Regioselective Conjugation of Chitosan with a Laminin-related Peptide, Tyr–Ile–Gly–Ser–Arg, and Evaluation of Its Inhibitory Effect on Experimental Cancer Metastasis[1)]

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A conjugate from the YIGSR peptide and chitosan has been prepared on the basis of a regioselective modification strategy of chitosan, and its antimetastatic activity has been assayed. Chitosan was converted to its organosoluble derivative, 6-*O*-trityl-chitosan, in 3 steps, and then coupled with the peptide portion containing a spacer amino acid, Ac–Tyr–Ile–Gly–Ser–Arg–βAla–OH [βAla; β-alanine]. The product was treated with $CHCl_2CO_2H$ to afford the desired conjugate, Ac–Tyr–Ile–Gly–Ser–Arg–βAla–chitosan, which proved to inhibit the experimental lung metastasis of B16BL6 melanoma cells in mice at lower doses than the parent peptide.

Key words YIGSR; chitosan; antimetastatic activity; conjugate; regioselective modification; laminin

Laminin, a 900 kDa trimeric glycoprotein in the basement membrane, is known to involve in invasion and metastasis of tumor cells.[2—4)] A synthetic pentapeptide corresponding to a partial sequence of the laminin β1 chain, H–Tyr–Ile–Gly–Ser–Arg–NH_2, was reported to inhibit experimental metastasis formation.[5)] It was also reported that peptides containing the Tyr–Ile–Gly–Ser–Arg (YIGSR) sequence inhibited angiogenesis and tumor growth.[6)] The YIGSR peptide has thus attracted attention as a potential candidate for the development of anticancer and antimetastatic agents, and a number of modifications have been attempted to enhance its activity and clinical utility. Poly(YIGSR) was shown to inhibit experimental metastasis more effectively than the monomer.[7)] Kawasaki and co-workers reported that hybrid compounds of the YIGSR peptide and poly(ethylene glycol) (PEG), a stable, bioinert, and only weakly immunogenic polymer, exhibited a much higher antimetastatic activity and stability to enzymatic degradation than the parent peptide.[8,9)] In addition, multimeric YIGSR derivatives,[10)] in which 4 to 6 YIGSR peptides were assembled on a branched lysine core, were found to be highly active antimetastatic agents. These results suggest that the coupling of the YIGSR peptide with appropriate polymers would be promising to develop highly active antimetastatic agents.

Chitin is an abundant amino polysaccharide consisting of *N*-acetyl-D-glucosamine, and chitosan can be readily obtained from chitin by simple deacetylation.[11,12)] Owing to their low toxicity, biodegradability, and abundance, their applications in various fields including the use as drug carriers are being studied extensively.[13)] Chitosan may be more favorable than chitin in conjugation with bioactive peptides due to the presence of reactive amino groups. Unlike PEG, chitosan has an amino function in every glucosamine residue, and therefore peptide molecules introduced at these amino groups would be ordered in a comb-like fashion, whereas the peptide–PEG conjugate is a linear molecule. A comb-like arrangement might be more advantageous than a linear one to increase the local concentration of the peptide. Furthermore, the degree of substitution (*d.s.*) of the chitosan-conjugate could be varied in a wider range than linear conjugates if necessary. The biodegradability and extremely low toxicity of chitosan are also attractive in view of possible use as a therapeutic agent for humans. Under these circumstances, we have focused on the potential of chitosan for use in conjugation with the YIGSR peptide. Chitosan is, however, insoluble in common reaction media, and is thus rather intractable. This report deals with the efficient and regioselective preparation of the YIGSR–chitosan conjugate, as shown in Fig. 1, *via* organosoluble derivatives of chitosan, and also with its antimetastatic activity.

To avoid two possible side reactions in the direct conjugation of YIGSR with chitosan, *i.e.*, epimerization and δ-lactam formation of the carboxy-terminal Arg,[14)] β-alanine (βAla) was employed as a spacer molecule. The peptide moiety containing the spacer, Ac–Tyr–Ile–Gly–Ser–Arg(HCl)–βAla–OH **1**, was synthesized by the conventional solution method.[15)] Carbodiimide-mediated coupling of peptide **1** with chitosan failed to yield the peptide–chitosan conjugate due to the heterogeneous condition. However, regioselective introduction of the peptide at the amino function of chitosan has been accomplished using an organosoluble chitosan derivative, 6-*O*-trityl(Trt)-chitosan, which has a protective group at the reactive C-6 hydroxy function.[16,17)] As shown in Fig. 2, chitosan was quantitatively converted to its *N*-phthaloyl (Pht) derivative by treating with phthalic anhydride in dimethylformamide at 130 °C, and the product was treated with chlorotriphenylmethane in pyridine at 80 °C to give *N*-Pht-6-*O*-Trt-chitosan. The *N*-Pht group was selectively removed with hydrazine hydrate to afford 6-*O*-Trt-chitosan.

The synthetic scheme for the chitosan conjugate from 6-*O*-Trt-chitosan is shown in Fig. 3. Peptide **1** was coupled with 6-*O*-Trt-chitosan by means of diphenylphophoryl azide (DPPA)[18)] in a homogeneous dimethylacetamide (DMAc) solution. The product was then treated with $CHCl_2CO_2H$ to afford the desired conjugate **2**.[19)] The IR spectrum of conjugate **2** demonstrated the regioselective introduction of **1** at the amino function of chitosan; clear amide bands and no ester bands were observed. The *d.s.* was confirmed to be 0.16 by amino acid analysis of the acid hydrolysate of **2**. This value means that the peptide was introduced into every 6.3 glucosamine residues.

The inhibitory activity of Ac–Tyr–Ile–Gly–Ser–Arg–βAla–chitosan **2**, as well as those of chitosan **3**[20)] and Ac–Tyr–Ile–Gly–Ser–Arg–βAla–OH **1**, on experimental lung metastasis was examined with B16BL6 melanoma cells in mice.[21)] As shown in Fig. 4, conjugate **2** showed a significant inhibitory activity at a dose of 0.08 mg/mouse, whereas chi-

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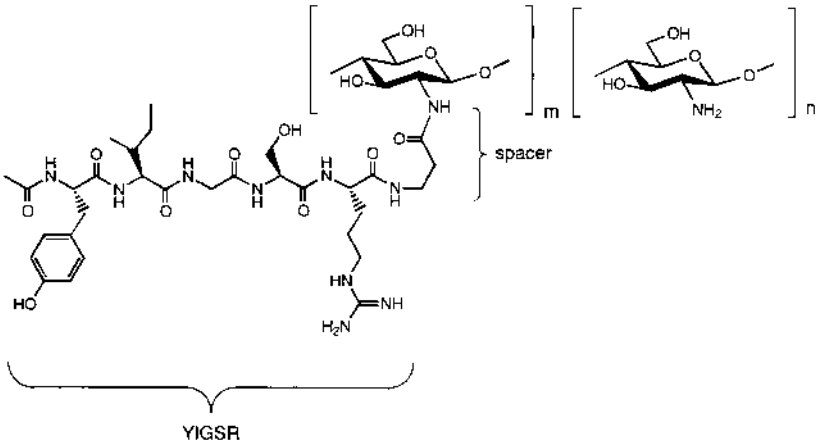

Fig. 1. Structure of the YIGSR–Chitosan Conjugate

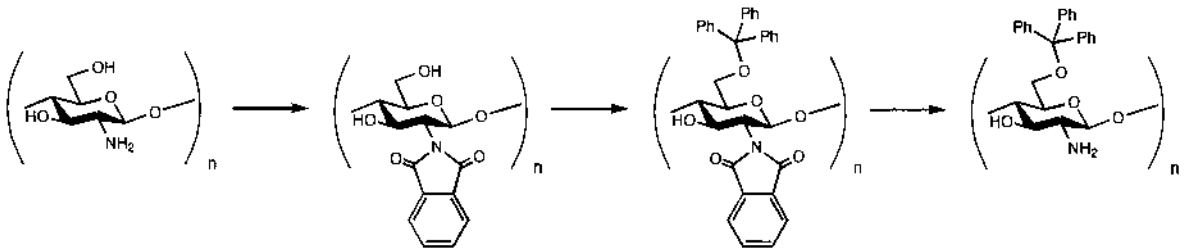

Fig. 2. Synthetic Scheme for 6-*O*-Trt-chitosan

Reagents and conditions: see Ref. 17.

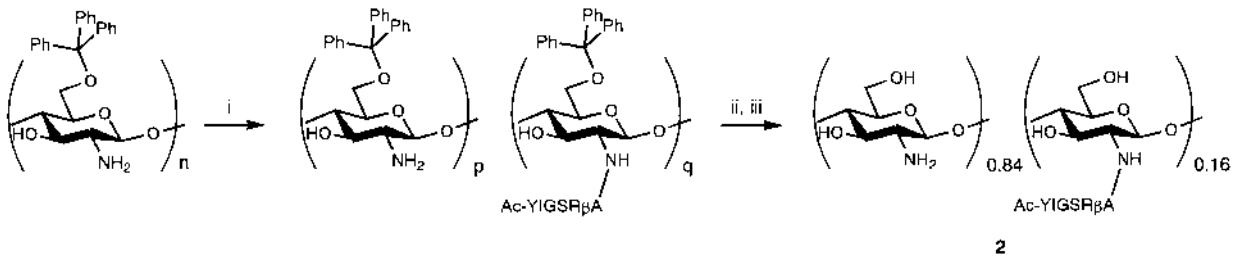

Fig. 3. Synthetic Scheme for the YIGSR–Chitosan Conjugate **2**

Reagents and conditions: i) **1** (1 equivalent for $-NH_2$), DPPA (1 equivalent for $-NH_2$), *N*,*N*-diisopropylethylamine (1 equivalent for $-NH_2$), DMAc (r.t. overnight); ii) $CHCl_2CO_2H$ (r.t., 20 min×2); iii) Sephadex G-25 (3% AcOH). *β*A: *β*Ala.

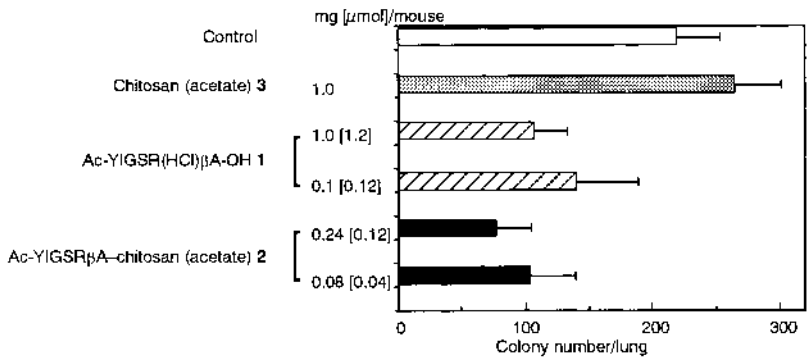

Fig. 4. Antimetastatic Activity of Peptide **1**, Peptide–Chitosan Conjugate **2**, and Chitosan **3**

B16BL6 melanoma cells (1.5×10^5/100 *μ*l) were inoculated *i.v.* into C57BL6 mice, and then **1**, **2**, or **3** was injected *i.v.* Mice were killed 2 weeks after tumor inoculation and tumor colonies of lungs were counted with a stereoscopic microscope. Each value represents the mean±S.E. *β*A: *β*Ala.

tosan **3** did not show any inhibition even at 1.0 mg/mouse. Injection of 1.0 mg/mouse of Ac–Tyr–Ile–Gly–Ser–Arg–βAla–OH **1** also decreased the number of colonies in the lung to *ca*. 50% of the control. Previously, 0.3 mg/mouse of the YIGSR–PEG conjugate was reported to decrease the number of colonies to *ca*. 50% of the control.[8,9] Based on the *d.s*., 0.08 mg of chitosan–conjugate **2** contains 0.04 μmol of the YIGSR peptide, while 0.3 mg of the PEG-conjugate contains 0.05 μmol peptide, and 1.0 mg of Ac–Tyr–Ile–Gly–Ser–Arg–βAla–OH **1** corresponds to 1.2 μmol. Thus chitosan-conjugate **2** has been confirmed to have a more potent antimetastatic activity than free peptide **1** and a comparable activity with the PEG-conjugate.

It is noteworthy that conjugate **2** exhibited higher activity than free peptide **1**. Generally, conjugation of small bioactive peptides with polymers has a possible drawback; a large polymer portion may hinder the peptide–receptor interaction. The results of antimetastatic assay in this study clearly indicate that the chitosan molecule does not severely hinder the YIGSR portion from interaction with the laminin receptor. Previously, the YIGSR–PEG conjugate was shown to be much more stable against enzymatic degradation than the free peptide.[9,21] This might be one reason for the high antimetastatic activity of the PEG-conjugate. Although, as described in the introductory remarks, the chitosan-conjugate is structurally different from the PEG-conjugate, a similar effect of protection against enzymatic digestion is expected, and may be partly responsible for the high activity of **2**. It is also notable that chitosan-conjugate **2** with a relatively low *d.s.* showed an antimetastatic potency comparable to that of the PEG-conjugate. If reaction conditions are appropriately controlled, the YIGSR peptide may be conjugated with chitosan at a higher *d.s*. A high *d.s*. is undoubtedly favorable to increase the local concentration of the peptide, and the chitosan-conjugate with a high *d.s*. may provide a higher antimetastatic activity than the PEG-conjugate.

In conclusion, the Ac–Tyr–Ile–Gly–Ser–Arg–βAla–chitosan conjugate has been successfully synthesized on the basis of regioselective modification strategy of chitosan, and has proved to exhibit higher antimetastatic activity than the parent peptide. The results obtained here suggest that chitosan is promising as a polymeric carrier for various bioactive peptides.

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References and Notes

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19) Selected data for conjugate **2**. IR (KBr) cm^{-1}: 1654, 1541, 1151—1000. Amino acid ratio in an acid hydrolysate (6 N HCl, 110 °C, 24 h): $Ser_{1.00}Gly_{1.00}Ile_{0.87}Tyr_{0.76}Arg_{0.97}\beta Ala_{0.78}$ (peptide content 0.43 mmol/g). Number-average molecular weight [determined by gel-permeation chromatography (GPC)]: 22.2×10^3.
20) To obtain chitosan with a molecular weight similar to that of the conjugate, chitosan **3** was regenerated by the treatment of 6-*O*-Trt-chitosan with $CHCl_2CO_2H$ followed by purification on a Sephadex G-25 column, which was equilibrated and eluted with 3% AcOH. IR (KBr) cm^{-1}: 1151—1000. Number-average molecular weight (determined by GPC): 13.8×10^3.
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