentiation of angiogenesis (10, 11), interaction with fibroblast growth factors (12, 13), and modulation of cell growth (14, 15). Interestingly, heparin can bind to characteristic sequences in the domains of adhesion molecules including those of fibronectin (16), laminin (17), and type IV collagen (18), which serve as substrates in vitro to promote the adhesion, spreading, and migration of tumor cells, and can inhibit experimental pulmonary metastases. Several attempts have been made to inhibit tumor metastasis experimentally by using heparin and its related compounds. Tsuuba et al. (19) have demonstrated that sulfated polysaccharides inhibited blood-borne metastasis 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 lodgment. On the other hand, chemically modified heparin without anticoagulant properties has been shown to successfully reduce the number of experimental lung metastases of melanoma cells and to inhibit the heparanase activity of murine metastatic melanoma cells (20, 21).

Chitin, (1→4)-linked polysaccharide composed of N-acetylglucosamine, is widely distributed in nature and has been reported to have medicinal and pharmaceutical applications (22, 23). CM-chitin, which is one of the chitin derivatives, has been shown to have no adsorption ability of blood components such as serum albumin, γ-globulin, and fibrinogen as compared with chitin, and no antigenicity (24–26). We recently reported that sulfated chitin derivatives, when administered intratumorally or intravenously at various days before or after surgical excision of the primary tumor, caused a marked decrease in the number of lung tumor colonies in a spontaneous lung metastasis model (27). Chemically modified chitin derivatives, in which 6-O-sulfate and 6-O-carboxymethyl groups were introduced, showed much lower levels of anticoagulant and antiplatelet aggregation activities than those of heparin.4

In this paper, we focus our attention on the tumor-invasive cascade in the metastatic process and examine the effect of sulfated chitin derivatives on the adhesion, motility (migration) of metastatic tumor cells, and enzymatic degradation of extracellular matrices.

MATERIALS AND METHODS

Cells. Highly metastatic B16-BL6 melanoma cells, obtained by an in vitro selection procedure for invasion, were kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, TX. Melanoma cells were maintained as monolayer cultures in MEM supplemented with 7.5% FBS, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine.

Sulfated Chitin Derivatives and Other Reagents. The chemical structures of chitin and its derivatives (chitin heparinoids) are given in Fig. 1. Chitin was prepared from Queen Crab shells by the method of Hackman (28) and powdered to 45–60 mesh before use. CM-chitin was prepared from chitin according to the method described previously (29), and the degrees of substitution used were 0.40, 0.56, and 0.80. The preparation was performed by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, TX. Melanoma cells were maintained as monolayer cultures in MEM supplemented with 7.5% FBS, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine.

The abbreviations used are: CM-chitin, 6-O-carboxymethyl chitin; MEM, Eagle’s minimal essential medium; PBS, Ca2++ and Mg2+-free phosphate-buffered saline; FBS, fetal bovine serum; I.T., intratumorally; BSA, bovine serum albumin; DME:F-12, Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium; HS, heparan sulfate, IFN-γ, γ-interferon; SCM-chitin, 6-O-sulfated and carboxymethyl chitin; S-chitin, 6-O-sulfated chitin; SCM-chitosan, 6-O- and N-sulfated but partially N-deacetylated chitin derivative; S-chitosan, 6-O-sulfated chitin derivative.

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sulfation of chitin and CM-chitin was carried out by the general method of Horton and Just (30). Briefly, chitin or CM-chitin was treated with distilled pyridine to remove water and was resuspended in pyridine. A 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 quantitative analyses for sulfur in the products (31). The molecular weights of sulfated chitin derivatives were estimated to be approximately 1 to 6 x 10^4 from viscosity measurements using an Ubbelohde-type viscometer by applying the equation proposed for heparin (32). The derivatives of chitin were dissolved in PBS before use. Heparin sodium salt (Lot TLP3856; specific activity, 197.1 units/mg) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Purified mouse fibronectin was purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Purified mouse laminin and basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan, and entactin) were obtained from Collaborative Research, Inc., Bedford, MA. All the reagents and media in this study were endotoxin free (approximately <1.0 ng/ml) as determined by a colorimetric assay (Pyrodidic; Seikagaku Kogyo Co., Ltd., Tokyo, Japan).

Microassay for Cell Adhesion. The cell attachment assay was carried out by the method described (33). B16-BL6 melanoma cells in the exponential growth phase were incubated for 24 h in MEM containing 5% FBS supplemented with 0.3 μCi/ml of [3H]dideoxyuridine (specific activity, 200 mCi/mmol; New England Nuclear, Boston, MA). The cells were washed twice in warm PBS to remove unbound radio-label, harvested by adding 0.02% EDTA for 1 min at 37°C, and resuspended in cold serum-free MEM to form a single suspension of cells. [3H]dideoxyuridine-labeled tumor cells (2 x 10^4) at a volume of 0.05 ml/well were added to microculture wells precoated with laminin. The cultures were incubated at 37°C for 30 min and then washed 4 times with PBS to remove unattached cells. The remaining substrate-bound tumor cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed by cotton swabs and monitored for radioactivity by γ counting. The binding capacity (number of cells bound/substrate) was expressed as follows.

\[
\text{Binding capacity} = \frac{\text{cpm of targets bound to substrate}}{\text{cpm of total tumor cells added}} \times \text{total no. of tumor cells added}
\]

Haptotactic Migration Assay. Tumor cell migration along a gradient of substratum-bound fibronectin or laminin was assayed in Transwell cell culture chambers (No. 3422; Costar, Cambridge, MA) according to the methods as reported by McCarthy et al. (34) with some modifications (35). Polyvinylpyrrolidone-free polycarbonate filters with an 8.0-μm pore size (Nucleopore, Pleasanton, CA) were precoated with either 5 μg of fibronectin or laminin 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 tumor cells were harvested with 1 ml EDTA in PBS, washed 3 times with serum-free MEM, and resuspended to a final concentration of 2 x 10^5/ml in MEM with 0.1% BSA. Cell suspensions (100 μl) with or without agents were added to the upper compartment and incubated for an appropriate number of hours at 37°C in a 5% CO2 atmosphere. 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 through the filter to various areas of the lower surface were manually counted under a microscope at a magnification of x400, and each assay was performed in triplicate.

Invasion Assay. The invasive activity of tumor cells was assayed according to the method reported by Albini et al. (36) with some modifications (35). Briefly, the lower surface of the filters was precoated with fibronectin or laminin as described above. The Matrigel was diluted to 100 μg/ml with cold PBS, applied to the upper surface of the filters (5 μg/filter), and dried at room temperature under a hood. The filters thus prepared were designated Matrigel/laminin- or Matrigel/fibronectin-coated filters, respectively. The following procedures were the same as those of the haptotactic migration assay. The cells that had invaded through Matrigel and filter to the lower surface of the filter were manually counted.

Heparin Binding Assay. The binding of [3H]heparin (specific activity, 0.49 mCi/mg; Du Pont-New England Nuclear Research Products, Boston, MA) to laminin and BSA was quantitated by a solid-phase radioimmunoassay in 96-well tissue culture plates (37). Laminin (3 μg) at a volume of 60 μl was added to each well and dried overnight at room temperature. BSA-containing buffer (2 mg/ml in 6 mM phosphate, 0.1 M NaCl, 68 μM CaCl2, pH 6.8) was added to each well, followed by a 2-h incubation at 37°C. After removal of the buffer, [3H]heparin (5 x 10^4 dpm/0.05 μg of heparin) at a volume of 50 μl was incubated for 2 h at 37°C in the absence or presence of unlabeled heparin or chitin derivatives. Unbound [3H]heparin was removed by washing 3 times with the buffer containing 0.1% (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate. Tritiated heparin was solubilized by incubation with 200 μl of 0.5 N NaOH containing 1% sodium dodecyl sulfate for 30 min at 37°C and quantitated in a liquid scintillation counter.

Heparanase Preparation. Heparanase was prepared from B16-BL6 melanoma cell extracts using heparin-Sepharose and concanavalin A-Sepharose chromatography as follows. B16-BL6 melanoma cells (2 x 10^6) were harvested from a subconfluent culture in a 1:1 mixture of DME:F12 containing 5% fetal bovine serum and extracted in 0.5% Triton X-100 in 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 50 mM Tris-HCl, pH 7.5 (Buffer 1) at 4°C for 30 min. The extract was centrifuged at 30,000 x g for 30 min, and the supernatant containing approximately 100 μg of protein was loaded at 30 ml/h on a heparin-Sepharose column (50-ml bed volume) equilibrated with Buffer 1. The column was sequentially washed with 200 ml ofBuffer 1, 200 ml of 0.2% Triton X-100:20 mM sodium acetate, pH 6.0 (Buffer 2), and with 300 ml of 0.15 mM sodium chloride:20 mM sodium acetate, pH 6.0 (Buffer 3) for 10 h. Heparin-binding proteins were eluted at 50 ml/h with a linear sodium chloride gradient (0.15 to 1.2 M) in 20 mM sodium acetate, pH 6.0. Heparanase-active fractions were collected and diazylated against Buffer 3 and then centrifuged at 30,000 x g for 30 min. The supernatant was loaded on a concanavalin A-Sepharose column (50-ml bed volume) equilibrated with Buffer 3. After washing the column with 200 ml of Buffer 3 for 4 h, concanavalin A-binding proteins were eluted at 50 ml/h with 200 ml of 1.0 M α-methyl-D-mannopyranoside in Buffer 3. The eluates were collected and concentrated with an Amicon Concentrator using a YM-10 membrane. The specific activity of the partially purified heparanase was determined by analysis of [3H]labeled HS degradation products as described in the heparanase inhibition assay. The heparanase fraction used in this study contained 0.89 mg/ml of protein and degraded 60 μg of HS/mg of protein/h at 37°C.

Heparanase Inhibition Assay. The heparanase inhibition assay was performed based on the methods previously described (38, 39). HS (M, 34,000) purified from bovine lung was partially N-desulfated and then

![Chemical structures of chitin derivatives.](image)
N-acetylated with [3H]acetic anhydride (38). [3H]-labeled HS (5 µg) was incubated at 37°C for 3 h with 26.7 µg of the partially purified heparanase in 50 µl of 0.2 M sodium acetate buffer, pH 5.6, in the presence or absence of various concentrations of chemically modified chitins. Incubation was terminated by heating at 100°C for 5 min, and the boiled samples were chilled and centrifuged at 18,000 × g for 5 min. [3H]HS degradation products in 25 µl of the supernatant were analyzed by size-exclusion chromatography using a Waters 600E high-pressure liquid chromatography system (Waters, Milford, MA) equipped with a Bio-Gel TSK-30-XL column (300 × 7.5 mm; Bio-Rad, Richmond, CA). Elution was performed at 23°C with 12.5 mM Tris-HCl:0.15 M sodium chloride, pH 7.5, at a flow rate of 0.5 ml/min. The eluates were collected every 30 s in 7-ml plastic vials and mixed with 3 ml of Liquscint (National Diagnostics, Manville, NJ), and the radioactivity of each fraction was measured using a LS2800 liquid scintillation counter (Beckman, Irvine, CA). Heparanase activity was determined by measuring the decrease in area of the first one half of intact [3H]HS peak on the chromatogram.

Preparation of [3H]-labeled Type IV Collagen. Type IV procollagen was purified from EHS tumors grown in C57BL/6 mice as described by Nakajima et al. (40) and acetylated using [3H]acetic anhydride. Type IV collagen solution (3 mg/ml) in 0.2 M acetic acid was neutralized with 2 M sodium acetate, pH 8.0, immediately mixed with 1 mCi of [3H]acetic anhydride (100 mCi/mmol; ICN Radiochemicals, Irvine, CA) in 50 µl of benzene, and then incubated for 16 h at 4°C. [3H]-labeled type IV collagen was solubilized in 3 ml of 0.5 M acetic acid and dialyzed against 4 liters of 0.2 M acetic acid for 6 h at 4°C. The dialysis was repeated 4 times. The radioactivity of [3H]-labeled type IV collagen used in this study was 2220 dpm/µg.

Type IV Collagenolyis Assay. [3H]-labeled type IV collagen (20 µg) was placed in each well of a 48-well tissue culture plate (Costar, Cambridge, MA) and dried in a laminar air flow hood overnight. The DME:F12 medium (500 µl) was added over the dried type IV collagen film and incubated for 3 h at 37°C. Then, the medium was replaced with fresh DME:F12 medium containing various sulfated chitin derivatives (400 µg/ml) and incubated for 3 h at 37°C. B16-BL6 (5 × 10⁴) cells suspended in 400 µl of DME:F12 medium containing 0.5% BSA were then added and incubated at 37°C in a humidified incubator (5% CO₂:95% air). After 48-h incubation, the culture supernatants were withdrawn, and undigested materials were precipitated by mixing with 100 µl of ice-cold 50% trichloroacetic acid and briefly centrifuged at 18,000 × g. Type IV collagenolytic activity was calculated from the radioactivity in the supernatant.

Statistical Analysis. The statistical significance of differences between groups was calculated by applying the Student two-tailed t test.

RESULTS

Inhibition of Tumor Cell Invasion by Chitin Derivatives. We first examined the direct effect of chitin derivatives and heparin on the growth of B16-BL6 melanoma cells in vitro. Table 1 shows that the incubation of tumor cells with 500 µg/ml of chitin derivatives did not affect the incorporation of [3H]thymidine into tumor cells. Recombinant IFN-γ (10³ units/ml) as a positive control potently inhibited the cell growth in vitro. We next examined the effect of chitin derivatives and heparin on the invasive capability of metastatic tumor cells through reconstituted basement membrane Matrigel (Table 2). Tumor cells were added to the upper compartment of Transwell chambers in the presence or absence of chitin derivatives or heparin. SCM-chitin II and III, which contain various amounts of sulfate and carboxymethyl groups at the C-6 position, as well as heparin could potently inhibit tumor cell invasion through Matrigel/fibronectin- and Matrigel/laminin-coated filters, whereas CM-chitin and an N-deacetylated chitin derivative, SCM-chitosan, could not.

Effect of Sulfated Chitin Derivatives on the Tumor Cell Attachment. Tumor cell adherence to extracellular matrices and basement membranes is considered to be an initial step in the invasive process for metastatic tumor cells. We next examined the influence of sulfated chitin derivatives on the adhesion of B16-BL6 cells to the substrates precoated with laminin, which is a major basement membrane component (Table 3). [125I]-labeled B16-BL6 cells were added to laminin-coated wells in the presence or absence of sulfated chitin derivatives. 6-O-sulfated chitin (S-chitin) as well as heparin significantly inhib-

### Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation of [3H]thymidine into the cells (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (medium)</td>
<td>13,266 ± 1,007*</td>
</tr>
<tr>
<td>Heparin</td>
<td>14,140 ± 1,225</td>
</tr>
<tr>
<td>CM-chitin</td>
<td>12,714 ± 2,629</td>
</tr>
<tr>
<td>S-chitin II (8.54)</td>
<td>13,005 ± 1,905</td>
</tr>
<tr>
<td>III (10.22)</td>
<td>11,725 ± 325</td>
</tr>
<tr>
<td>SCM-chitin I (1.43)</td>
<td>12,217 ± 1,554</td>
</tr>
<tr>
<td>(II.70)</td>
<td></td>
</tr>
<tr>
<td>III (7.66)</td>
<td>11,399 ± 911</td>
</tr>
<tr>
<td>S-chitosan (11.89)</td>
<td>11,133 ± 2,204</td>
</tr>
<tr>
<td>SCM-chitosan (5.90)</td>
<td>12,582 ± 1,617</td>
</tr>
<tr>
<td>IFN-γ, 10³ units/ml</td>
<td>2,871 ± 48</td>
</tr>
</tbody>
</table>

* Mean ± SD.

### Table 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Matrigel/fibronectin</th>
<th>Matrigel/laminin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>115 ± 11*</td>
<td>73 ± 5</td>
</tr>
<tr>
<td>SCM-chitin I (1.43)</td>
<td>116 ± 11</td>
<td>47 ± 7</td>
</tr>
<tr>
<td>III (6.70)</td>
<td>58 ± 10*</td>
<td>9 ± 5*</td>
</tr>
<tr>
<td>III (7.66)</td>
<td>46 ± 8*</td>
<td>7 ± 2*</td>
</tr>
<tr>
<td>CM-chitin</td>
<td>111 ± 9</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>SCM-chitosan (5.90)</td>
<td>107 ± 19</td>
<td>68 ± 9</td>
</tr>
<tr>
<td>Heparin</td>
<td>52 ± 5*</td>
<td>45 ± 6*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

### Table 3.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Binding capacity (no. of cells bound/substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4411 ± 319*</td>
</tr>
<tr>
<td>SCM-chitin I (1.43)</td>
<td>4439 ± 945</td>
</tr>
<tr>
<td>III (6.70)</td>
<td>3261 ± 593 [26]*</td>
</tr>
<tr>
<td>III (7.66)</td>
<td>2501 ± 117 [43]*</td>
</tr>
<tr>
<td>CM-chitin</td>
<td>5145 ± 468</td>
</tr>
<tr>
<td>S-chitin II (8.54)</td>
<td>2666 ± 510 [40]*</td>
</tr>
<tr>
<td>SCM-chitosan (5.90)</td>
<td>4337 ± 977</td>
</tr>
<tr>
<td>Heparin</td>
<td>2887 ± 450 [35]*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Numbers in parentheses, degree (percentage) of sulfation.

P < 0.001 by Student’s two-tailed t test.

P < 0.01.
tumor cells to laminin-coated wells (P < 0.01, respectively), whereas CM-chitin did not. SCM-chitin I, II, and III could inhibit the tumor cell adhesion in parallel with the increased degrees of sulfation. In contrast, SCM-chitosan was not effective for the inhibition of tumor cell adhesion to laminin-coated substrates, although it was fully 6-O- and N-sulfated.

Effect of Sulfated Chitin Derivatives on the Migration of Tumor Cells. To investigate the effect of sulfated chitin derivatives and heparin on the migration of tumor cells along the gradient of a substratum-bound insolubilized laminin or fibronectin (haptotaxis), we first performed checkerboard analyses with Transwell chamber filters precoated on the lower surface with laminin by placing various concentrations of heparin above and below each filter. As shown in Fig. 2, each square of the checkerboard represents a different gradient condition for heparin, and each number represents tumor cell migration to laminin-coated filters in the presence of the given concentrations of heparin in the upper and lower compartments. The control level of cell migration to laminin-coated filters was determined by the absence of heparin in the lower and upper compartments. Under conditions with various net negative gradients (more heparin in the upper than lower compartments) and with various net positive gradients (more heparin in the lower than upper compartments), tumor cell migration was inhibited by the presence of heparin in the lower compartment. Cell movement under nongradient conditions (equal heparin in lower and upper compartments, i.e., along the diagonal dotted line) was also inhibited by heparin in a dose-dependent fashion. We therefore examined the effects of sulfated chitin derivatives on the haptotactic migration of tumor cells to laminin- or fibronectin-coated filters by adding them in the lower compartment (Table 4). S-chitins and SCM-chitins as well as heparin in the lower compartment could significantly inhibit the migration of tumor cells in parallel with the increased degree of sulfation (P < 0.001, respectively). However, CM-chitin and N-deacetylated chitin derivatives, S-chitosan and SCM-chitosan, did not inhibit migration. Fig. 3 shows that SCM-chitin III inhibited the haptotactic migration of B16-BL6 cells to laminin-coated filters in a concentration-dependent manner. CM-chitin did not have any effect at the concentration of 500 µg/ml. Since SCM-chitin III caused a marked reduction of lung colonization of B16-BL6 melanoma in the experimental and spontaneous metastases model (27), we mainly investigated SCM-chitin III in the following experiments.

Influence of Tumor Cell Migration by the Treatment of Tumor Cells or Laminin-Substrate with SCM-Chitin III. We next examined whether the pretreatment of tumor cells or laminin-substrate with SCM-Chitin III caused the inhibition of tumor cell migration or not (Table 5). Tumor cell migration to laminin immobilized on the lower surface of the filter was blocked by the addition of SCM-chitin III or heparin in the lower compartment of the chamber as explained above. The pretreatment substrate with SCM-chitin III caused the inhibition of tumor cell migration or not (Table 5).

\[
\begin{array}{c|ccc|ccc}
\text{Heparin concentration (µg/ml)} & 0 & 250 & 500 \\
\hline
\text{Upper compartment (µg/ml)} & 0 & 126 & 115 & 131 \\
250 & 93 & 79 & 67 \\
500 & 39 & 34 & 32 \\
\end{array}
\]

Fig. 2. Effect of heparin on haptotactic migration of B16-BL6 melanoma on laminin-coated filters. B16-BL6 cells (2 × 10⁵) in 0.1% BSA-medium were seeded in the upper compartment. The lower surface of the filter in a Transwell chamber was precoated with 5 µg of laminin. Various concentrations of heparin were dispensed into the lower and upper compartments. After 6-h incubation, the migrant cells on the lower surface were visually counted. The number indicates the mean of triplicate determinations per high-power field.

\[
\begin{array}{c|c|c|c}
\text{Treatment} & \text{Laminin} & \text{Fibronectin} \\
\hline
\text{Untreated} & 66 ± 11* & 70 ± 7 \\
\text{S-chitin} & 33 ± 5 & NT*
\end{array}
\]

\[
\begin{array}{c|c|c|c|c|c|c}
\text{Addition (µg/ml) in lower compartment} & 50 & 100 & 500 \\
\hline
\text{SCM-chitin III, 250} & 23 ± 6 & <0.001 \\
\text{CM-chitin, 250} & 65 ± 3 \\
\text{Heparin, 250} & 27 ± 6 & <0.001 \\
\end{array}
\]

\[
\begin{array}{c|c|c|c}
\text{Treatment (µg/ml) of laminin-substrate with} & 11 ± 3 & <0.001 \\
\text{SCM-chitin III, 250} & 47 ± 10 \\
\text{CM-chitin, 250} & 10 ± 2 & <0.001 \\
\text{Heparin, 250} & 45 ± 11 \\
\text{Treatment (µg/ml) of tumor cells with} & 52 ± 12 \\
\text{SCM-chitin III, 250} & 46 ± 3 \\
\text{CM-chitin, 250} & 45 ± 11 \\
\text{Heparin, 250} & 45 ± 11 \\
\end{array}
\]

\* Compared with the control by Student's two-tailed t test.
\# Mean ± SD.
\% Chitin heparinoids were added into the lower compartment of Transwell chambers.
\& Heparin-coated filters were pretreated for 2 h at room temperature.
\% Heparin was not tested.
\*= P < 0.001, compared with the control by Student's two-tailed t test.

Table 4 Effect of sulfated chitin derivatives on haptotactic migration of B16-BL6 melanoma on laminin- or fibronectin-coated filters.

Table 5 Inhibition of haptotactic migration by B16-BL6 melanoma cells on laminin-coated filters by SCM-chitin III.

Table 6 Effect of sulfated chitin derivatives on haptotactic migration of B16-BL6 melanoma on laminin- or fibronectin-coated filters.

* Numbers in parentheses, degree (percentage) of sulfation.
NT, not tested.
\[P < 0.001,\] compared with the control by Student's two-tailed t test.

Fig. 3. Dose-response of SCM-chitin III on haptotactic migration of tumor cells. B16-BL6 cells (2 × 10⁵) were seeded on the filters precoated on the lower surface with 5 µg of laminin. Various concentrations of SCM-chitin III were added into the lower compartment of Transwell chambers. After a 6-h 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.
of laminin-coated filters with SCM-chitin III or heparin resulted in the inhibition of tumor cell movement, whereas the pretreatment of tumor cells with them did not. CM-chitin had no inhibitory effect by any treatment modalities.

Competition of [3H]Heparin Binding to Laminin by Sulfated Chitin Derivatives. We observe in preliminary form that [3H]-heparin binding to laminin increased as a function of time and was saturable by 2 h after incubation (data not shown). In this assay, [3H]heparin was added to laminin-coated wells in the presence or absence of unlabeled heparin or sulfated chitin derivatives as an inhibitor. As shown in Fig. 4, unlabeled heparin or SCM-chitin III competitively inhibited [3H]heparin binding to laminin in a concentration-dependent manner, whereas SCM-chitin I and CM-chitin, which had no inhibitory effect on the invasion, adhesion, and migration of tumor cells (Tables 2 to 4), did not. We also found that 70 to 80% of laminin-bound [3H]heparin was removed within 1 h by the addition of SCM-chitin III or unlabeled heparin, implying that the bindings of SCM-chitin III and heparin to laminin are reversible.

Effect of Sulfated Chitin Derivatives on Enzymatic Degradation of Extracellular Matrices by Tumor Cells. During the invasive cascade, proteases and glycosidases produced by tumor cells have been also suggested to be major factors in metastatic cell penetration through several connective tissue barriers consisting of extracellular matrices such as fibronectin and sulfated glycosaminoglycans (1-7). Interestingly, heparin is known to inhibit heparan sulfate degradation by melanoma cells (4). We therefore investigated whether or not a sulfated chitin derivative of low anticoagulant properties, SCM-chitin III, can inhibit the enzymatic degradation of extracellular matrices by tumor cells. SCM-chitin III as well as heparin inhibited HS degradation by partially purified heparanase from B16-BL6 cells in a concentration-dependent manner (Fig. 5). CM-chitin, which showed no inhibition of tumor cell invasion to the reconstituted basement membrane Matrigel (Table 1), did not inhibit HS degradation at the indicated concentrations except 1000 µg/ml. On the other hand, the effects of SCM-chitin III on type IV collagenolysis were further investigated directly using 3H-labeled type IV collagen film treated at 37°C for 3 h with 26.7 µg of the partially purified heparanase in 50 µl of 0.2 M sodium acetate buffer, pH 5.6, in the presence or absence of chitin derivatives (4). 3H-labeled type IV collagen film was treated at 37°C for 3 h with or without chitin derivatives and incubated with B16-BL6 cells (5 x 10⁴) suspended in 400 µl of medium containing 0.5% BSA for 48 h.

DISCUSSION

During the sequential steps of metastasis, metastasizing tumor cells encounter various host cells (platelets, lymphocytes, or endothelial cells) and/or extracellular matrix and basement membrane components. Platelet aggregation by circulating tumor cells is also thought to be one of the important events to facilitate tumor cell adhesion to vascular endothelium. As a result of adhesive interaction, this encounter may result in an embolus formation which can subsequently enhance the survival, arrest, or invasiveness of tumor cells. Tsubura et al. (19) have indicated that anticoagulants, heparin, and other sulfated polysaccharides reduced blood-borne pulmonary metastasis in rats, probably by the inhibition of tumor emboli formed by platelet aggregation together with activation of the coagulation cascade. We recently reported that a sulfated derivative of 6-O-carboxymethyl chitin (SCM-chitin III) and heparin were effective for the inhibition of lung metastasis when administered i.v. or i.t. in a spontaneous metastasis model (27). SCM-chitin III was also observed to possess much less anticoagulant and antiplatelet aggregation activities than heparin.

We found that heparin led to a decrease in the arrest and retention of tumor cells in the lung over 24 h after its coinjection with radiolabeled tumor cells but that SCM-chitin III was much less effective than heparin. The mechanism for the inhibitory effect of SCM-chitin III on spontaneous and experimental lung metastases may be other than the anticoagulation effects on tumor metastasis. We demonstrated here that SCM-chitin of low anticoagulant properties (i.e., SCM-chitin II and III) potentially inhibited the invasion of metastatic tumor cells through basement membrane Matrigel and filters, whereas nonsulfated CM-chitin did not (Table 2). In contrast, SCM-chitosan was not effective for the inhibition of tumor invasion, irrelevant to the degree of sulfation. These results suggest that 6-O-sulfation and N-acetylation of glucosamine units are associated with the expression of inhibitory effects on tumor invasion of sulfated chitin derivatives as well as heparin. In addition, the inhibitory
effect of chitin derivatives may not be due to their direct cytotoxicity against tumor cells (Table 1).

Invasion is a complex process involving the attachment of tumor cells, secretion of enzymes by tumor cells that cause the degradation of adjacent membranes, and the migration of the cells into target tissue (1–7). To further analyze the inhibitory effect of sulfated chitin derivatives on tumor cell invasion, we investigated their influence on the adhesion and haptotactic migration of tumor cells to laminin-coated substrates. S-chitins and SCM-chitins as well as heparin inhibited the adhesion and migration of tumor cells, whereas CM-chitin and SCM-chitosan did not (Tables 3 and 4). SCM-chitin III, a derivative with a high degree of sulfation, could inhibit the haptotactic migration to laminin-coated substrates in a dose-dependent manner (Fig. 3). Haptotactic migration of tumor cells was also inhibited by the pretreatment of laminin-substrates with SCM-chitin III or heparin but not by the pretreatment of tumor cells with them (Table 5). In addition, SCM-chitin III and heparin competitively inhibited [3H]heparin binding to immobilized laminin in a concentration-dependent manner, but SCM-chitin I and CM-chitin did not (Fig. 4). These results indicate that the inhibition of adhesion, haptotactic migration, and invasion of tumor cells may result in the specific binding of sulfated chitin derivatives and heparin to laminin molecules (presumably the heparin-binding domain) in Matrigel or for coating the filter. We recently observe that monoclonal antibodies specific for the heparin-binding domain of fibronectin, in place of heparin or SCM-chitin III, were also able to inhibit tumor cell migration to fibronectin. Further detailed study will be needed to determine the binding property and affinity of SCM-chitin III in comparison with those of heparin.

The ability of tumor cells to degrade extracellular matrices in vitro correlates well with in vivo metastatic potential (3–5, 41–44). Degradation of extracellular matrices during tumor invasion involves a number of enzymes, including heparanase (endo-β-glucuronidase), collagenase, and their proteolytic activators. Heparin is known to inhibit heparanase-mediated digestion of sulfated glycosaminoglycan heparan sulfate by melanoma cells (4). As shown in Fig. 5, SCM-chitin III and heparin could block 3H release from labeled heparan sulfate by purified heparanase digestion in a dose-dependent manner, but CM-chitin at the concentrations ranging from 1 to 100 μg/ml could not. Neutral metalloproteinases capable of specifically degrading type IV collagens have also been postulated to play a role in tumor cell invasion and metastasis (45). Liotta and coworkers (46–49) have reported a correlation between spontaneous metastatic potential and the type IV collagen-degradative activity of tumor cells. Heparin could block 3H release from labeled type IV collagen by about 20%, while SCM-chitin III blocked the release by about 80% (Fig. 5). CM-chitin showed no inhibitory effect on type IV collagenolysis. We also reported that 1,10-phenanthroline and bestatin, which are considered to be inhibitors of metalloproteinases, could inhibit the tumor cell invasion through reconstituted basement membrane Matrigel, but other enzyme inhibitors such as pepstatin, leupeptin, and soybean trypsin inhibitor did not (35). Further study will be necessary to fully understand the mechanism of inhibitory effect of SCM-chitin III on enzymatic degradation by tumor cells.

In summary, we demonstrated that SCM-chitin III, a sulfated chitin derivative of low anticoagulant properties, as well as heparin inhibited the invasion of B16-BL6 melanoma cells through Matrigel including laminin and heparan sulfate proteoglycan. SCM-chitin III was also shown to block the adhesion and migration of tumor cells to laminin, which is one of the important components of extracellular matrices. Degradation of heparan sulfate by heparanase was inhibited by SCM-chitin III and heparin, while SCM-chitin III inhibited type IV collagenolytic activity of tumor cells more potently than heparin. Thus, nontoxic SCM-chitin III may provide a therapeutic benefit for the prevention of lung tumor metastasis.

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REFERENCES

ANTIINVASIVE ACTIVITY OF SULFATED CHITIN DERIVATIVES

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