J. Pharm. Pharmacol. 2000, 52: 67–73
Received April 7, 1999
Accepted August 31, 1999

Facile Synthesis of a Chitosan Hybrid of a Laminin-related Peptide and Its Antimetastatic Effect in Mice

KEIKO HOJO, MITSUKO MAEDA, YU MU*, HARUHIKO KAMADA*, YASUO TSUTSUMI*, YASUHIRO NISHIYAMA†, TOMOKO YOSHIKAWA†, KEISUKE KURITA†, LAWRENCE H. BLOCK‡, TADANORI MAYUMI* AND KOICHI KAWASAKI

*Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Ikawadani-cho, Nishi-ku, Kobe 651-2180, Japan, *Faculty of Pharmaceutical Sciences, Osaka University, Yamadaoka 1-6, Suita 565-0871, Japan, †Faculty of Engineering, Seikei University, Musashino-shi, Tokyo 180-8633, Japan, and ‡Graduate School of Pharmaceutical Sciences, Duquesne University, Mellon Hall of Science, Pittsburgh, PA 15282-1504, USA*

Abstract

Laminin, a cell adhesion protein, consists of three peptide chains (α-1, β-1 and γ-1). The β-1 chain contains a Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence that has been found to inhibit experimental metastasis in mice. We have prepared a hybrid of a water-soluble chitosan and a laminin-related peptide, and have examined its inhibitory effect on experimental metastasis in mice.

A laminin-related peptide, acetyl-Tyr-Ile-Gly-Ser-Arg-βAla-OH (Ac-YIGSRβA-OH), was prepared by a solid-phase method. Ac-YIGSRβA-OH was then reacted with a water-soluble chitosan. βAla is a spacer and was placed to avoid racemization of the Arg residue when the peptide was coupled with chitosan. Although chitosan has amino groups, they did not react with the peptide. Four methods were tried to achieve a coupling reaction, the diphenylphosphoryl azide method, the diisopropylcarbodiimide/1-hydroxybenzotriazole method, the water-soluble carbodiimide (WSC), and the 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) method, but all four methods were unsuccessful. Therefore, a small spacer, *tert*-butyloxycarbonyl-Gly, was intercalated in chitosan, by the TBTU method, to facilitate its coupling with the peptide. After removal of the protecting group, the Gly-chitosan was coupled with Ac-YIGSRβA-OH by the water-soluble carbodiimide method to give Ac-YIGSRβAG–chitosan.

Conjugation of the peptide with the larger chitosan molecule did not reduce the inhibitory effect of the peptide on experimental metastasis in mice, it actually potentiated the antimetastatic effect, demonstrating that chitosan may be effective as a drug carrier for peptides.

Bioactive peptides are presumed to be useful drugs, but their biological half-lives are often too short to permit clinical use. Polymer hybrids of bioactive materials (especially proteins) are the focus of many studies in drug delivery due to the resultant improvement in drug delivery and the enhancement of the therapeutic effect. We have studied poly (ethylene glycol) (PEG) hybrids of bioactive peptides (such as fibronectin-related peptides and laminin-related peptides) and have successfully prepared PEG hybrids of peptides (such as Arg-Gly-Asp (RGD) derived from fibronectin (Pierschbacher & Ruoslahti 1984) and Tyr-Ile-Gly-Ser-Arg (YIGSR) from laminin (Iwamoto et al 1987) that resulted in the potentiation of activity of the parent peptides (Kawasaki et al 1991a, b). Laminin (Timpl et al 1979), a cell adhesion protein that promotes adhesion and growth of epithelial and tumour cells (Kleinman et al 1985), contains three peptide chains (α-1, β-1 and γ-1). The β-1 chain contains YIGSR that has been found to inhibit experimental metastasis in mice (Iwamoto et al 1987). The objective of this research was to study a new synthetic strategy for preparation of a peptide–chitosan hybrid (acetyl-YIGSRβAG–chitosan; Figure 1) and to examine its inhibitory effect on experimental metastasis in mice.

Correspondence: K. Kawasaki, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Ikawadani-cho, Nishi-ku, Kobe 651-2180, Japan.
E-Mail: kawasaki@pharm.kobegakuin.ac.jp

This paper summarizes our research on chitosan as a polymer carrier of a laminin-related peptide, acetyltyrosylisoleucylglycylserylarginyl-β-alanine (Ac-YIGSRβA-OH), and the hybrid's antimetastatic activity.

Chitosan, a polymer of glucosamine (2-amino-2-deoxy-D-glucose), is a partially deacetylated chitin. Chitin, a principal component of the shells of crustaceans, is of limited commercial use even though chitin and its derivative chitosan are biodegradable biopolymers of low toxicity with a number of unique potential applications, e.g. artificial skin, sutures. Crustacean shells are treated as waste and, as such, have been implicated in some environmental problems. Therefore, it is desirable to find an effective application for chitin and chitosan. Murata et al (1989) reported that 6-O-sulphated and 6-O-carboxymethylated chitin had antimetastatic effect. Komazawa et al (1993) reported that the antimetastatic activity of a fibronectin-related peptide (Arg-Gly-Asp-Ser, RGDS) was potentiated occasionally by conjugation with 6-O-sulphated and 6-O-carboxymethylated chitin. They acylated RGDS peptide with the carboxymethyl group of the carboxymethylated chitin derivative. The antimetastatic activity of the chitin–RGDS was dependent on substituent groups of the chitin skeleton.

Chitin, in which most amino groups are acetylated, is an insoluble material, but chitosan is soluble in aqueous media depending on its degree of deacetylation. Sannan et al (1975, 1976) reported that chitosan with 40–60% of its acetyl groups is soluble in water at neutral pH. This current study focussed on the preparation of a hybrid of a water-soluble chitosan and a laminin-related peptide.

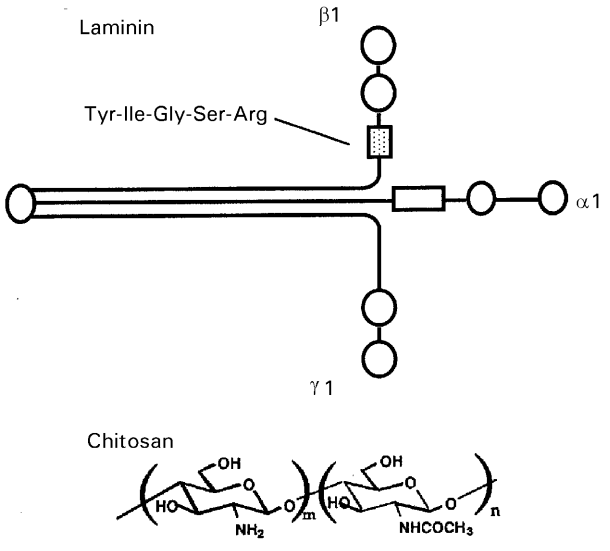

Figure 1. Structures of laminin and chitosan.

Materials and Methods

Chemistry

Water-soluble chitosan was purchased from Wako Pure Chemical Industries, Ltd. According to the manufacturer, the molecular weight of the chitosan was 2000–5000. The average molecular weight of the chitosan, determined by gel permeation chromatography, was 2500. Chloromethylated polystyrene resin and coupling reagents were purchased from Watanabe Chemical Industries, Ltd. Amino acid compositions of acid hydrolysates were determined with a Waters Pico.TAG amino acid analyser. The RP-HPLC was conducted with a Waters 600 on a DAISOPAK column using gradient systems of acetonitrile/water containing 0·05% trifluoroacetic acid (TFA). Matrix assisted laser desorption ionization time of flight mass spectra (MALDI-TOF-MS) were measured with a Shimazu/Kratos Kompact MALDI IV mass spectrometer.

Ac-YIGSRβA-OH. This hexapeptide was prepared by the manual solid-phase method using *tert*-butyloxycarbonyl groups as α-amino-protecting groups. Introduction of *tert*-butyloxycarbonyl-βAla-OH on chloromethylated polystyrene resin was performed by the caesium salt method (Gisin 1973). The resulting resin (*tert*-butyloxycarbonyl-βAla-resin, βAla content: 0·58 mmol g^{-1}, 3 g) was treated with 50% TFA/dichloromethane containing 5% anisole to remove the *tert*-butyloxycarbonyl group. The following amino acid derivatives were used for the stepwise elongation of a peptide chain: *tert*-butyloxycarbonyl-Tyr(2,6-dichlorobenzyl)-OH, *tert*-butyloxycarbonyl-Ile-OH, *tert*-butyloxycarbonyl-Gly-OH, *tert*-butyloxycarbonyl-Ser(benzyl)-OH, *tert*-butyloxycarbonyl-Arg(tosyl)-OH. Dimethylformamide was used as a reaction solvent and dichloromethane was used as a washing solvent. *tert*-Butyloxycarbonyl groups were removed with 50% TFA/dichloromethane containing 5% anisole. The resulting TFA salt was neutralized with *N*-methylmorpholine. Coupling reactions were performed by the diisopropylcarbodiimide/1-hydroxybenzotriazole method (König & Geiger 1970). *tert*-Butyloxycarbonyl-amino acids (3-times excess in molar ratio to the βAla content on the resin) were pre-activated with diisopropylcarbodiimide/1-hydroxybenzotriazole for 10 min and then reacted with the resin. Each coupling

reaction was checked by the Kaiser test (Kaiser et al 1970). The synthetic protocol for the solid-phase peptide synthesis is shown in Table 1.

The resulting H-Tyr(2,6-dichlorobenzyl)-Ile-Gly-Ser(benzyl)-Arg(tosyl)-βAla-resin was treated with acetic anhydride (1 mL, 10·5 mmol) and 0·5 M *N*-methylmorpholine/dichloromethane (20 mL) in dimethylformamide for 30 min and washed with dimethylformamide (×3) and dichloromethane (×3). Yield of the resulting Ac-Tyr(2,6-dichlorobenzyl) - Ile -Gly-Ser(benzyl)-Arg(tosyl)-βAla-resin was 4·22 g. The resin (2·11 g) was treated with hydrofluoric acid (50 mL) containing 5% anisole at 0°C for 40 min after which the hydrofluoric acid was removed in-vacuo. The residue was washed with ether (five times) and extracted with 5% acetic acid. The extract was lyophilized and the resulting powder (799 mg) was purified by Sephadex G-25 column (3 × 140 cm) chromatography using 3% acetic acid as an eluent. The product was converted to its hydrochloride by lyophilization from HCl-containing water. Yield 399 mg. The product exhibited a single peak by analytical RP-HPLC (DAISOPAK SP-125-5-ODS-B, 4·6 × 250 mm) using a gradient system of acetonitrile and water. Amino acid ratios in an acid hydrolysate: Tyr 0·93, Ile 1·00, Gly 1·00, Ser 0·92, Arg 0·87, βAla 0·95 (average recovery 94%).$[\alpha]_D^{20} -13{\cdot}9^\circ$ ($c = 1{\cdot}0$, H_2O). MALDI-TOF-MS m/z 708·3 $(M+1)^+$ (matrix, sinapinic acid).

Chitosan. Chitosan (600 mg) was purified by Sephadex G-25 column (3 × 140 cm) chromatography using water as an eluent. Fractions (17 g each) were checked by absorbance at 220 nm. Fractions 26–35 were pooled and the solvent was removed in-vacuo, followed by lyophilization. The purified material was converted to its hydrochloride by lyophilization from HCl-containing water. Yield 187 mg (28%).

Attempt to couple Ac-YIGSRβA-OH and chitosan. Coupling reactions between Ac-YIGSRβA-OH and chitosan were attempted (according to the reported procedures) in a mixture of dimethylformamide and water by four different methods. These methods were the diphenylphosphoryl azide (DPPA) method (Shioiri et al 1972), the diisopropylcarbodiimide/1-hydroxybenzotriazole method (König & Geiger 1970), the water-soluble carbodiimide (WSC) method (Scheehan et al 1961), and the 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) method (Knorr et al 1989). Since the reactions did not proceed with any of the methods used, the desired material (Ac-YIGSRβA–chitosan) was not obtained.

H-Gly-Chitosan. *tert*-butyloxycarbonyl-Gly-OH (283 mg, 1·62 mmol), 1-hydroxybenzotriazole (338 mg, 2·5 mmol), and TBTU (803 mg, 2·5 mmol) were dissolved in dimethylformamide (2 mL) at 37°C and the solution was combined with a fine powder suspension of chitosan hydrochloride (300 mg) in dimethylformamide (1 mL). The mixture was adjusted to pH 8–9 with Et_3N and stirred overnight at 37°C. The mixture became a clear solution. The solvent was removed in-vacuo and the residue was purified by Sephadex LH-20 column (3 × 140 cm) chromatography using dimethylformamide as an eluent. Fractions (15 g each) were checked by the ninhydrin test. Fractions 20–28 (which showed negative ninhydrin test) were pooled and the solvent was removed in-vacuo. Yield 300 mg. The material was treated with 5% anisole/TFA (10 mL) at 0°C for 2·5 h and the TFA was removed in-vacuo. The residue was washed with ether (×4), and extracted with water, followed by lyophilization. Yield 350 mg. The material was purified by Sephadex G-25 column (3 × 140 cm) chromatography using 3% acetic

Table 1. The synthetic protocol for the solid-phase peptide synthesis.

Step	Reagent	Reaction time (min)	Times
1	0·5 M *N*-Methylmorpholine/dimethylformamide	3	×3
2	Dimethylformamide	3	×4
3	*tert*-Butyloxycarbonyl-amino acid, diisopropylcarbodiimide/1-hydroxybenzotriazole	60	
4	Dimethylformamide	3	×3
5	Kaiser test		
6	Dichloromethane	3	×3
7	50% TFA/dichloromethane	5	×1
		45	×1
8	Dichloromethane	3	×2
9	Dimethylformamide	3	×3

acid as an eluent. Fluffy powder 287 mg. The material was lyophilized from HCl-containing water to facilitate its conversion to the hydrochloride. Yield 239 mg. Gly content: 2·08 mmol g^{-1}.

Ac-YIGSRβAG-chitosan. To a solution of H-Gly-Chitosan HCl (100 mg) in water (2 mL), Ac-YIGSR(HCl)βA-OH (100 mg, 134 μmol) and WSC HCl (32 mg, 168 μmol) were added and the mixture was stirred for 12 h at room temperature. A small amount of the precipitate was removed by centrifugation and the supernatant was applied to a Sephadex G-25 column (3 × 140 cm). Water was used as an eluent and the absorbance of each fraction (17 g) was checked at 220 nm. Fractions 24–33 were collected and lyophilized. Yield 90 mg. The material was purified by preparative HPLC twice. Yield 55 mg. Amino acid ratios in an acid hydrolysate: Tyr 1·04, Ile 0·99, Gly 3·67, Ser 1·01, Arg 1·00, βAla 1·18. Peptide content calculated from recoveries of amino acids: 0·40 mmol g^{-1}. IR (KBr, cm^{-1}): 3294 (–OH), 1681 (–CONH–).

Colony formation assay

The viability of B16–BL6 melanoma was assessed when admixed with samples of H-Gly-chitosan, Ac-YIGSRβA-OH and Ac-YIGSRβAG-chitosan. Cells in modified Eagle's medium containing 0·1% BSA (2×10^4 mL^{-1}) and samples (10 mg mL^{-1}) were admixed at the ratio of 1 : 1. Then the cells were seeded onto culture dishes, and colonies were counted after one week.

Metastasis assay

The metastasis-inhibiting effect of synthetic materials was examined as reported by Kawasaki et al (1991a, b). Briefly, B16–BL6 melanoma cells (1 × 105/0·1 mL) and a synthetic peptide (1 mg/0·1 mL/mouse) were intravenously administered (as separate injections) to C57BL/6 mice. The mice were killed 14 days after tumour inoculation and the lungs were removed. The number of surface melanoma colonies on the lungs was counted with the aid of a stereoscopic microscope.

Results and Discussion

YIGSR, a partial sequence peptide of laminin, has been reported by Iwamoto et al (1987) to be an inhibitor of experimental metastasis in mice. We have attempted to prepare its chitosan hybrid. A coupling reaction of an *N*-blocked YIGSR peptide and chitosan may induce partial racemization of the Arg residue at the *C*-terminal. βAla was intercalated as a non-chiral spacer, to avoid racemization, resulting in Ac-YIGSRβA-OH. The peptide was prepared by the solid-phase method using a *tert*-butyloxycarbonyl-strategy. The α-amino group was protected with the *tert*-butyloxycarbonyl group that was removable by TFA treatment. Side chains of constitutional amino acids were protected with benzyl type protecting groups (for Tyr and Ser) and tosyl group (for Arg). *tert*-Butyloxycarbonyl-Tyr (2, 6-dichlorobenzyl) -Ile-Gly-Ser (benzyl) -Arg (tosyl)-βAla-resin was prepared and treated with TFA to remove the *tert*-butyloxycarbonyl group. Then the peptide–resin was treated with acetic anhydride to give Ac-Tyr(2,6-dichlorobenzyl)-Ile-Gly-Ser(benzyl)-Arg(tosyl)-βAla-resin. The resulting peptide–resin was treated with hydrofluoric acid to give Ac-YIGSRβA-OH. The peptide was purified by Sephadex G-25 column (3 × 140 cm) chromatography using 3% acetic acid as an eluent and the purified peptide was lyophilized from HCl-containing water to convert the guanidino group of Arg to its hydrochloride (Ac-Tyr-Ile-Gly-Ser-Arg(HCl)-βAla-OH). It is necessary to protect the guanidino group of Arg by forming the hydrochloride when the peptide is activated for reaction, since acylation by the activated peptide on the guanidino group may occur. Furthermore, the guanidino group of Arg was converted to acetate during the Sephadex column chromatography (using 3% acetic acid), since hydrofluoric acid is a weak acid in water. Acetylation might occur on the guanidino group of Arg by the carbodiimide activation of the acetic acid of acetate. Therefore, acetate must be converted to hydrochloride to avoid acetylation before a coupling reaction. Commercial chitosan was purified by Sephadex G-25 column

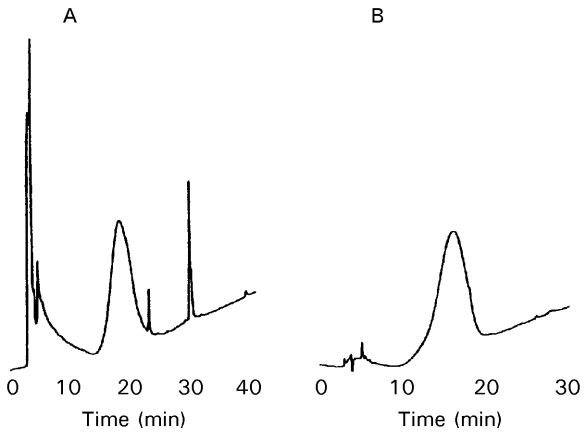

Figure 2. Chromatographic profiles of commercial chitosan (A) and chitosan after purification by Sephadex G-25 column chromatography (B).

chromatography before the coupling reaction. Commercial chitosan contained many impurities, which could be removed by molecular sieve gel chromatography (Figure 2).

Four methods were used to try to react the peptide, Ac-Tyr-Ile-Gly-Ser-Arg(HCl)-βAla-OH, with the water-soluble chitosan in a mixture of dimethylformamide and water. Neither the DPPA method (Shioiri et al 1972), the diisopropylcarbodiimide/1-hydroxybenzotriazole method (König & Geiger 1970), the WSC method (Scheehan et al 1961), nor the TBTU method (Knorr et al 1989) resulted in the desired material (Ac-YIGSRβA–chitosan). Assuming that steric hindrance was responsible, in part, for the low reactivity of these coupling reactions, a small spacer was placed on chitosan to facilitate the coupling reaction. Gly was selected as the spacer and intercalated by reacting *tert*-butyloxycarbonyl-Gly-OH with chitosan using the TBTU method (Figure 3). The coupling reaction was successful and the product was purified by Sephadex LH-20 column chromatography. The yield was 61%. The *tert*-butyloxycarbonyl group was removed by TFA treatment to give H-Gly-chitosan trifluoroacetate. Since chitosan has amino groups and hydroxyl groups, both amide bond formation and esterification may occur by acylation. However, it is reported that amide formation is fast and esterification is quite slow by the TBTU method. Furthermore, acylation of the water-soluble chitosan had been proven to take place selectively at the amino group in the presence of dicyclohexylcarbodiimide in aqueous dimethylformamide (Kurita et al 1977). IR spectrum of H-Gly-chitosan did not show the absorbance of ester bond. To avoid trifluoroacetylation, H-Gly-chitosan (G-chitosan) trifluoroacetate was converted to its hydrochloride and coupled with Ac-YIGSRβA-OH HCl in water by the WSC method. The product, Ac-YIGSRβAG–chitosan, was purified by Sephadex G-25 column chromatography, followed by preparative HPLC (twice). The HPLC profiles of the hybrid are shown in Figure 4. The preparative HPLC profile of the product showed multi-peak behaviour. The hybrid obtained from the multi-peak region (as shown by the arrow) by preparative HPLC showed a homogeneous broad polymer-type peak by analytical HPLC (Figure 4C). We observed the same phenomenon when chitosan (purified by Sephadex column chromatography) was examined by the same preparative HPLC column and by the same analytical HPLC column. The chitosan exhibited a broad homogeneous peak by the analytical HPLC column (Figure 2), but exhibited a

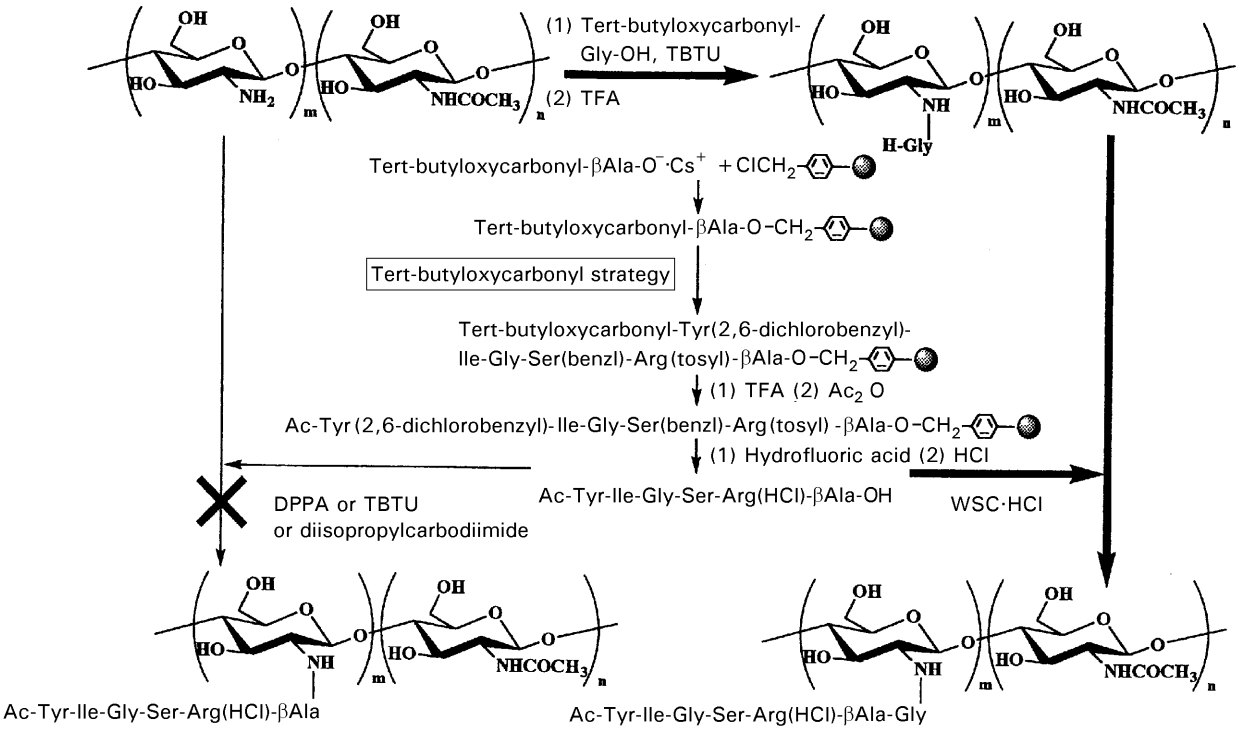

Figure 3. Synthetic scheme for a chitosan hybrid of a laminin-related peptide. DPPA = diphenylphosphoryl azide. TBTU = 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate. WSC.HCl = water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] hydrochloride. TFA = trifluoroacetic acid.

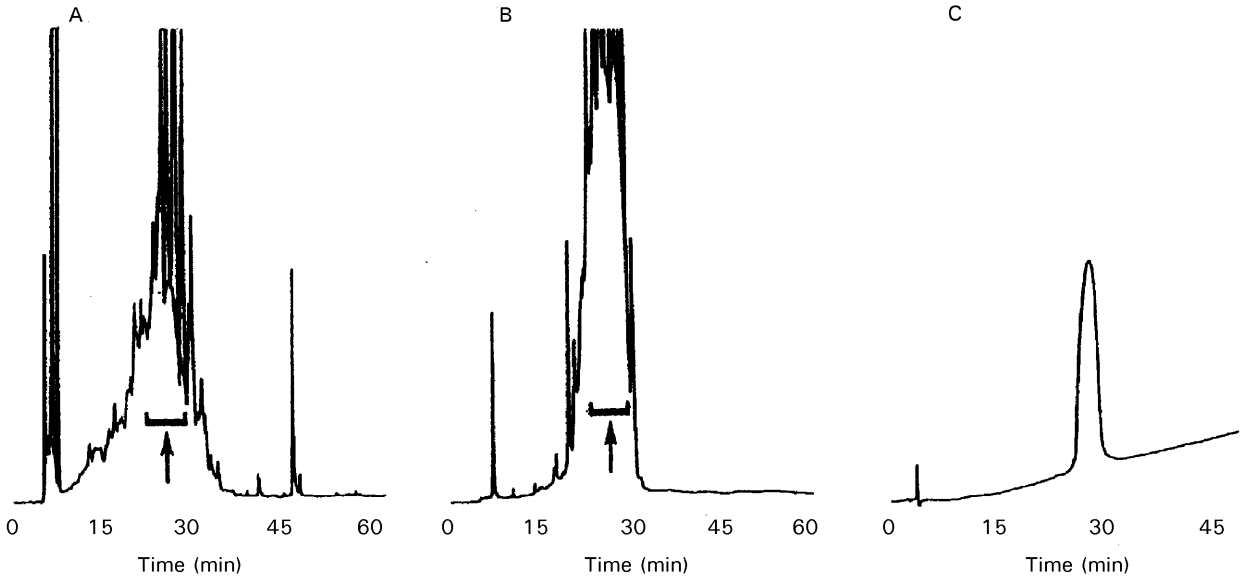

Figure 4. HPLC profiles of crude and purified chitosan-peptide hybrids. A. Preparative HPLC profile of crude hybrid. Column: DAISOPAK SP120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL min^{-1}. Eluent: (1) 0·05% TFA/water, (2) 0·05% TFA/acetonitrile. Gradient of 1 : 2 = 98 : 2→50 : 50 (40 min). Eluate indicated by an arrow was collected. Absorbance: 220 nm. B. Preparative HPLC profile of re-purified hybrid. The hybrid obtained by the first HPLC purification was purified again by HPLC. The column and other conditions were the same as those for A. Eluate indicated by an arrow was collected. C. Analytical HPLC profile of the purified hybrid. Column: DAISOPAK SP120-5-ODS-B (4·6 × 250 mm). Flow rate: 1 mL min^{-1}. Gradient of 1 : 2 = 98 : 2→50 : 50 (50 min).

Table 2. Viability of B16–BL6 melanoma admixed with samples of H-Gly–chitosan, Ac-YIGSRβA-OH and Ac-YIGSRβAG–chitosan. The cells were seeded onto culture dishes, and the colonies were counted after one week.

	Dose (mg)	Peptide content (μmol)	Colony number
Control	–	–	119
H-Gly–chitosan	1·0	1·2	115
Ac-YIGSRβA-OH	0·3	0·4	129
	0·1	0·12	124
Ac-YIGSRβAG–chitosan	0·3	0·12	116
	0·1	0·04	130

Each value represents the mean ± s.e.

Table 3. Inhibitory effect of Ac-YIGSRβAG–chitosan on experimental metastasis of B16–BL6 melanoma in mice. B16–BL6 cells and synthetic Ac-YIGSRβAG–chitosan hybrid were injected separately into five mice per group. Control mice received the same amount of melanoma B16–BL6 cells. Lung tumour colonies were counted after 14 days.

	Dose (mg)	Peptide content (μmol)	Colony number
Control	–	–	324·88 ± 16·11
Ac-YIGSRβA-OH	1·0	1·2	291·50 ± 11·99
	0·3	0·4	340·00 ± 20·04
	0·1	0·12	303·83 ± 23·88
Ac-YIGSRβAG–chitosan	1·0	0·2	207·17 ± 20·71**
	0·3	0·12	225·60 ± 19·21*
	0·1	0·04	328·75 ± 29·84

*$P < 0·05$, **$P <$ 0·01 compared with control.

multi-peak region by the preparative HPLC column (data are not shown). The reason for these phenomena is not clear, but it might depend on the number of theoretical plates of the columns and heterogeneous molecular size of the chitosan. The preparative column has a high number of theoretical plates, so the chitosan (a heterogeneous mixture of molecular sizes) might exhibit multi-peak behaviour depending on the size distribution of the molecules. The peptide content of the hybrid was 0·4 mmol g^{-1}.

The viability of the B16-BL6 melanoma cells incubated with Gly–chitosan and the hybrid were examined, before the metastasis assay, and the results are shown in Table 2. The results indicate that Gly–chitosan and the hybrid are not cytotoxic.

The inhibitory effect of the Ac-YIGSRβAG-chitosan on experimental metastasis was examined in mice and results are shown in Table 3.

B16-BL6 melanoma cells and the hybrid were intravenously administered (as separate injections) to mice. Separate injections may increase the variability in response, but will best isolate the antimetastatic effect of the sample, avoiding other antimetastatic factors (such as cytotoxic effects of a peptide in-vivo). The mice were killed 14 days after tumour inoculation, and the lungs were removed. The number of surface melanoma colonies on the

lungs was counted with the aid of a stereoscopic microscope.

As shown in Table 3, 1 mg Ac-YIGSRβA-OH did not show significant antimetastatic effect. The hybrid, however, exhibited a dose-dependent inhibitory effect. The inhibitory effect of 0·3 mg of the hybrid (peptide content 0·12 μmol) was more potent than that of 1 mg of the parent peptide (1·2 μmol). No antimetastatic effect was observed by administration of Gly–chitosan at 1 mg/mouse (data not shown). Thus, hybrid formation of the peptide with the larger chitosan molecule apparently does not reduce, but actually potentiates the activity of the parent peptide. Previously we reported that the potentiation of the antimetastatic effect of YIGSR by hybrid formation with PEG was due to a longer half-life in blood (Kaneda et al 1995). The potentiation of the antimetastatic effect of a peptide by hybrid formation with a polymer may be based on slower enzymatic degradation and more stable receptor-binding of the hybrid as compared with the parent peptide, although its mechanism is not yet fully understood.

In conclusion, we have developed a facile procedure to prepare a peptide–chitosan hybrid using two spacers. One is a small amino acid spacer to be put on chitosan and the other is an amino acid spacer (to avoid racemization of a constitutional amino acid) which does not have a chiral centre. We also demonstrated that the bulky chitosan moiety did not reduce the biological activity of the parent peptide, but actually potentiated the inhibitory effect of the parent peptide, i.e. chitosan may be used as a drug carrier for peptides.

Acknowledgements

This work was supported in part by Grant-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture.

References

Gisin, B. F. (1973) The preparation of Merrifield-resins through total esterification with cesium salts. Helv. Chim. Acta 56: 1476–1482

Iwamoto, Y., Robey, F. A., Graf, J., Sasaki, M., Kleinman, H. K., Yamada, Y., Martin, G. R. (1987) YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. Science 238: 1132–1134

Kaiser, E., Colescotto, R. L., Bossinger, C. D., Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. 34: 595–598

Kaneda, Y., Yamamoto, S., Kihira, T., Tsutsumi, Y., Nakagawa, S., Miyake, M., Kawasaki, K., Mayumi, T. (1995) Synthetic cell-adhesive laminin peptide YIGSR conjugated with polyethylene glycol has improved antimetastatic activity due to a longer half-life in blood. Invasion Metastasis 15: 156–162

Kawasaki, K., Namikawa, M., Murakami, T., Mizuta, T., Iwai, Y., Hama, T., Mayumi, T. (1991a) Amino acids and peptides. XIV. Laminin related peptides and their inhibitory effect on experimental metastasis formation. Biochem. Biophys. Res. Commun. 174: 1159–1162

Kawasaki, K., Namikawa, M., Yamashiro, Y., Hama, T., Mayumi, T. (1991b) Preparation of [Arg-Gly-Asp]-[amino-poly(ethylene glycol)] hybrids and their inhibitory effect on experimental metastasis. Chem. Pharm. Bull. 39: 3373–3375

Kleinman, H. K., Cannon, F. B., Laurie, G. W., Hassel, J. R., Aumailley, M., Terranova, V. P., Martin, G. R., DuBois-Dalcq, M. (1985) Biological activities of laminin. J. Cell. Biochem. 27: 317–325

Knorr, R., Trzeciak, A., Bannwarth, W., Gillessen, D. (1989) New coupling reagents in peptide chemistry. Tetrahedron Lett. 30: 1927–1930

Komazawa, H., Saiki, I., Nishikawa, N., Yoneda, J., Yoo, Y. C., Kojima, M., Ono, M., Itoh, I., Nishi, N., Tokura, S., Azuma, I. (1993) Inhibition of tumor metastasis by Arg-Gly-Asp-Ser (RGDS) peptide conjugated with sulfated chitin derivative, SCM–chitin–RGDS. Clin. Exp. Metastasis 11: 482–491

König, W., Geiger, R. (1970) Eine neue Methode zur Synthese von Peptiden: Aktivierung der Carboxylgruppe mit Dicyclohexylcarbodiimid unter Zusatz von 1-Hydroxy-benzotriazolen. Chem. Ber. 103: 788–798, 2024–2033

Kurita, K., Sannan, T., Iwakura, Y. (1977) Graft copolymerization of γ-methy-L-glutamate NCA onto water-soluble chitin. Makromol. Chem. 178: 2595–2602

Murata, J., Saiki, I., Nishimura, S., Nishi, N., Tokura, S., Azuma, I. (1989) Inhibitory effect of chitin heparinoids on the lung metastasis of B16–BL6 melanoma. Jpn. J. Cancer Res. 80: 866–872

Pierschbacher, M. D., Ruoslahti, E. (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309: 30–33

Sannan, S., Kurita, K., Iwakura, Y. (1975) Studies on chitin, 1: solubility change by alkaline treatment and film casting. Macromol. Chem. 176: 1191–1195

Sannan, S., Kurita, K., Iwakura, Y. (1976) Studies on chitin, 2: effect of deacetylation on solubility. Macromol. Chem. 177: 3589–3600

Scheehan, J. C., Cruickshank, P. A., Boshrt, G. L. (1961) A convenient synthesis of water-soluble carbodiimides. J. Org. Chem. 26: 2525–2531

Shioiri, T., Ninomiya, K., Yamada, S. (1972) Diphenylphosphoryl azide. A new convenient reagent for a modified curtius reaction and for the peptide synthesis. J. Am. Chem. Soc. 94: 6203–6206

Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., Martin, G. R. (1979) Laminin, a glycoprotein from basement membranes. J. Biol. Chem. 254: 9933–9937