Carbohydrate Polymers **21** (1993) 299–307

The conjugation of RGDS peptide with CM-chitin augments the peptide-mediated inhibition of tumor metastasis

Hiroyuki Komazawa, Ikuo Saiki,* Yu Igarashi, Ichiro Azuma

Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060, Japan

Seiichi Tokura

Department of Polymer Science, Faculty of Science, Hokkaido University, Kita-10, Nishi-8, Kita-ku, Sapporo 060, Japan

Masayoshi Kojima, Atsushi Orikasa, Mitsunori Ono & Isamu Itoh

Fuji Photo Film Co., Ltd, Ashigara Research Laboratories 210, Nakanuma, Minamiashigara 250-01, Japan

(Received 15 October 1992; revised version received 18 March 1993; accepted 20 March 1993)

A water soluble 6-*O*-carboxymethyl chitin (CM-chitin) containing cell adhesive Arg–Gly–Asp–Ser (RGDS) sequence, i.e. CM-chitin–RGDS conjugate was synthesized, and the inhibitory effects of this compound on lung or liver metastasis of lung-metastatic B16-BL6 melanoma or liver-metastatic L5178Y-ML25 lymphoma cells in mice was examined. CM-chitin–RGDS showed the inhibitory effects on lung metastasis of melanoma cells in a dose-dependent manner (ranging from 100 to 1000 μg) and on liver metastasis of lymphoma cells. A mixture of CM-chitin and RGDS peptide or CM-chitin alone did not show any inhibitory effect on experimental lung metastasis as compared with the conjugate CM-chitin–RGDS on a molar basis. GRGDS peptide, however, required a higher dose (3000 μg) to obtain a sufficiently antimetastatic effect. The in-vitro tumor invasion study showed that CM-chitin–RGDS was apparently more effective for the inhibition of tumor cell penetration into reconstituted basement membrane Matrigel than RGDS or the mixture of RGDS and CM-chitin on a molar basis. Intermittent i.v. administration of CM-chitin–RGDS after the inoculation of B16-BL6 cells caused significant inhibition of spontaneous lung metastasis produced by intrafootpad injection of tumor cells as compared with the multiple administration of RGDS, CM-chitin or untreated control. These results demonstrate the importance of the conjugation of RGDS peptide with CM-chitin as a polymeric carrier for the increased therapeutic potential to cancer metastasis, thus implying a possibility that RGDS–polymer conjugation may lead to the prolongation of antimetastatic action of RGDS peptide *in vivo*.

INTRODUCTION

Metastasis is one of the major cause of mortality in cancer. During the sequential steps of metastasis, metastasizing tumor cells encounter various host cells (platelets, lymphocytes or endothelial cells), extracellular matrix and basement membrane components such as fibronectin, laminin and collagen (Hart, 1982; Liotta *et al.*, 1983; Fidler, 1984; Nicolson, 1987). This interaction may lead to enhancement of the survival, arrest or invasiveness of tumor cells (Liotta *et al.*, 1983; Karpathlan & Pearlstein, 1984; McCarthy & Furcht, 1984; Terranova *et al.*, 1986; Nicolson, 1987). Specific incidents of tumor interaction with host cells or components are, therefore, fundamental events in the metastatic process. Consequently, both adhesion and

*To whom correspondence should be addressed.

Carbohydrate Polymers 0144-8617/93/$06.00

detachment of cells are probably of prime importance in achieving control of the cellular functions of diverse cell types, including highly metastatic tumor cells.

Several studies have sugested that some synthetic peptides derived from adhesion molecules that are present in extracellular matrices, basement membranes or plasma can modulate the mechanism involved in the metastasizing function of tumor cells (Pierschbacher & Rouslahti, 1982; Rouslahti & Pierschbacher, 1986). In attempts to regulate the mechanism involved in cell adhesion during the metastatic process, fibronectin-derived peptides such as RGDS (Humpries *et al.*, 1986*a, b*) and CS1 of alternative splicing type III connecting segment (Humphries *et al.*, 1987; Saiki *et al.*, 1990) and purified 33 kDa heparin-binding fragment (McCarthy *et al.*, 1986 & 1988) have been used to inhibit experimental tumor metastasis in murine tumor systems. However, a high dose of RGD-containing oligopeptide was needed to obtain acceptable effects because of the rapid clearance of the peptides from the circulation and their low affinity for cell surface receptors. Therefore, it may be important to find improved methods to control tumor metastasis. We have previously reported that poly(RGD), which contains the repetitive RGD sequence, inhibited experimental and spontaneous tumor metastasis of different tumors, as well as cell-adhesive properties more effectively than RGD-containing oligopeptides and that the radiolabeled polymeric peptide was biphasically cleared from the circulation by the i.v. injection more slowly than the oligopeptide (Saiki *et al.*, 1989*a, b, c*). This suggested that polymerization of the RGD sequence was able to augment the inhibition of tumor metastasis and that the inhibitory effect was partially due to the slower rate of circulatory clearance and subsequent decomposition of peptides by serum.

Chitin, a (1 → 4) linked polysaccharide composed of 2-acetamide-2-deoxy-β-D-glucopyranose residues, is distributed widely in nature, and has been reported to have some beneficial medicinal (Muzzarelli, 1978) and pharmaceutical (Miyazaki *et al.*, 1981) applications. 6-*O*-Carboxymethyl-chitin (CM-chitin) has been shown to severely decrease the adsorption of blood components such as serum albumin, γ-globulin and fibrinogen as compared with chitin (Nishimura *et al.*, 1987). Also, no antibody induction against CM-chitin was detected (Tokura *et al.*, 1987). Chitin and CM-chitin are known to be susceptible to lysozyme in the human body (Tokura *et al.*, 1983). Miyazaki *et al.* (1981) reported that sustained release of indomethacin and papaverine hydrochloride was obtained from dried gels of chitin and chitosan. The applicability of chitosan as a vehicle for sustained release of propranolol hydrochloride was examined by Sawayanagi *et al.* (1982). We have also demonstrated that CM-chitin gel was easily prepared by the addition of iron chloride into a CM-chtin solution (Watanabe *et al.*, 1990), and anticancer drug-incorporated CM-chitin gel inhibited lung and liver metastases or murine tumor cells more effectively than the drug alone, with decreased toxicity against the host (Saiki *et al.*, 1992; Watanabe *et al.*, 1992). Thus, in order to suppress the toxic side effect of anticancer drugs and to improve their efficiency, an attempt to conjugate drugs to polymeric carriers appears to be a promising approach. The conjugation of drugs with a polymer would be expected to provide some advantageous features such as preferable tissue distribution of the drug, prolonged half life of the drug in plasma, controlled drug release and reduction of drug toxicity, etc. (Ringsdorf, 1975). Therefore, many polymeric drugs have been extensively investigated (Hirano *et al.*, 1985; Duncan *et al.*, 1988; Yokoyama *et al.*, 1990).

In the present study, we report the synthesis of a water-soluble CM-chitin conjugated with the cell adhesive Arg–Gly–Asp–Ser (RGDS) peptide, i.e. CM-chitin–RGDS and the effects of this compound on experimental lung or liver metastasis in mice, as well as the therapeutic effects on spontaneous lung metastasis in mice. We have also tested CM-chitin–RGDS in an in-vitro tumor cell invasion assay.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all commercially available solvents and reagents were used without further purification. *N,N*-Dimethylformamide(DMF) was stored over molecular sieves (3 Å) for several days before use. *t*-Butoxycarbonyl(Boc-) amino acids and Gly–Arg–Gly–Asp–Ser pentapeptide (GRGDS) were purchased from Kokusan Chemical Works Ltd. CM-chitin was a kind gift from Yaizu Suisan Co., Ltd. CM-chitin was dialyzed against deionized water using Visking tube for 1 day and precipitated with acetone, washed with ethanol followed by ether, dissolved in water again and dried *in vacuo* before use.

^{1}H-NMR spectra were recorded with a Bruker AC-200 (200 MHz) spectrometer in chloroform-*d*, dimethyl-d_6 sulfoxide or deuterium oxide, using tetramethylsilane (TMS) or 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS) as an internal standard. Gel permeation chromatography (GPC) was carried out on a Shimadzu LC-9A HPLC system which was calibrated with pullulan standard, and data analysis was performed using a Shimadzu Chromatopac C-R4A. TOSOH G3000PW$_{XL}$ and G4000PW$_{XL}$ columns were connected in series, and 0·2 M phosphate buffer at pH 7·4 was used as the eluent. The modified CM-chitin was hydrolyzed with 6 N HCl for 24 h at 110°C. Amino acid analysis of the hydrolysate was performed using a Beckman System 7300. Mass spectra were obtained on a JEOL DX-303 with either EI or FAB ionization. Reac-

tions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel $60F_{256}$ (Merck).

Synthesis of 6-*O*-carboxymethyl chitin (CM-chitin) conjugated with RGDS (CM-chitin-RGDS)

Boc–Ser(Bzl)–OBzl

In a typical preparation, to 60 g (0·2 mol) of Boc–Ser(Bzl) in 400 ml ethyl acetate was added 21 g (0·20 mol) of triethylamine and 35·4 g (0·20 mol) of benzyl bromide, and the solution was refluxed for 4 h (Scheme 1). After cooling, triethylammonium bromide was removed by filtration. The filtrate was washed with 9% aqueous $NaHCO_3$, followed by 36% aqueous NaCl, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. 54 g (68%) Boc–Ser(Bzl)–OBzl (**1**) was obtained as colourless solid.

Mass spectrum (FAB) (M + H) + 386.

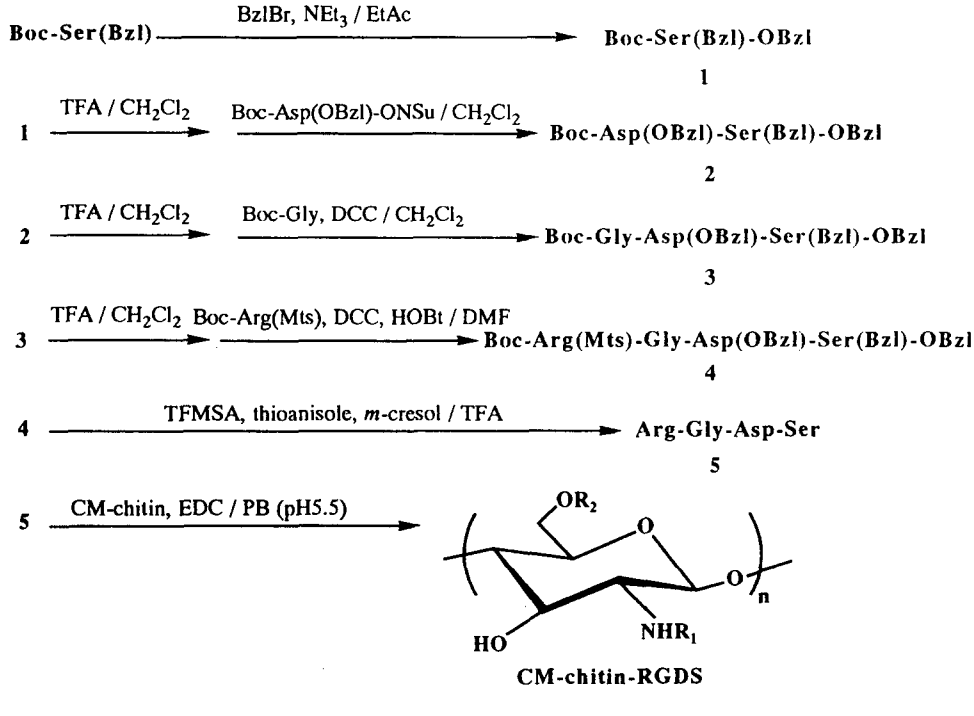
Boc–Asp(OBzl)–Ser(Bzl)–OBzl

To 30 g of **1** in 100 ml CH_2Cl_2, 100 ml of trifluoroacetic acid (TFA) was added, and the solution stirred at room temperature for 1 h. After removing the TFA and CH_2Cl_2 by evaporation, the TFA salt was dissolved in ethyl acetate, washed with 9% aqueous $NaHCO_3$ followed by 39% aqueous NaCl. The solution was dried over Na_2SO_4 and evaporated to dryness. To the residue in 500 ml CH_2Cl_2, 32·8 g (78 mmol) Boc–Asp (OBzl)*N*–hydroxysuccinimide ester [Boc–Asp(OBzl)–ONSu] was added and stirred at room temperature overnight. After evaporation of the CH_2Cl_2 under reduced pressure, the residue was dissolved in ethyl acetate followed by washing with 9% aqueous $NaHCO_3$, 1 M citric acid and 36% aqueous NaCl. The solution was dried over Na_2SO_4 and evaporated to dryness. 41 g (89%) Boc–Asp(OBzl)–Ser(Bzl)–OBzl (**2**) was obtained as a colourless solid. The protected dipeptide **2** was used for the next condensation reaction without further purification.

Mass spectrum (EI) *m/Z* 590.

Boc–Gly–Asp(OBzl)–Ser(Bzl)–OBzl

To 35 g (59 mmol) of **2** in 100 ml CH_2Cl_2, 100 ml TFA was added and stirred at room temperature for 1 h. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate, washed with 9% aqueous $NaHCO_3$ followed by 36% aqueous NaCl and dried over Na_2SO_4. Evaporation of the solution to dryness gave Asp(OBzl)–Ser(Bzl)–OBzl. To a mixture of 9·8 g (59 mmol) Boc–Gly and Asp(OBzl)–Ser(Bzl)–OBzl in 150 ml CH_2Cl_2, 12·2 g dicyclohexylcarbodiimide (DCC) at 0°C was added and the solution stirred for 3 h at 0°C (ice bath). The ice bath was then removed and the reaction continued at room temperature overnight. DC urea was removed by filtration, and the filtrate evaporated to dryness. The crude



Scheme 1. Synthetic procedure for the CM-chitin–RGDS.

product was dissolved in ethyl acetate, washed with 9% aqueous $NaHCO_3$, 1 M citric acid, 36% aqueous NaCl and then dried over Na_2SO_4. The solvent was removed under reduced pressure, and the product was purified by silica gel column chromatography using ethyl acetate-*n*-hexane (50/50 (*v*/*v*)) as an eluent; 30·5 g (75%) Boc–Gly–Asp(OBzl)–Ser(Bzl)–OBzl (**3**) (colourless solid) was obtained.

Mass spectrum (EI) *m*/Z 647.

Boc–Arg(Mts)–Gly–Asp(OBzl)–Ser(Bzl)–OBzl

To 25 g (39 mmol) of **3** in 100 ml CH_2Cl_2, 100 ml TFA was added and stirred at room temperature for 1 h. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate, washed with 9% aqueous $NaHCO_3$, followed by 36% aqueous NaCl and dried over Na_2SO_4. Evaporation of the solution to dryness gave Gly–Asp (OBzl)–Ser(Bzl)–OBzl. To a mixture of 17·8 g (39 mmol) Boc–Arg(Mts) and Gly–Asp(OBzl)–Ser(Bzl)–OBzl in 400 ml DMF was added 8·0 g (39 mmol) DCC and 6·8 g 1-hydroxybenzotriazole (HOBt) (45 mmol) at 0°C, and the solution was stirred for 3 h at 0°C (ice bath), and then at room temperature overnight. DC urea was removed by filtration, and the filtrate was evaporated to dryness. The crude product was dissolved in ethyl acetate, washed with 9% aqueous $NaHCO_3$, 1 M citric acid, 36% aqueous NaCl, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the product was recrystallized from a small amount of ethyl acetate; 19·5 g (50%) Boc–Arg(Mts)–Gly–Asp(OBzl)–Ser(Bzl)–OBzl (**4**) was obtained.

Mass spectrum (FAB) (M + H) + 986.

Anal. Calcd. for $C_{50}H_{63}N_7O_{12}S_1$ (986·1): C, 60·89; H, 6·43; N, 9·94; S, 3·25. Found C, 60·47; H, 6·43; N, 9·87; S, 3·15.

Deprotection of Boc–Arg(Mts)–Gly–Asp(OBzl)–Ser(Bzl)–OBzl

To 5·0 g of **4** in 25 ml TFA at 0°C, trifluoromethanesulfonic acid (TFMSA) (25 g), thioanisole (19·7 ml) and *m*-cresol (17·4 ml) in 90 ml TFA was added and stirred for 1 h. The solution was precipitated with ether, washed several times with ether, dissolved in water and applied to an Amberlite IRA-400(Cl form) column. The ninhydrin positive fractions were collected and 2·10 g (88%) Arg–Gly–Asp–Ser (**5**) was obtained by lyophilization. If necessary the product can be further purified by reprecipitation with acetonitrile to remove trace amounts of deprotection reagents.

Mass spectrum (FAB) (M + H) + 434.

^{1}H-NMR $\delta(D_2O)$, 1·73 (*m*, 2H, Arg γCH_2), 1·95 (*m*, 2H, Arg βCH_2), 2·92 (*m*, 2H, Asp βCH_2), 3·25 (*m*, 2H, Arg δCH_2), 4·05 (*m*, 2H, Gly αCH_2), 4·53 (*m*, 1H, Asp αCH).

Synthesis of CM-chitin–Arg–Gly–Asp–Ser

To the solution of 0·50 g CM-chitin containing 1·49 mmol carboxyl groups in 40 ml (pH 5·5) phosphate buffer solution (PB), 231 mg (1·49 mmol) of 1-ethyl-3,3-(dimethylaminopropyl)-carbodimide in 5·0 ml PB was added and stirred at 0°C for 1·5 h. Then 1·29 g (3·0 mmol) Arg–Gly–Asp–Ser (**5**) in 5·0 ml PB was added to the solution and allowed to react at 0°C for 1·5 h then at room temperature for 2 days. The modified CM-chitin was purified by dialysis against deionized water using Spectra/Por7 (cutoff mol. wt 8000) and lyophilized. A colourless solid (1·00 g) was obtained. The RGDS content of the product was calculated from the results of amino acid analysis.

Amino acid analysis: 256 μg RGDS/1000 μg CM-chitin–Arg–Gly–Asp–Ser.

GPC data: Mn 400 000.

Mice

Specific pathogen-free female C57BL/6, and CDF_1 (BALB/c × DBA/2) mice, 7–10 weeks old, were purchased from Japan SLC, Inc., Hamamatsu, Japan. The mice were maintained in the Laboratory for Animal Experiments, Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. All mice used in this study were sex-matched.

Cells

Highly metastatic B16-BL6 melanoma cells, obtained by an in-vitro selection procedure for invasion (Hart, 1979), were kindly provided by Dr I.J. Fidler, M.D. Anderson Cancer Center, Houston, TX. Liver metastatic L5178Y-ML25 cells (partially metastasizing to the spleen), were kindly provided by Dr A. Okura, Banyu Pharmaceutical Co., Tokyo (Watanabe *et al.*, 1988).

B16-BL6 cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7·5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. L5178Y-ML25 cells were maintained in RPMI-1640 supplemented with 7·5% FBS and L-glutamine.

Assay for experimental and spontaneous lung metastases of melanoma cells

C57BL/6 mice were given i.v. injection of B16-BL6 melanoma cells (5×10^4) admixed with compounds. Fourteen days after the inoculation of the tumor cells, the mice were killed and the number of lung tumor colonies was recorded (experimental metastasis). In a spontaneous lung metastasis assay, mice were given s.c. injections of B16-BL6 melanoma cells (5×10^5) into the right hind footpad. The compounds were administered i.v. on various days after tumor inoculation and the

surgical excision of primary tumors was carried out on day 21. Mice were killed 14 days after the surgical excision. The lungs were fixed in Bouin's solution and the lung tumor colonies were counted under a dissecting microscope.

Assay for liver metastasis of lymphoma cells

CDF_1 mice were given i.v. injection of L5178Y-ML25 T-lymphoma cells (4×10^4) with or without compounds in phosphate-buffered saline (PBS). Fourteen days later, the mice were killed and the liver weights were recorded to evaluate tumor metastasis as previously described (Watanabe *et al.*, 1988).

Invasion assay

The invasive activity of tumor cells was assayed in a Transwell cell culture chamber (Costar No. 3422, Cambridge, MA) according to the methods described previously (Saiki *et al.*, 1990). Polyvinylpyrrolidone-free polycarbonate filters with an 8·0 μm pore size (Nucleopore, Pleasanton, CA) were precoated with 5 μg of fibronectin in a volume of 50 μl on their lower surfaces, and dried at room temperature. Reconstituted basement membrane Matrigel (Collaborative Research Inc., MA) was diluted to 100 μg/ml with cold PBS, applied to the upper surfaces of the filter (5 μg/filter), and dried overnight at room temperature under a hood. The filters thus prepared were designated Matrigel/fibronectin-coated filters. 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 mM EDTA in PBS, washed three times with serum-free MEM, and resuspended to a final concentration of 2×10^6/ml in MEM with 0·1% bovine serum albumin (BSA). Cell suspensions (100 μl) with or without agents were added to the upper compartment and incubated for the appropriate number of hours at 37°C in a 5% CO_2 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 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, and each assay was performed in triplicate.

Statistical analysis

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

RESULTS

Effect of CM-chitin–RGDS on experimental lung metastasis of B16-BL6 melanoma

When CM-chitin–RGDS was co-injected i.v. with B16-BL6 melanoma cells into C57BL/6 mice and the lung tumor colonies were counted 14 days after tumor inoculation (Fig. 1(A)), it drastically reduced the number of tumor colonies in the lungs in a dose-dependent manner (ranging from 100 to 1000 μg per mouse). CM-chitin or GRGDS peptide at a dose of 1000 μg per mouse did not show the antimetastatic activity. However, GRGDS peptide at a dose level of 3000 μg significantly inhibited lung metastasis. In addition, 1000 μg of CM-chitin–RGDS, which contains equivalent amounts of 744 μg of CM-chitin and 256 μg of RGDS peptide, inhibited lung metastasis more effectively than RGDS, CM-chitin or their mixture (Fig. 1(B)). These results indicate that the conjugation of RGDS peptide with CM-chitin as a polymeric carrier

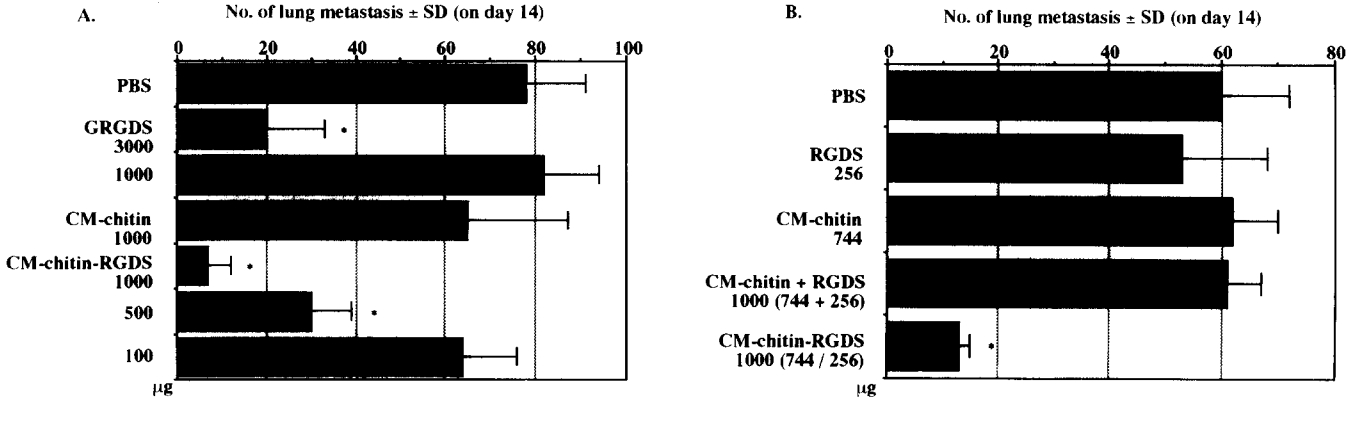


Fig. 1. Effect of CM-chitin–RGDS on experimental lung metastasis produced by i.v. injection of B16-BL6 melanoma cells. Five C57BL/6 mice per group were injected i.v. with B16-BL6 melanoma cells (5×10^4) admixed with or without CM-chitin–RGDS and its related compounds at the indicated doses. Mice were killed two weeks after tumor inoculation and lung tumor colonies were manually counted. $^*P < 0.001$ as compared with control (PBS) by Student's two-tailed *t*-test.

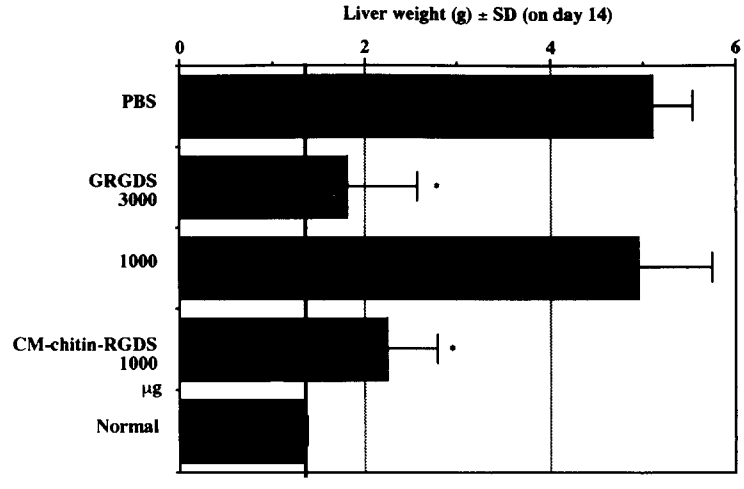


Fig. 2. Effect of CM-chitin–RGDS on liver metastasis produced by i.v. injection of L5178Y-ML25 T-lymphoma cells. Five CDF_1 mice per group were injected i.v. with L5178Y-ML25 T-lymphoma cells (4×10^4) admixed with or without CM-chitin–RGDS and its related compounds at the indicated doses. Mice were killed two weeks after tumor inoculation and liver weights were measured. $^*P < 0.001$ as compared with control (PBS) by Student's two-tailed *t*-test.

may lead to the augmentation of RGDS-mediated inhibition of lung metastasis.

Effect of CM-chitin–RGDS on experimental liver metastasis of L5178Y-ML25 T-lymphoma

We next examined the effect of CM-chitin–RGDS on liver metastasis by the i.v. coinjection of L5178Y-ML25 T-lymphoma cells (Fig. 2). When the tumor cells were injected i.v. into CDF_1 mice, liver weights of the mice were increased approximately four-fold as compared to those of normal mice. The co-injection of tumor cells with 1000 μg of CM-chitin–RGDS significantly inhibited liver metastasis. In contrast, GRGDS exhibited the inhibition of liver metastasis of L5178Y-ML25 lymphoma at a dose of 3000 μg per mouse, but it had no effect at 1000 μg per mouse. CM-chitin–RGDS was more effective for the inhibition of liver metastasis of lymphoma cells as well as lung metastasis of melanoma cells than GRGDS pentapeptide.

Inhibition of spontaneous lung metastasis by systemic administration of CM-chitin–RGDS

We also investigated the therapeutic effect on spontaneous lung metastasis of the multiple systemic administration of CM-chitin–RGDS. The first i.v. administration of CM-chitin–RGDS began on day 7 or day 17 after tumor inoculation, and the treatments were carried out 7 times at 1-day interval. Primary tumors were surgically removed on day 21 after tumor inoculation. Seven intermittent i.v. administrations of CM-chitin–RGDS from day 7 after tumor inoculation caused a marked decrease of lung tumor colonies at a dose of 100 or 250 μg per mouse (Exp. I and II of Table 1). However, CM-chitin or RGDS peptide did not show any inhibition of spontaneous lung metastasis. As shown in Exp. III of Table 1, seven intermittent treatments of CM-chitin–RGDS from day 17 after tumor inoculation resulted in the significant inhibition of lung metastasis of melanoma cells, whereas the treatments of RGDS peptide had no effect. This suggests that the conjugation of RGDS with CM-chitin can lead to a sustained effect for RGDS-mediated inhibition of tumor metastasis. In these experiments, multiple i.v. administrations of CM-chitin–RGDS did not affect the primary tumor growth (size) at the time of removal on day 21. The incubation of B16-BL6 melanoma cells with 250 μg/ml of CM-chitin–RGDS, CM-chitin or RGDS *in vitro* did not affect the incorporation of [^{3}H]-thymidine into tumor cells (data not shown). This result indicates that CM-chitin–RGDS was not directly cytotoxic nor did it inhibit cell growth.

Effect of CM-chitin–RGDS on the invasion of B16-BL6 melanoma cells

Tumor cell invasion into extracellular matrices and basement membranes is a crucial step in the complex multistage process of metastasis. Therefore, we examined the effect of antimetastatic CM-chitin–RGDS on tumor cell invasion of Matrigel (reconstituted basement membrane components). B16-BL6 cells were added to the upper compartment of Transwell chambers in the presence or absence of CM-chitin–RGDS, CM-chitin or RGDS peptide. The invasion of tumor cells through the Matrigel/fibronectin-coated filters was inhibited by CM-chitin–RGDS, RGDS and a mixture of RGDS and CM-chitin [at a similar molar ratio to CM-chitin–RGDS] in a concentration-dependent manner of RGDS, whereas CM-chitin alone showed no anti-invasive activity (Fig. 3). These results indicate that the

Table 1. Effect of CM-chitin–RGDS on spontaneous lung metastasis produced by intrafootpad injection of B16-BL6 melanoma cells

Treatment	Timing (on day)	Dose (μg)	Primary tumor size on day 21: Mean ± SD (mm)	No. of lung tumor colonies on day 35		p
				Mean ± SD	(Range)	
Exp. I	7, 9, 11, 13, 15, 17, 19					
PBS		—	12 ± 2	92 ± 11	(67–104)	
RGDS		250 × 7	12 ± 2	101 ± 20	(81–138)	
CM-chitin		250 × 7	11 ± 2	81 ± 18	(59–105)	
CM-chitin–RGDS		250 × 7	12 ± 2	55 ± 11	(37–71)	< 0·001
Exp. II	7, 9, 11, 13, 15, 17, 19					
PBS		—	12 ± 2	81 ± 20	(45–119)	
RGDS		100 × 7	12 ± 2	78 ± 20	(59–119)	
CM-chitin–RGDS		100 × 7	10 ± 2	31 ± 7	(23–42)	< 0·01
Exp. III	17, 19, 22, 24, 26, 28, 30					
PBS		—	12 ± 2	64 ± 24	(45–104)	
RGDS		250 × 7	11 ± 2	58 ± 14	(23–86)	
CM-chitin–RGDS		250 × 7	11 ± 2	13 ± 4	(6–17)	< 0·01

Five C57BL/6 mice per group were administered i.v. with the compounds at the indicated time after intrafootpad injection of B16-BL6 melanoma cells (5×10^5 per 50 μl). Primary tumors were surgically removed on day 21. Mice were killed 2 weeks after tumor excision and tumor colonies in the lungs were manually counted.

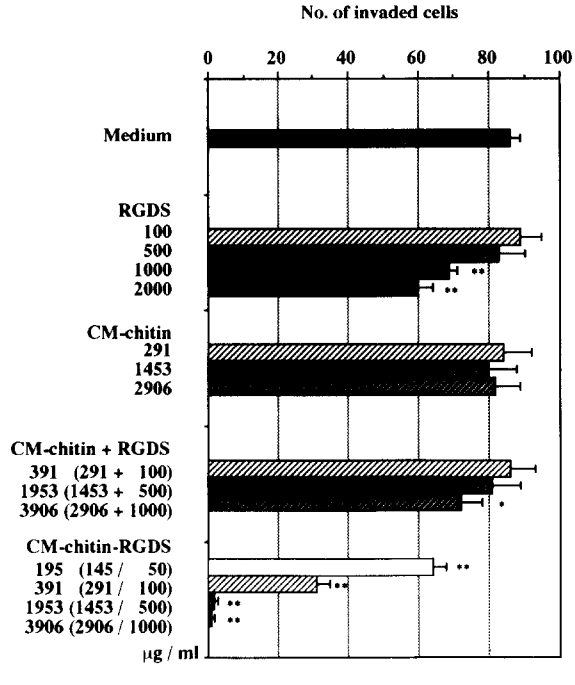


Fig. 3. Effect of CM-chitin–RGDS on the invasion of B16-BL6 melanoma cells into Matrigel/fibronectin-coated filters. Filters were precoated with 5 μg of fibronectin on their lower surfaces, and with Matrigel (5 μg) on their upper surfaces. B16-BL6 melanoma cells (2×10^5/well) in 0·1% BSA medium were seeded with RGDS (100, 500, 1000, 2000 μg/ml), CM-chitin (291, 1453, 2906 μg/ml), CM-chitin + RGDS (291 + 100, 1453 + 500, 2906 + 1000 μg/ml) or CM-chitin–RGDS (CM-chitin/RGDS = 145/50, 291/100, 1453/500, 2906/1000 μg/ml) into the upper compartment of the Transwell cell culture chamber. After 4 h of incubation, the invaded cells on the lower surfaces were visually counted. $*P < 0·01$, $**P < 0·001$ as compared with control (medium) by Student's two-tailed t-test.

inhibitory effect on tumor cell invasion was substantially due to the RGDS sequence. CM-chitin–RGDS conjugate was much more effective for the inhibition of tumor invasion *in vitro* as well as tumor metastasis *in vivo* than a mixture of CM-chitin and RGDS peptide, or either alone.

DISCUSSION

We have attempted to control the mechanism involved in the cell function such as adhesion, migration and invasion of tumor cells during the metastatic process. Poly(RGD), which contains a repetitive structure of RGD sequence derived from fibronectin, showed anti-metastatic effect on experimental and spontaneous tumor metastasis more effectively than RGD-containing oligopeptide on a weight basis and the prolongation of the survival rate of the mice when it was administered i.v. after tumor inoculation (Saiki *et al.*, 1989*a*, *b*, *c*). These results suggest that RGD-containing peptide could prevent the cancer metastasis through RGD-dependent mechanisms for the inhibition of cellular adhesive interactions between tumor and host. Our data also suggests that the potent antimetastatic effect of polymerized RGD peptide might be related to the relatively (approximately six-fold) slow clearance in the circulation as compared with RGD oligopeptide (Saiki *et al.*, 1989*a*). In general, most peptides, such as RGD-containing peptides as well as cytokines or anticancer drugs, have very short half lives in the circulation, which results in a decrease in their therapeutic and biological potential *in vivo*. An increase in the half-life of the drug

in circulation without its toxicity may lead to the augmentation of its biological effect.

In this study, we synthesized a water-soluble chitin derivative containing a cell adhesive RGDS sequence (CM-chitin–RGDS), to test the inhibitory effect on experimental and spontaneous metastasis in mice and on invasiveness of tumor cells *in vitro*. CM-chitin–RGDS achieved the inhibition of lung metastasis by co-injection with B16-BL6 melanoma cells more effectively than GRGDS pentapeptide on a weight basis. CM-chitin alone showed no effect on tumor metastasis as described previously (Murata *et al.*, 1989). CM-chitin–RGDS inhibited lung metastasis in a dose-dependent manner, and the minimum effective dose was 500 μg/mouse (Fig. 1(A)). CM-chitin–RGDS containing an equivalent amount of 256 μg RGDS peptide exhibited an inhibitory effect on experimental lung metastasis, but 256 μg RGDS tetrapeptide alone or a mixture of 256 μg RGDS peptide and 744 μg CM-chitin at a similar molar ratio to CM-chitin–RGDS did not result in the reduction of lung tumor colonization (Fig. 1 (B)). These results suggest that covalent conjugation of CM-chitin and cell adhesive RGDS peptide was important for the expression of antimetastatic effect *in vivo*.

CM-chitin–RGDS exhibited potent inhibition of liver metastasis of L5178Y-ML25 T-lymphoma cells as compared with GRGDS pentapeptide (Fig. 2), as well as the inhibition of lung metastasis of B16-BL6 melanoma. In the spontaneous lung metastasis produced by intrafootpad inoculation of B16-BL6 melanoma cells, CM-chitin–RGDS caused significant inhibition of lung metastasis by multiple systemic administration after tumor inoculation, whereas CM-chitin or RGDS peptide did not show any inhibitory effect (Table 1). These results indicate that the conjugation of RGDS peptide and CM-chitin might lead to the prolongation of the cell adhesive RGDS action.

The invasion of B16-BL6 melanoma cells through the Matrigel/fibronectin-coated filters was inhibited by CM-chitin–RGDS, RGDS and a mixture of RGDS and CM-chitin (at a similar molar ratio to CM-chitin–RGDS) in a concentration-dependent manner of RGDS, whereas CM-chitin alone showed no anti-invasive activity (Fig. 3). This result clearly indicates that the inhibition of tumor invasion is RGDS dependent. CM-chitin–RGDS conjugate was much more effective for the inhibition of tumor invasion *in vitro* as well as tumor metastasis *in vivo* than a mixture of CM-chitin and RGDS peptide, or either alone, and that multivalent units of the RGDS sequence of CM-chitin–RGDS could augment the biological activities more effectively than the monovalent unit of RGDS tetrapeptide. In general, when multivalent ligands such as antibody or lectins bind to specific plasma membrane proteins exposed on the surface of cells, they tend to cross-link them into large clusters. β_1-Integrin complexes were also localized in focal adhesion plaques on fibronectin substrate. CM-chitin–RGDS has plural RGDS peptides, and might be able to concentrate the RGDS sequence around cell surface integrin receptors as compared with monovalent RGDS. Therefore β_1-integrin complexes might be clustered by binding with multivalent RGDS of CM-chitin–RGDS. Consequently, CM-chitin–RGDS can block more effectively than monovalent RGDS tetrapeptide. On the other hand, it might be possible that the conformational properties of the RGDS sequence of CM-chitin–RGDS may contribute to the enhancement of the specific interaction between ligands and cell surface receptors. The conjugation of RGDS peptide with CM-chitin may also be attributable to augmentation or sustaining of peptide-mediated antimetastatic effect.

Further study will be needed to examine the detailed mechanisms of CM-chitin–RGDS conjugate for the inhibition of tumor metastasis and invastion, the dosage schedules for the prevention of spontaneous metastasis, and the behavior of the conjugate in the circulation, etc. However, it seems probable that the application of the conjugation of antimetastatic peptide with water-soluble chitin derivative as a representative carrier might provide a promising basis for the treatment of cancer metastasis.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from Japanese Ministry of Education, Science and Culture; from the Japanese Ministry of Health and Welfare for Comprehensive 10-Year Strategy for Cancer Control; and for Scientific Research from the Japanese Ministry of Education, Science and Culture; by Grants-in-Aid for Special Project Research from Hokkaido University, Japan; and also by NHK Public Welfare Organization, Japan. We also thank Ms. M. Sato for typing the manuscript.

REFERENCES

Duncan, R., Kopeckova, P., Strohalm, J., Hume, I.C., Lloyd, J.B. & Kopecek, J. (1988). *Br. J. Cancer*, **57**, 147–56.

Fidler, I.J. (1984). In *Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects*, ed. G.L. Nicolson & L. Milas. Raven Press, New York, USA, pp. 5–26.

Hart, I.R. (1979). *Am. J. Pathol.*, **97**, 587–92.

Hart, I.R. (1982). *Cancer Metastasis Rev.*, **1**, 5–16.

Hirano, T., Ohashi, S., Morimoto, S., Tsuda, K., Kobayashi, T. & Tsukagoshi, S. (1985). *Makromol Chem.*, **187**, 2815–24.

Humphries, M.J., Olden, K. & Yamada, K.M. (1986*a*). *Science*, **233**, 467–70.

Humphries, M.J., Yamada, K.M. & Olden, K. (1986*b*). *J. Clin. Invest.*, **81**, 782–90.

Humphries, M.J., Komoriya, A., Akiyama, S.K., Olden, K. & Yamada, K.M. (1987). *J. Biol. Chem.*, **262**, 6886–92.

Karpathlan, S. & Pearlstein, E. (1984). In *Hemostasis*

Mechanisms and Metastasis, ed. K.V. Honn & B.F. Sloane. Martinus Nijhoff, Boston, USA, pp. 139–69.
Liotta, L.A., Rao, C.V. & Barsky, S.H. (1983). *Lab. Invest.*, **49**, 636–49,
McCarthy, J.B. & Furcht, L.T. (1984). *J. Cell Biol.*, **98**, 1474–80.
McCarthy, J.B., Hagen, S.T. & Furcht, L.T. (1986). *J. Cell Biol.*, **102**, 179–88.
McCarthy, J.B., Skubitz, A.P.N., Palm, S.L. & Furcht, .L.T. (1988). *J. Natl Cancer Inst.*, **80**, 108–16.
Miyazaki, S., Ishii, K. & Nadai, T. (1981). *Chem. Pharm. Bull.*, **29**, 3067–9.
Murata, J., Saiki, I., Nishimura, S., Nishi, N., Tokura, S. & Azuma, I. (1989). *Jpn J. Cancer Res.*, **80**, 866–72.
Muzzarelli, R.A.A. (ed) (1978). In *Chitin*, Pergamon Press, Oxford, UK, pp. 139–52.
Nicolson, G.L. (1987). *Cancer Res.*, **47**, 1473–87.
Nishimura, S., Ikeuchi, Y. & Tokura, S. (1984). *Carbohydr. Res.*, **134**, 305–12.
Pierschbacher, M.D. & Rouslahti, E. (1982). *Nature*, **309**, 30–3.
Ringsdorf, H. (1975). *J. Polym. Sci., Polym. Symp.*, **51**, 135–53.
Rouslahti, E. & Pierschbacher, M.D. (1986). *Cell*, **44**, 517–18.
Saiki, I., Iida, J., Murata, J., Ogawa, R., Nishi, N., Sugimura, K., Tokura, S. & Azuma, I. (1989*a*). *Cancer Res.*, **49**, 3815–22.
Saiki, I., Murata, J., Nishi, N., Sugimura, K. & Azuma, I. (1989*b*). *Brit. J. Cancer*, **59**, 194–7.
Saiki, I., Murata, J., Iida, J., Sakurai, T., Nishi, N., Matsuno, K. & Azuma, I. (1989*c*). *Brit. J. Cancer*, **60**, 722–8.
Saiki, I., Murata, J., Makabe, T., Matsumoto, Y., Ohdate, Y., Kawase, Y., Taguchi, Y., Shimojo, T., Kimizuka, F., Kato, I. & Azuma, I. (1990). *Jpn J. Cancer Res.*, **81**, 1003–11.
Saiki, I., Watanabe, K., Tokura, S. & Azuma, I. (1992). In *Chitin Derivatives in Life Science*, ed. S. Tokura & I. Azuma. Japanese Society for Chitin/Chitosan, Chiba, Japan, pp. 20–6.
Sawayanagi, Y., Nambu, N. & Nagai, T. (1982). *Chem. Pharm. Bull.*, **30**, 4213–15.
Terranova, V.P., Hujanen, E.S. & Martin, G.R. (1986). *J. Natl Cancer Inst.*, **77**, 311–6.
Tokura, S., Nishi, N., Nishimura, S. & Somorin, O. (1983). *Sen-i Gakkaishi*, **39**, T-507–11.
Tokura, S., Hasegawa, O., Nishimura, S., Nishi, N. & Takatori, T. (1987). *Anal. Biochem.*, **161**, 117–22.
Watanabe, Y., Okura, A., Naito, K. & Kobayashi, M. (1988). *Jpn J. Cancer Res.*, **79**, 1208–16.
Watanabe, K., Saiki, I., Uraki, Y., Tokura, S. & Azuma, I. (1990). *Chem. Pharm. Bull.*, **38**, 506–9.
Watanabe, K., Saiki, I., Matsumoto, Y., Azuma, I., Seo, H., Uraki, Y., Miura, Y. & Tokura, S. (1992). *Carbohydr. Polym.*, **17**, 29–37.
Yokoyama, M., Miyauchi, M., Yamada, N., Okano, T., Sakurai, Y., Kataoka, K. & Inoue, S. (1990). *J. Controlled Release*, **11**, 269–78.