Nutritional Sciences 7(3), August, 2004, pp. 144-150

Inhibition of Tumor Invasion and Metastasis by Calcium Spirulan (Ca-SP), a Novel Sulfated Polysaccharide Derived from a Blue-Green Alga *Spirulina Platensis*[*]

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We have investigated the effect of calcium spirulan (Ca-SP) isolated from a blue-green alga *Spirulina platensis*, which is a sulfated polysaccharide chelating calcium and mainly composed of rhamnose and fructose, on invasion of both B16-BL6 melanoma cells, Colon 26 carcinoma and HT-1080 fibrosarcoma cells through reconstituted basement membrane (Matrigel). Ca-SP significantly inhibited the invasion of these tumor cells through Matrigel/fibronectin-coated filters in a concentration-dependent manner. Ca-SP also inhibited the haptotactic migration of tumor cells to laminin, but it had no inhibitory effect on tumor cell migration to fibronectin-coated filters. Ca-SP prevented the adhesion of B16-BL6 cells to Matrigel- and laminin-substrates but did not affect the adhesion to fibronectin. The pretreatment of tumor cells with Ca-SP inhibited the adhesion to laminin in a concentration-dependent fashion, while the pretreatment of laminin-substrates did not. Ca-SP had no effect on the production and activation of type IV collagenase in gelatin zymography. In contrast, Ca-SP significantly inhibited degradation of heparan sulfate by purified heparanase. The experimental lung metastasis was significantly reduced by co-injection of B16-BL6 cells with Ca-SP in a dose-dependent manner. Seven intermittent i.v. injection of 100 μg of Ca-SP caused a marked decrease of lung tumor colonization of B16-BL6 cells in a spontaneous lung metastasis model. These results suggest that Ca-SP, a novel sulfated polysaccharide, could reduce the lung colonization of B16-BL6 melanoma cells in experimental metastasis model, by inhibiting the tumor invasion of basement membrane Matrigel, probably through the prevention of the adhesion and migration of tumor cells to laminin-substrate and of the heparanase activity.

Key words : Tumor invasion, Metastasis, Calcium spirulan (Ca-SP)

INTRODUCTION

A complex series of steps is required to permit the successful establishment of tumor metastasis.[1)] In some tumors, the formation of tumor emboli arised from the platelet aggregation and the activation of the coagulation cascade[2-5)] facilitates the penetration of tumor cells through endothelial cells[6,7)] and their underlaying subendothelial matrix.[8,9)] Tumor invasion of basement membrane is also one of the important steps comprised of adhesion and migration of tumor cells to extracellular matrix (ECM) components and degradation of ECM by degrading enzymes such as heparanase and matrix metalloproteinases.[10)]

Several attempts have been made to inhibit tumor metastasis by using sulfated polysaccharides to prevent the formation of tumor emboli and tumor invasion of basement membrane. Suemasu and Ishikawa[11)] have shown that heparin and dextran sulfate significantly inhibited the experimental lung metastasis of Sato lung carcinoma, and the inhibitory mechanism might be partially associated with its effect in changing the ionic properties of tumor cell surface. Tsubura et al.[12)] have demonstrated that sulfated polysaccharides could reduce the blood-borne pulmonary metastasis in rats by interfering with the formation of tumor emboli caused by platelet aggregation. On the other hand, Irimura et al.[13)] have found that chemically modified heparin analogue without anticoagulant activity inhibited both experimental lung metastasis and heparanase activity of murine melanoma cells. We reported previously that sulfated chitin derivatives which were composed of N-acetylglucosamine residues (SCM-chitin) successfully inhibited

* This work was supported in part by Grants-in-Aid for Cancer Research from the Japanese Ministry of Education, Science, Sports and Culture (No. 06282122 & 07273106), and by a Grant from the Hokuriku Industrial Advancement Center, and by the Proposal-Based Advanced Industrial Technology R & A Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, and by Grant-in-Aid for Special Project Research from Toyama Medical and Pharmaceutical University, Japan.

Accepted : June 3, 2004

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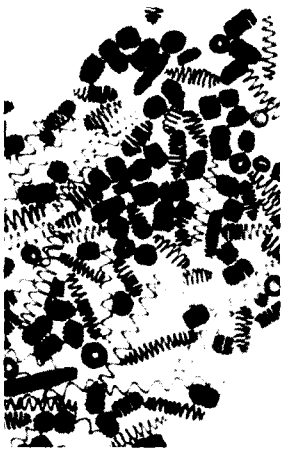

Fig. 1 *Spirulina platensis*
- Blue-green alga (Cyanobacterium)
- Length: 0.2-0.5 mm, Width (thickness): 6-8 μm
- Distribution: Aflican and Central and South American lakes rich in salts
- Pharmacological activities
  ① Reduction of hypercholesterolemia, ② Reduction of hypertension, ③ Enhancement of immune functions, ④ Improvement of intestinal bacterial flora, ⑤ Protection of HIV-1 infection *in vitro*, ⑥ Prevention of oral cancer etc.

the lung colonization of B16-BL6 melanoma cells in experimental and spontaneous metastasis model.[14] In addition, the inhibition may be due to the prevention of tumor invasion of basement membrane and suppression of tumor-induced angiogenesis through the inhibition of cell migration to fibronectin and laminin and of the degrading activity of tumor-derived enzymes, such as heparanese and type IV collagenase.[15,16]

Spirulina platensis, which belongs to blue-green alga (Fig. 1), originally has been used as a source of nutritious food in some African area.[17] It also exhibits a variety of biological properties such as reduction of hypercholesterolemia,[18] enhancement of immune functions,[19] and prevention of oral cancer.[20] Calcium spirulan (Ca-SP), isolated from *Spirulina platensis*, is a sulfated polysaccharide mainly containing rhamnose, fructose and calcium.[21] Previous study have shown that Ca-SP inhibited the replication of several enveloped viruses, including Herpes simplex virus type I (HSV-1), human cytomegalovirus, measles virus, mumps virus, influenza A virus, and human immunodeficiency virus-1 (HIV-1).[21,22] However, other biological properties of Ca-SP, such as an effect on tumor invasion and metastasis, are still unknown. In the present study, we investigated the effect of Ca-SP on the invasion of Matrigel *in vitro* and experimental lung metastasis produced by B16-BL6 melanoma cells in mice.

MATERIALS AND METHODS

1. Animals

Inbred 7- to 10-week old female C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The mice were maintained in the Laboratory for animal experiments, Institute of Natural Medicine, Toyama Medical & Pharmaceutical University, under laminar air flow conditions.

2. 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. Lung metastatic line of Colon 26 carcinoma (Colon 26 M 3.1) was obtained by the *in vivo* selection method of Fidler. These murine tumor cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal calf serum (FCS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine.

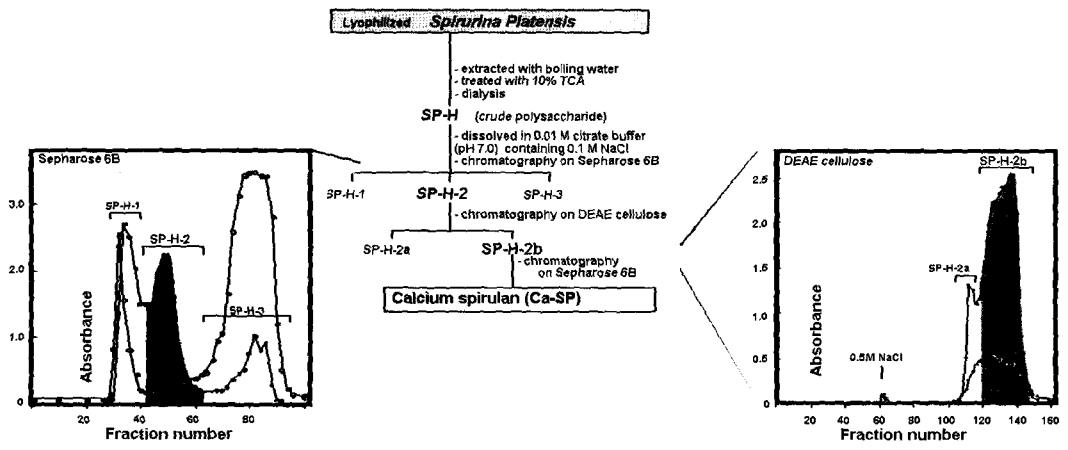

Fig. 2 The isolation method of calcium spirulian (Ca-SP) from a blue-green alga *Spirulina platensis*

3. Calcium spirulan (Ca-SP)

Ca-SP was isolated from a blue-green alga *Spirulina platensis* according to the methods described previously (Fig. 2).[21]

Briefly, freeze-dried powder of *Spirulina* grown in outdoor open tanks was extracted with boiling water, and the hot water extract was treated with 10% trichloroacetic acid (TCA). The crude polysaccharide (SP-H) was obtained from the TCA-soluble fraction by dialysis against distilled water followed by lyophilization. Gel filtration of SP-H on Sepharose 6B gave three fractions. The second fraction (SP-H-2) with anti-HSV-1 activity was then subjected to an ion exchange column chromatography on DEAE cellulose. After elution with water, the second elute with 0.5 M NaCl was further purified by a column chromatography on Sepharose 6B to give colorless polysaccaride (Ca-SP). The molecular weight of Ca-SP was estimated to be 2.6×10^5 and 3.1×10^5 by gel filtration on Sepharose 6B and light scattering experiments, respectively. Acid hydrolysates of Ca-SP were suggested to contain rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid and galacturonic acid by HPLC analysis. A high ash content (12.17%) and detection of sulfur (3.24%) in Ca-SP suggested the presence sulfate ester, which was confirmed by an S=O stretching absorption band at 1265 cm-1 in its infrared (IR) spectrum. X-ray microanalysis of Ca-SP revealed the presence of calcium as well as sulfur. These results suggested that Ca-SP is a sulfated polysaccharide chelating calcium ion and mainly composed of rhamnose and fructose.

4. Invasion Assay

The invasive activity of tumor cells was assayed in a Transwell cell culture chamber (Costar No.3422, Cambridge, MA) according to methods described previously.[23] Polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μm pore size (Nucleopore, Pleasanton, CA) were precoated with 1 μg fibronectin (IWAKI Glass Co. Ltd., Tokyo, Japan) in a volume of 40 μL on lower surfaces, and dried at room temperature. Reconstituted basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin, Collaborative Research Inc., Bedford, MA) was diluted to 500 μg/mL with cold PBS, applied to the upper surfaces on the filters (5 μg/filter), and dried at room temperature under a hood. These filters were designated Matrigel/fibronectin-coated filters. The coated filters were washed thoroughly in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 1mM EDTA in PBS, washed with serum-free MEM, and resuspended to give a final concentration of 3×10^6/mL (B16-BL6), 4×10^6/mL (Colon26 M3.1) in MEM with 0.1% bovine serum albumin (BSA). Cell suspension (100 μL), with or without Ca-SP, were added to the upper compartment and incubated for 3.5 - 4.0 h at 37 ℃ in a 5.0% CO_2 atmosphere. The filters were fixed with methanol and then stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping them with a cotton swab. The cells that had invaded through Matrigel and filter to the lower surface were manually counted under a microscope in 5 predetermined fields at a magnification of ×400.

5. Haptotactic Migration Assay

Tumor cell migration along a gradient of substratum-bound fibronectin was assayed in a Transwell cell culture chamber according to the methods reorted previously.[23] The filters with an 8.0 μm pore size were precoated with 1 μg fibronectin in a volume of 40 μL on their lower surface, as described above. The subsequent procedures were the same as those of the invasion assay.

6. Microassay for Cell Adhesion

The cell attachment assay was carried out by a method described previously[24] with some modifications. B16-BL6 melanoma cells in an exponential growth phase were washed with PBS, harvested by adding 1 mM EDTA in PBS, and resus- pended in cold serum-free MEM to form a single-cell suspension. The tumor cell suspensions (2×10^4), with or without Ca-SP in a volume of 100 μL/well, were added to microculture wells precoated with 50 μL of 5 μg/mL fibronectin, 50 μL of 10 μg/mL Matrigel, or 50 μL of 40 μg/mL laminin (Collaborative Research Inc., Bedford, MA). The cultures were incubated at 37 ℃ for 30 min. The wells were washed two times with warm PBS to remove unattached cells, then the attached cells were stained with 0.5% crystal violet in 20% methanol for 1 h. After a washing with water, the residual stained cells were lyzed with 25 μL of 30% acetic acid and the absorbance of the lysates were measured at 590 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon Inter-Med. K.K., Tokyo, Japan).

7. Gelatin zymography

HT-1080 human fibrosarcoma cells (5×10^4) were cultured in 6-well plates in the presence or absence of Ca-SP for 24 h. The cells at 80% confluency were rinsed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and incubated in D-MEM/F12. After a 24 h-incubation, the supernatant was collected and concentrated 10-fold using a Centricon-10 (Amicon, Danvers, MA, USA). The conditioned medium was used for detection of gelatinase activity. Gelatin zymographs were performed according to the method as reported by

Heussen and Dowdle[25] with some modifications. Briefly, samples were applied to SDS-polyacrylamide gels (7.5%, w/v) containing 0.1% (w/v) gelatin. After electrophoresis the gels were rinsed twice (30 min each) in 50 mM Tris-HCl, pH 7.6, containing 2.5% Triton X-100, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.05% NaN_3 to remove SDS, and incubated for 12 h at 37 ℃ in incubation buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.05% NaN_3). In some experiments, Ca-SP was added into incubation buffer to evaluate the effect on gelatinolytic activity. The gelatin gels were stained with 0.1% Coomassie blue, containing 10% acetic acid, 10% isopropanol, destained in 10% acetic acid, 10% isopropanol, and dried. Enzyme-digested regions were identified as white bands against a blue background, and quantitated by Master Scan gel analysis system (Scanalytics, Billerica, MA, USA). The optical density (OD) of a spot (band) is calculated as follows;

$OD = -\log (I/Im)$

where I is the pixcel intensity and Im is the maximal pixel intensity value handled by the image acquisition device; for eight bit devices this would correspond to 255.

8. Heparanase assay

Heparanase was assayed by the method as previously described[26] with some modifications. For FITC-labeling of heparan sulfate, 5 mg of heparan sulfate (Na salt) and 5 mg of fluorescein isothiocyanate (FITC) were dissolved in 1 mL of 0.1 M sodium carbonate (pH 9.5), and incubated with gentle mixing at 4 ℃ overnight. Free FITC was removed from the mixture using a fast-desalting column: PD-10, and fractionated FITC-labeled high molecular weight heparan sulfate by gel filtration using a Sephacryl S-300HR column (2.6×60 cm) equilibrated with 25 mM Tris-HCl, 150 mM NaCl, pH 7.5. A molecular ratio of FITC/ heparan sulfate was determined by measuring FITC and uronic acid contents. For the measurement of heparanase activity, 10 mg/mL FITC-labeled heparan sulfate was mixed with 5 ng of partially purified heparanase derived from B16-BL6 cells in 100 mM sodium acetate, pH 4.2, 0.5% NP-40 and various concentrations of Ca-SP, and incubated at 37 ℃ for 2 h. The enzyme reaction was terminated by addition of 10 μL of 10 mg/mL heparin and heating at 100 ℃ for 5 min. The insoluble materials were precipitated by centrifugation at 10,000×g for 5 min at 4 ℃. The incubation products in the supernatant were analyzed by gel-filtration column: G3000SWXL equipped with fluorescence detector.

9. Cell Growth Assay

Cell growth was assessed by WST-1 Cell Counting Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Briefly, B16-BL6 cells (5×10^3/well) in MEM containing 5% FCS were seeded into 96-well plates. After a 24-h incubation, various concentrations of Ca-SP was added to the well, and the plates were incubated at 37 ℃ for an additional 24 h. Doxorubicin hydrochloride (DOX, Kyowa Hakko Co. Ltd., Tokyo, Japan) was used as a positive control. WST-1 solution was added to each well and incubated at 37 ℃ for 4 h before the termination. The absorbance at 450 nm was measured in an immuno-reader.

10. Assay of Experimental Lung Metastasis of Tumor Cells

Log-phase cell cultures of B16-BL6 melanoma cells were harvested with 1mM EDTA in Ca^{2+}-and Mg^{2+}-free phosphate buffered saline (PBS), washed with serum-free MEM, and resuspended to give appropriate concentrations in PBS. C57BL/6 mice were given by i.v. injection of B16-BL6 (5×10^4/100 μL) cells, admixed with various concentrations of Ca-SP in PBS (100 μL). The mice were killed 14 days after inoculation of the tumor cells, and the lungs were fixed in Bouin's solution. The number of tumor colonies in each lung was recorded under a dissecting microscope.

11. Statistical Analysis

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

RESULTS

1. Effect of Ca-SP on tumor cell invasion and migration

We first examined the effect of Ca-SP on the invasion of two different tumor cells, B16-BL6 melanoma and Colon26 M3.1 adenocarcinoma cells into reconstituted basement membrane components Matrigel in the Trans-

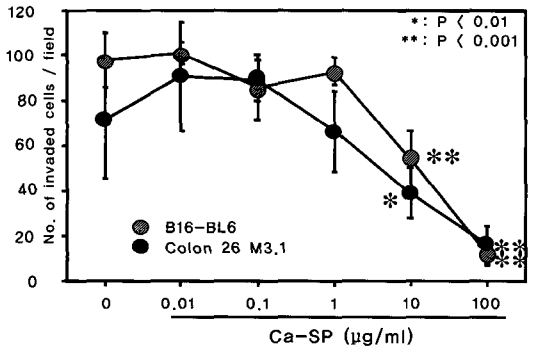

Fig. 3 Effect of Ca-SP on the invasion of B16-BL6 melanoma or Colon 26-M3.1 carcinoma cells into Matrigel

well cell culture chambers. The polycarbonate filters were precoated with Matrigel on the upper surface and with fibronectin on the lower surface in a Transwell chamber. Fig. 3 shows that the invasion of both tumor cells into the Matrigel/fibronectin-coated filters was inhibited by Ca-SP in a concentration-dependent fashion, and achieved 50% reduction at 10 μg/mL.

We also observed that Ca-SP could reduce the haptotactic migration of both B16-BL6 and Colon26 M3.1 cells to laminin in a concentration-dependent manner, but it did not inhibit the migration of both cell lines to fibronectin-coated filters.

2. Effect of Ca-SP on tumor cell adhesion

Since tumor cell adhesion to extracellular matrix (ECM) is considered to be a fundamental step in tumor invasion, we next investigated the effect of Ca-SP on the adhesion of B16-BL6 cells to ECM components. The tumor cells were mixed with Ca-SP at concentrations ranging from 0.1 to 100 μg/mL. Ca-SP significantly inhibited the tumor cell adhesion to Matrigel or laminin-coated substrates in a concentration-dependent manner, but did not affect the cell adhesion to fibronectin-coated substrates. In cell adhesion to other Matrigel components, B16-BL6 cells could not bind directly to heparan sulfate and Ca-SP had no effect on the adhesion to type IV collagen (data not shown).

In a next set of experiments, we further examined the inhibitory mechanism of Ca-SP on tumor cell adhesion to laminin-substrate. B16-BL6 cells were incubated for 30 min on ice with Ca-SP at concentrations ranging from 0.1 to 100 μg/mL, and then washed with PBS. Alternatively, laminin-coated substrates were incubated for 3 h at 37 ℃ with Ca-SP, and then washed with PBS. The pretreatment of tumor cells with Ca-SP prevented the cell attachment to the laminin-substrate in a concentration-dependent manner. However, the pretreatment of the substrates with Ca-SP had no inhibitory effect.

3. Effect of Ca-SP on production and activity of degradation enzymes of tumor cells

Since B16-BL6 cells produced quite low amount of type IV collagenase, we here examined the effect of Ca-SP on production of type IV collagenase from HT-1080 fibrosarcoma cells instead of B16-BL6 cells by gelatin zymography. Ca-SP could not inhibit the production of 92-kD type IV collagenase (MMP-9) from HT-1080 human fibrosarcoma cells even after a 24 h-pretreatment. Interestingly, Ca-SP at 100 μg/mL enhanced the production from HT-1080 cells. Ca-SP also did not affect the production of MMP-2 and gelatinolytic activity of both MMP-2 and -9 in HT-1080 cells (data not shown). On the other hand, Ca-SP inhibited degradation of heparan sulfate by partially purified heparanase from B16-BL6 cells in a concentration-dependent manner. The IC50 value of Ca-SP was about 56 ng/mL, whereas that of suramin was 65 μg/mL.[26]

4. Effect of Ca-SP on the growth of B16-BL6 melanoma

Ca-SP had no inhibitory effect on the growth of B16-BL6 cells at concentrations used in this study (0.01-100 μg/mL). Ca-SP also did not affect the number of cells or the viability after a 24-h incubation (data not shown). On the other hand, DOX as a positive control potently inhibited the cell growth *in vitro*. These results indicated that the inhibition of tumor cell adhesion and invasion is not due to the cytotoxicity of Ca-SP.

5. Effect of Ca-SP on experimental and spontaneous lung metastasis of B16-BL6 melanoma

Since the above results demonstrated that Ca-SP resulted in a marked reduction of tumor invasion *in vitro*, we examined the effect of Ca-SP on the experimental lung metastasis produced by intravenous injection of B16-BL6 melanoma cells. The co-injection of B16-BL6 melanoma cells with Ca-SP achieved significant reduction of number of tumor colonies in lungs in a dose-dependent manner (Fig. 4).

We also investigated the therapeutic effects of Ca-SP on spontaneous lung metastasis of B16-BL6 cells. Ca-SP was administered i.v. into the lateral tail vain of mice after tumor inoculation, but before amputation of primary tumors. Seven intermittent i.v. injections of Ca-SP caused a marked decrease of lung tumor colonies in a dose-dependent manner. In particular, intermittent administrations of 100 μg of Ca-SP achieved a statistically significant reduction of lung tumor colonies ($p<0.005$).

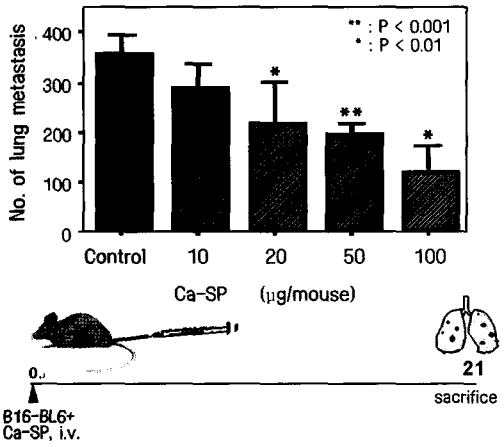

Fig. 4 Effect of Ca-SP on lung metastasis produced by i.v. injection of B16-BL6 melanoma cells

Intravenous administrations of Ca-SP had no effect on the primary tumor size at the amputation time (on day 20).

DISCUSSION

In this study, we showed that Ca-SP successfully inhibited experimental lung metastasis produced by co-injection with B16-BL6 melanoma cells in a dose-dependent manner (Fig. 4) and the therapeutic benefit was suggested in the spontaneous lung metastasis model. Some sulfated polysaccharide such as heparin and heparin-like molecules have been shown to possess the prevention of tumor embolus formation which arised from platelet aggregation and activation of coagulation cascade[2)-5)] and consequently lead to the inhibition of tumor metastasis.[11),12),27)] Our preliminary study observes that Ca-SP did not affect the platelet aggregation elicited by B16-BL6 melanoma cells and blood coagulation (data not shown), but that heparin caused a marked reduction of platelet aggregation.[27)] This suggests that the inhibition of tumor metastasis by Ca-SP may not be associated with interference with tumor embolus formation.

Tumor cell invasion is a complex process involving the attachment of tumor cells to ECM, secretion of enzymes by tumor cells that cause the degradation of adjacent membranes, and the cell migration into target tissue. We here demonstrated that Ca-SP inhibited the invasion of reconstituted basement membrane Matrigel by B16-BL6 cells *in vitro* (Fig. 3). As a result of detailed examination of the anti-invasive mechanism, Ca-SP was selectively able to inhibit the adhesion and migration of B16-BL6 cells to a ECM component laminin in a concentration-dependent manner (Fig. 5). Since short term pretreatment of tumor cells with Ca-SP blocked the adhesion to laminin, the inhibition of tumor cell adhesion to laminin-substrate by Ca-SP would be associated with the binding of Ca-SP to the tumor cell surface rather than laminin-substrate. The detailed mechanism is still unclear. One plausible explanation includes the possibility that Ca-SP may bind to laminin-receptors such as integrins and 67-kDa protein on tumor cell surface, and consequently lead to the inhibition of tumor cell adhesion and invasion. On the other hand, Gabius et al.[28)] have demonstrated that specific receptors for rhamnose were present in lung-metastasizing tumor cells from different types of primary tumor sites, such as sarcoma, melanoma and adenocarcinoma in human tissue. Since a major component in Ca-SP is rhamnose, it is also conceivable that Ca-SP may be recognized by such receptor for rhamnose on the surface. Further study will be needed to determine these points.

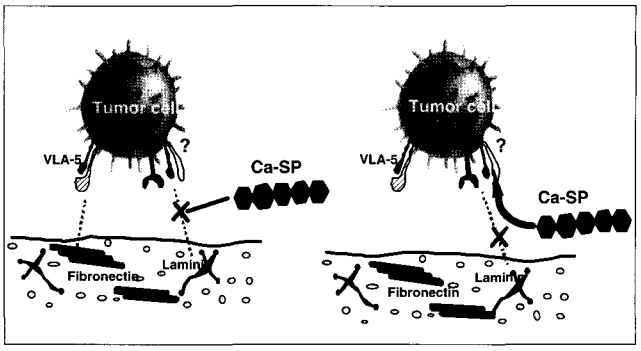

Fig. 5 The anti-invasive mechanism of Ca-SP

We have previously reported that SCM-chitin, a sulfated chitin derivative with anti-invasive and anti-metastatic property, was able to inhibit both heparanase and type IV collagenase activity of tumor cells.[15)] Although Ca-SP failed to prevent both type IV collagenolytic activity (data not shown) and secretion of type IV collagenase in HT-1080 fibrosarcoma cells, it exhibited the significant effect on the inhibition of degradation of heparan sulfate by purified heparanase as compared with suramin. These results suggest that the other possible mechanism of the inhibition of tumor invasion of Matrigel by Ca-SP is the prevention of heparanase digestion of heparan sulfate.

In conclusion, a novel sulfated polysaccharide Ca-SP prevented the tumor invasion of Matrigel probably through the inhibitory mechanism by interfering the adhesion and migration of tumor cells to laminin-substrate and the heparanase digestion of heparan sulfate, and it achieved the inhibition of experimental and spontaneous lung metastasis by B16-BL6 melanoma cells. We are now investigating the effect of Ca-SP on tumor-induced angiogenesis in mice.

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