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Bioconjugation of Laminin Peptide YIGSR with Poly(Styrene Co-maleic Acid) Increases Its Antimetastatic Effect on Lung Metastasis of B16-BL6 Melanoma Cells

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A comb-shaped polymeric modifier, SMA [poly(styrene comaleic anhydride)], which binds to plasma albumin in blood was used to modify the synthetic cell-adhesive laminin peptide YIGSR, and its inhibitory effect on experimental lung metastasis of B16-BL6 melanoma cells was examined. YIGSR was chemically conjugated with SMA via formation of an amide bond between the N-terminal amino group of YIGSR and the carboxyl anhydride of SMA. The antimetastatic effect of SMA-conjugated YIGSR was approximately 50-fold greater than that of native YIGSR. When injected intravenously, SMA-YIGSR showed a 10-fold longer plasma half-life than native YIGSR *in vivo*. In addition, SMA-YIGSR had the same binding affinity to plasma albumin as SMA, while native YIGSR did not bind to albumin. These findings suggested that the enhanced antimetastatic effect of SMA-YIGSR may be due to its prolonged plasma half-life by binding to plasma albumin, and that bioconjugation of *in vivo* unstable peptides with SMA may facilitate their therapeutic use.

Key Words: **peptide modification; YIGSR; poly(styrene-co-maleic anhydrid); plasma half-life; albumin; binding affinity.**

Tumor metastasis is one of the major causes of mortality in cancer (1–3) and the interaction of tumor cells with various components of the extracellular matrix (ECM) such as laminin and fibronectin plays a crucial role in tumor metastasis (4,5). Therefore, agents which can selectively block these interactions would be potentially useful as antimetastatic drugs.

Laminins, a family of glycoproteins in basement membranes, are active in adhesion, migration, spreading and differentiation of various cell types, typified by endothelial and tumor cells (6). We previously reported that YIGSR, a core sequence located in the β1 chain of laminin, markedly inhibited experimental lung metastasis of B16 melanoma cells (7). However, a large amount of YIGSR is needed to obtain the inhibitory effect, because of its enzymatic degradation and rapid rental excretion from blood. These problems are common to other bioactive peptides and cytokines (8). Thus, for the therapeutical use of YIGSR as an antimetastatic agent, the development of an appropriate drug delivery system (DDS) is required to improve its *in vivo* stability that allows administration at a low dose. An increase in the blood residency of YIGSR could augment its inhibitory effect against metastasis distributed through the vascular circulation. Several strategies such as peptide-cyclization (9) and D-amino acid substitution (10) have been reported to enhance the therapeutic potency of antimetastatic peptides (YIGSR, etc.) perhaps due to improvement of the physicochemical stability of the steric structure and biological stability by inhibiting enzymatic degradation. However, even these approaches could not overcome rapid renal excretion from the circulation, and thus the therapeutic use of YIGSR has remained limited. We previously reported a new approach in which bioconjugation of cytokines (TNFα and IL-6) with water-soluble polymeric modifier (polyethylene glycol (PEG) (11, 12) and divinylether maleic anhydride (DIVEMA) (13) markedly increased their blood-residency, resulting in effective augmentation of their therapeutic potency

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(TNFα; anti-tumor activity, IL-6; thrombopoietic activity). This prolonged blood-residency by bioconjugation with polymeric modifiers was attributed to blocking of the attack by proteinases and a decrease in their renal excretion rate due to the increased molecular size and steric hindrance, both of which resulted from polymeric modifiers attached to cytokines.

Recently, we attempted to enhance the *in vivo* stability and blood residency of YIGSR by bioconjugation with PEG (PEGylation) to increase its antimetastatic potency (14). We found that PEGylated YIGSR showed more potent antimetastatic activity than native YIGSR, while the *in vitro* specific activity of PEGylated YIGSR relative to that of native YIGSR was markedly decreased, probably due to the inhibition of receptor-binding by steric hindrance caused by attached PEG. In addition, the enhanced inhibitory effect of PEGylated YIGSR was due to its increased stability against various peptidases in the blood but not to the prolonged plasma half-life for rapid renal excretion (15). Therefore, an appropriate polymeric modifier for the enhancement of blood-residency other than PEG is required for the development of a more potent YIGSR as an antimetastatic agent.

The amphipathic polymer poly(styrene-co-maleic anhydride) (SMA) has been shown to bind to plasma albumin (16) SMA-conjugated neocarzinostatin (SMANCS) binds rapidly to plasma albumin when injected intravenously (17). SMANCS showed much longer plasma half-lives than native NCS, resulting in an increase in its antitumor potency. In this study, we designed SMA-conjugated YIGSR to increase its antimetastatic effect by enhancement of its blood residency. This study provided fundamental information which will facilitate the design of conjugated bioactive peptides with polymeric modifiers applicable to therapeutic use.

MATERIALS AND METHODS

Materials. Poly(styrene co-maleic anhydride) (SMA) was obtained from EIF Atochem North America. INC. (Philadelphia, PA) and was separated into fractions (number-average molecular weight: 6000) by gel filtration chromatography (G2500HHR, Tosoh, Tokyo, Japan) in THF. Synthetic peptide YIGSR was prepared by a conventional solid-phase method as reported previously (18). The other reagents and solvents used were of analytical grade.

Preparation of SMA-conjugated YIGSR. YIGSR was chemically conjugated with SMA via the formation of an amide bond between the terminal amino group of YIGSR and carboxylanhydride of SMA. Briefly, 0.1 ml of SMA (12 μmol/ml) dissolved in DMSO was added to 12 ml of YIGSR (0.5 μmol/ml) dissolved in 0.5 M borate buffer (pH 8.5) and stirred at room temperature for 3 hr. The reaction mixture was dialyzed to remove free YIGSR, followed by lyophilization. The product (SMA-conjugated YIGSR; SMA-YIGSR) was obtained as an amorphous powder. The content of YIGSR in SMA-YIGSR thus obtained was measured with a DC Protein Assay Kit (Bio-Rad). The reaction efficiency of SMA-YIGSR was determined by measuring the amounts of amino groups of uncoupled YIGSR by fluorescamine assay (19). The molecular weight of SMA-YIGSR was assessed by gel filtration chromatography (GFC analysis).

Experimental pulmonary metastasis assay. Highly metastatic and invasive B16-BL6 melanoma cells were suspended in Eagle's minimal-essential medium (MEM) containing 0.1% BSA (1.5×10^6 cells/ml). Specific-pathogen-free male C57BL/6 mice were intravenously inoculated with cell solution (1.5×10^5 cells/0.1 ml). Following tumor inoculation, native YIGSR (1.5 μmol/0.1 ml) and SMA-YIGSR (0.03 μmol/0.1 ml) were injected intravenously. Mice were sacrificed two weeks after tumor inoculation. Lungs were stained with Bouin's solution, and colonies on the surface of lung were counted under a stereoscopic microscope.

Evaluation of plasma clearance. SMA-YIGSR and native YIGSR were radiolabeled with ^{125}I by the chrolamine-T method (20) yielding ^{125}I-SMA-YIGSR and ^{125}I-native YIGSR with specific activities of 0.68 μCi/mg peptide. The inhibitory effect of ^{125}I labeled SMA-YIGSR and native YIGSR on migration of B16-BL6 melanoma cells against laminin were indistinguishable from those of non-radiolabeled SMA-YIGSR and YIGSR (data not shown). C57BL/6 mice (male, 6 weeks) were intravenously injected with ^{125}I-labeled SMA-YIGSR and YIGSR (1×10^6 cpm/200 μl/mouse) and blood was collected from the tail vein at various time points. The blood radioactivity was measured with a γ-counter. Elimination of radioactivity from the circulation was evaluated by curve fitting with a nonlinear least-squares program (MULTI).

Analysis of albumin-binding affinity. The affinities of SMA, YIGSR and SMA-YIGSR to albumin were examined with BIAcore Biosensor (BIAcore 2000; Pharmacia Biotech). Mouse serum albumin (MSA; 10 μg/ml) in 10 mM acetate buffer (pH 4.0) was immobilized to sensorchip CM5 at a flow rate of 5 μl/min over a period of 7 min with an amino coupling kit (Pharmacia Biotech). The final level of MSA immobilization was about 3206 resonance units. The analytes were injected at a rate of 20 μl/min over a period of 3 min. Immobilized MSA was regenerated after each cycle by injection of 20 μl of 0.5% SDS solution. All experiments were performed at a temperature of 25°C.

RESULTS AND DISCUSSION

A molecule of SMA, a comb-shaped polymeric modifier, can bind to multiple YIGSR, while a molecule of PEG, a chain-shaped polymeric modifier, can only react with one or two YIGSR molecules. SMA-conjugated NCS (SMANCS) showed longer plasma half-lives than native NCS, because SMA attached to NCS rapidly bound to plasma albumin. The laminin cell-adhesive peptide YIGSR has been suggested to be useful as a novel antimetastatic agent. However, YIGSR was quickly cleared from blood circulation when intravenously injected for the treatment of vascular metastasis, and therefore its clinical application has been limited. We designed SMA- conjugated-YIGSR to enhance the blood residency of YIGSR to augment its clinical use as an antimetastatic agent.

SMA-YIGSR was synthesized via the formation of an amide bond between the amino group of YIGSR and anhydride group of SMA with number-average molecular weight of 6000 (Fig. 1a). We previously reported that there is an optimum molecular weight (about 5000~6000) of polymeric modifier to enhance the therapeutic potency of cytokines, and the antimetastatic potency of YIGSR was also augmented by bioconjugation with PEG with an average molecular weight of 6000 (21) The synthetic reaction of SMA-YIGSR was

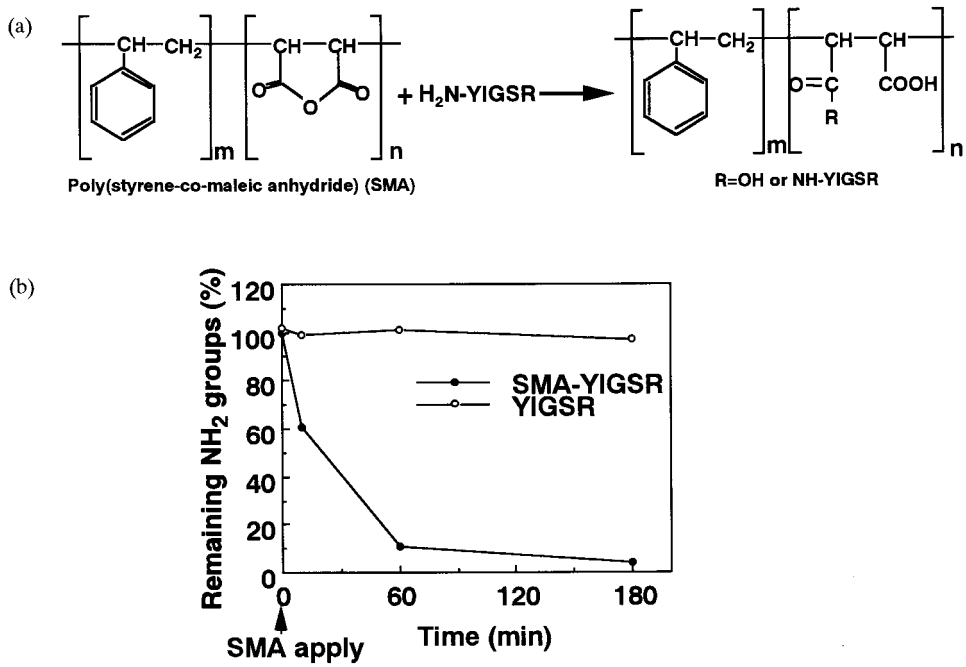

FIG. 1. Chemical coupling of SMA to YIGSR. (a) The coupling reaction was carried out at room temperature by adding of SMA solution in DMSO to the YIGSR solution in borate buffer while the solution was stirred. The ratio of m and n of SMA with a molecular weight 6000, is 1. (b) The reaction of the amino group of YIGSR was quantified by the fluorescamin method. Before adding SMA, the amount of amino group was designed as 100% and the remaining amino groups were determined at indicated times (closed circles). At the same time the amino group of YIGSR solution without adding SMA was also measured (open circles).

found to progress time-dependently by measuring the N-terminal amino groups of unreacted YIGSR, and 95% of added YIGSR was coupled to SMA during the initial 180 min of this synthesis reaction (Fig. 1b). The number average molecular weight of SMA-YIGSR thus obtained was shown to be about 9000 by GFC analysis, and the SMA-YIGSR molecule was found to consist of one SMA molecule and five YIGSR molecules by DC protein assay. This molar content of YIGSR to SMA-YIGSR, assessed by DC protein assay was in good agreement with that calculated from the molecular weight of SMA-YIGSR and synthetic efficiency of SMA-YIGSR (Fig. 1b).

The antimetastatic effects of SMA-YIGSR and native YIGSR on experimental lung metastasis of B16-BL6 melanoma cells were evaluated by separate injection of the peptides from B16-BL6 melanoma cells; i.e., SMA-YIGSR and native YIGSR were injected iv followed by iv inoculation of B16-BL6 melanoma cells (Fig. 2). We previously assessed the antimetastatic potencies of various anti-adhesive peptides by iv coinjection of mixtures of these peptides and B16-BL6 melanoma cells (22). However, on co-injection the initial interaction between these peptides and the cells *in vitro*, such as cytotoxicity of peptides themselves typified by acidic peptides, reduced the number of metastatic colonies of B16-BL6 melanoma cells. Thus, this separate injection method was used to assess the antimetastatic potencies of anti-adhesive peptides. Native YIGSR at a dose of 1.5 μmol/mouse reduced the number of colonies by up to 50% of that seen in controls, while YIGSR did not show any cytotoxic effects on B16-BL6 melanoma cells *in vitro*. In contrast, SMA-YIGSR at a dose of only 0.03 μmol/mouse showed a comparable inhibitory effect to native YIGSR at a dose of 1.5 μmol/mouse. SMA alone did not exhibit any inhibitory effect (data not shown). Thus, SMA-YIGSR had approximately 50-fold more antimetastatic activity than native YIGSR. We previously reported that PEG-conjugated YIGSR was only

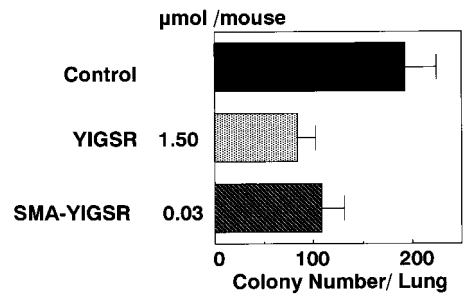

FIG. 2. Inhibitory effects of YIGSR and SMA-YIGSR on lung metastasis of B16-BL6 melanoma cells. The peptides were iv injected into C57BL/6 mice followed with injection of B16-BL6 melanoma cells. The number of colonies was counted under a stereoscopic microscope 14 days after tumor inoculation. Each value represents the mean ± SE.

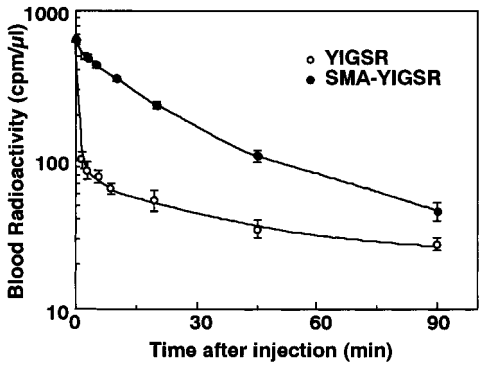

FIG. 3. Clearance of ^{125}I-labeled SMA-YIGSR and YIGSR after iv injection in C57BL/6 mice. SMA-YIGSR (●), YIGSR (○). Blood radioactivity was counted with a γ counter. Mice were used in groups of five. Each value represents the mean ± SE.

20-fold more potent than native YIGSR, although the antimetastatic effect of PEG-YIGSR was assessed by the coinjection method (15). These results suggested that SMA-YIGSR is more useful as an antimetastatic agent than native YIGSR and PEG-YIGSR.

To clarify the mechanism of the enhanced antimetastatic effect of SMA-YIGSR, the elimination profiles of YIGSR and SMA-YIGSR from the circulation after iv injection were studied at the same dosage (Fig. 3). Native YIGSR rapidly disappeared from the circulation, and its plasma half-life was only 1 min. Native YIGSR was quickly degraded by plasma peptidases and mainly distributed to the kidney and excreted via the urine (data not shown). In contrast, the plasma-clearance of SMA-YIGSR was decreased relative to that of native YIGSR, and its plasma half-life (10 min) was 10-fold longer than that of native YIGSR. In addition, SMA-YIGSR was shown to be more resistant to proteolysis than the corresponding native YIGSR and was mainly present in circulation (data not shown). We previously reported that PEG-YIGSR showed the same elimination profile as native YIGSR (15) These findings indicated that SMA is more useful as a polymeric modifier by prolonging plasma half-life. The prolonged half-life was likely because of the increased molecular size by the binding of SMA to plasma albumin.

Binding of SMA-YIGSR to albumin was assessed using BIAcore 2000 Biosensor which measures the ligand-analyte interaction directly in real time without the use of label. Sensorgrams depicting the binding curves obtained for SMA derivatives to immobilized albumin are shown in Figs. 4A and 4B. Various concentrations of SMA and SMA-YIGSR were analyzed, ranging from 0.125 to 1 μg/m. An increase in resonance unit (RU) was observed with increasing analyte concentration, while no significant resonance signals were obtained for native YIGSR (data not shown). These results indicated that the binding of SMA-YIGSR to albumin is due to SMA and not YIGSR.

We analyzed the data in the Langmuir binding model (23, 24). In this model, the detector response, RU, corresponds to the amount of complex formed and the maximum response, R_{max}, is proportional to the

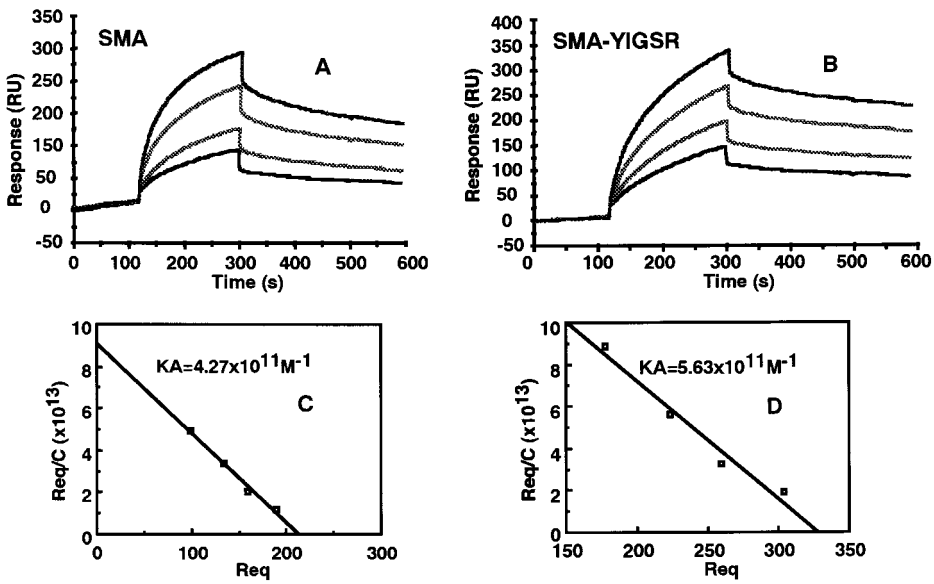

FIG. 4. (A and B) Binding of SMA and SMA-YIGSR to immobilized MSA (mouse serum albumin). SMA or SMA-YIGSR was injected at four different concentrations ranging from 0.125 to 1 μg/ml. Buffer flow rate was 5 μl/min. Equilibrium binding levels were reached within 7 min. (C and D) Scatchard analysis of the binding of SMA and SMA-YIGSR to albumin. Plots are from the data in A and B, respectively, after correction for nonspecific binding; Req is the corrected equilibrium response at a given concentration C. The plots are linear with correlation coefficients of 0.985 and 0.963, respectively. The apparent KA for the SMA-MSA interaction is 4.27×10^{11} M^{-1} and for the SMA-YIGSR-MSA interaction it is 5.63×10^{11} M^{-1}.

surface concentration of the receptor. The rate equation may therefore be expressed as:

dR/dt = KaCn(Rmax-R)-KdR, at equilibrium, dR/dt = 0. Thus, $KaCn(R_{max} - Req) = KdReq$. Hence, a plot of Req/Cn vs Req will give a slope of KA (association constants, Ka/Kd). In this study, Scatchard analysis (a plot of Req/Cn vs Req) was performed with the data shown in Figs. 4A and 4B, and maximum binding (intercept on the X axis) and KA were calculated after correction for nonspecific binding (Figs. 4C and 4D). KA of SMA and SMA-YIGSR were 3.8×10^{11} 1/M and 4.5×10^{11} 1/M, respectively. These results indicated that SMA-YIGSR can bind to plasma albumin as strongly as SMA in agreement with our original assumption.

In conclusion, this is the first report which describes the preparation of a conjugated bioactive peptide with SMA, a comb-shaped macromolecule. SMA-YIGSR showed prolonged blood residency and increased the antimetastatic activity compared with native YIGSR. This prolonged blood residency is attributable to plasma albumin binding of SMA-YIGSR through SMA, but not through the YIGSR portion.

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