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Facile Synthesis of Laminin-Related Peptide–Poly(Ethylene Glycol) Hybrids by the Solid Phase Method

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Poly(ethylene glycol) (PEG) has been used as a drug-carrier for proteins, but not for small peptides. Laminin, a cell adhesive protein, has Tyr–Ile–Gly–Ser–Arg (YIGSR) sequence and peptides containing this sequence inhibit experimental metastasis. We have studied PEG hybrids of YIGSR and other small laminin-related peptides. In a previous paper, we reported preparation of PEG–YIGSR hybrids by combination of the solid phase method and the solution method, but the synthetic procedure was problematic. Here we report a facile synthesis of PEG hybrids of YIGSR (PEG–YIGSR, YIGSR–PEG, PEG–YIGSR–PEG) by the solid phase method.

Key words poly(ethylene glycol); peptide–poly(ethylene glycol) hybrid; laminin; antimetastatic peptide; peptide synthesis

Laminin, a cell adhesion protein consisting of three peptide chains, promotes the adhesion and growth of epithelial and tumor cells. It contains Tyr–Ile–Gly–Ser–Arg (YIGSR) sequence and peptides containing that sequence was found to be an inhibitor of experimental metastasis in mice by lwamoto et al. 1)

Since poly(ethylene glycol) (PEG) has low toxicity, low immunogenicity and good solubility in both aqueous and organic solvents, it appears to be a promising drug-carrier. “Pegylation” (hybrid formation with PEG) 2) is a relatively new concept and is growing in popularity. PEG has been used for modification of proteins and relatively few studies have focused on the synthesis and characterization of PEG hybrids with small peptides. The previous lack of interest in this approach may be based on the assumption that the modification of a small peptide with a large molecule such as PEG would result in a loss of activity. In a previous paper, we were successful in forming a biologically active PEG hybrid of YIGSRG (Gly was a spacer). 3) We prepared it by combination of the solid phase method and the solution method. Fmoc–Tyr(BzI)–Ile–Gly–Ser(BzI)–Arg(NO2)–Gly–methylbenzhydrolamine resin (Fmoc: fluorenylmethyloxycarbonyl) 4) was prepared and treated with HF. The resulting Fmoc–BzlTyr–Ile–Gly–Ser(Bzl)–Arg(NO2)–Gly–methylbenzhydylamine resin (Fmoc strategy and treated it with 20% piperidine/DMF to remove the Fmoc group, followed by acetylation with acetic anhydride. An acetylated peptide may be more stable to hydrolysis by aminopeptidases.

First, PEG–YIGSR was prepared. Pegylation of a peptide on a resin was performed according to the general procedure reported by Lu and Felix. 3) They pegylated the peptide-resin with carboxymethylated PEG (cPEG). We prepared cPEG from PEG monomethyl ether (mPEG, average molecular weight, 2000) using the general procedure of Leonard et al. 5) Although they used naphthalene sodium as a reagent, we used sodium hydride, which is much easier to control. The reaction of mPEG with ethyl iodoacetate in a presence of sodium hydride and subsequent saponification yielded carboxymethylated PEG methyl ether (cPEG). The conjugate cPEG–YIGSR was prepared by the Fmoc strategy on a Rink amide resin. 3) Fmoc–Arg(Pmc)–OH (Pmc: 2,2,5,7,8-penta- methylchroman-6-sufonyl), Fmoc–Ser(Bu’)-OH (Bu’: tert-buty1), Fmoc–Gly–OH, Fmoc–Ile–OH, and Fmoc–Tyr(Bu’)-OH were introduced on the resin in a stepwise manner by the diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) method. 9) Fmoc groups were removed by treatment with 20% piperidine/dimethylformamide (DMF). The reaction with cPEG was slow, the reaction was repeated 3 times using a 3 equivimolar excess of cPEG. Synthetic cPEG–Tyr(Bu’)-Ile–Gly–Ser(Bu’)-Arg(Pmc)–resin was treated with trifluoracetic acid (TFA) to give cPEG–Tyr–Ile–Gly–Ser–Arg–NH2 (cPEG–YIGSR) which was purified by HPLC. The peptide content of the purified hybrid was 0.25 mmol/g.

Next, YIGSR–PEG was prepared. Recently, resins that contain PEG as a spacer have become commercially available. Among them, TentaGel NH2 is a unique resin which liberates a peptide–PEG hybrid at the final cleavage reaction of a synthetic peptide-resin. The average molecular weight of the PEG is 3000 according to the manufacturer’s specifications. We prepared Fmoc–Tyr(Bu’)-Ile–Gly–Ser(Bu’)-Arg(Pmc)–TentaGel NH2 by the solid phase method using the Fmoc strategy and treated it with 20% piperidine/DMF to remove the Fmoc group, followed by acetylation with acetic anhydride. An acetylated peptide may be more stable to hydrolysis by mamphetamine in vivo compared to a peptide with a free amino group. The synthetic peptide-resin was treated with TFA and the product, Ac–YIGSR–PEG, was purified by HPLC. The peptide content of the purified hybrid was 0.19 mmol/g.

The above synthetic intermediate, H–Tyr(Bu’)-Ile–Gly–Ser(Bu’)-Arg(Pmc)–TentaGel NH2, was used for conjugation with cPEG. Since the reaction of cPEG was slow, the reac-
tion was repeated 3 times using a 3 equimolar excess of cPEG. The synthetic cPEG–Tyr(Bu’–Ile–Gly–Ser(Bu’–Arg(Pmc)–TentaGel NH₂ was treated with TFA and the product, cPEG–YIGSR–PEG, was purified by HPLC. Peptide content of the purified product was 0.14 mmol/g.

Prior to the metastasis assay, the viability of B16-BL6 incubated with synthetic hybrids was examined and the results are shown in Fig. 3. Although there is a wide variation in colony counts, the data in Fig. 3 indicates that the synthetic hybrids are not cytotoxic.

The inhibitory effect of the hybrids on experimental metastasis in mice was examined in mice and the results are shown in Fig. 4. B16-BL6 cells and the hybrids 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. The mice were sacrificed 14d after tumor inoculation, and the lungs were removed. The number of surface melanoma colonies on the lungs was counted with a stereoscopic microscope. As shown in Fig. 4, when compared to control values, hybrids did not appear to have a significant inhibitory effect at doses used in these experiments. Ac–YIGSR was slightly inhibitory at a dose of 1.33 mmol/mouse and cPEG–YIGSR displayed some inhibitory effect at 0.13 and 0.44 mmol/mouse. Ac–YIGSR–PEG did not show an inhibitory effect even at 0.44 mmol/mouse. These results may indicate that N-terminal pegylation of YIGSR is more effective than C-terminal pegylation. Since the molecular weights of the PEGs of these hybrids were different, there is a possibility that the differential
effects depend, at least in part, upon molecular weight differences in the conjugates. Tentagel NH₂, containing PEG with a molecular weight of 2000, is not commercially available, and PEG monomethyl ether with a molecular weight 3000 is also not commercially available. This precluded a comparison of PEG hybrids which have an equal size of PEG at the C-terminal and N-terminal of YIGSR. cPEG–YIGSR–PEG displayed a dose-dependent inhibitory effect. At 0.44 mmol, cPEG–YIGSR–PEG had a greater inhibitory effect than Ac–YIGSR. The effect of 0.44 mmol of cPEG–YIGSR–PEG is nearly equal to that of 0.44 mmol of cPEG–YIGSR and 1.33 μmol of Ac–YIGSR. From these results, PEG may activate YIGSR, depending upon the conjugate formed. A flexible large molecule such as PEG does not inhibit the action of YIGSR and may potentiate the inhibitory effect by preventing enzymatic degradation of YIGSR portion.

Experimental
Acid hydrolysates were performed in constant-boiling HCl at 110 °C for 24 h in evacuated tubes. Amino acid compositions of acid hydrolysates were determined with a Waters Pico-TAG amino acid analyzer and RP-HPLC was performed using a Waters 600 with a DAISOPAK column and gradient systems of CH₃CN/water containing 0.05% TFA. Time of flight (TOF)-mass spectra were obtained from a SHIMAZU/KRATOS KOMPACT MALDI IV spectrometer. Rink amide resin, amino acids and coupling reagents were purchased from Watanabe Chemical Industries, Ltd. PEG monomethyl ether (average molecular weight, 2000) was purchased from Sigma-Aldrich Japan Inc. Tentagel NH₂ resin was purchased from Bio-Medical System, Shimadzu Scientific Research Inc. According to the manufacturer, the average molecular weight of the PEG portion of Tentagel NH₂ is approximately 3000.

General Procedure for Peptide Synthesis by the Solid Phase Method
Ac–YIGSR–NH₂ and the hybrids were prepared on Rink amide resin by a manual method according to the procedure shown below. Coupling reactions were checked by the Kaiser test (ninhydrin test).
cPEG monomethyl-PEG (average molecular weight 2000, 10 g, 5 mmol) was used in a reaction with ethyl iododecylate (3.2 g, 15 mmol) and 60% sodium hydride/oil (400 mg, 10 mmol) in tetrahydrofuran (150 ml) in a nitrogen atmosphere at room temperature for 4 h. The solvent was removed in vacuo and the residue was washed with ether. The residue was dissolved in 1 N NaOH (25 ml) and stirred for 2 h at 40°C. The mixture was acidified with formic acid and extracted with CHCl₃. The CHCl₃ layer was washed with saturated NaCl solution and dried with MgSO₄. The solvent was concentrated and ether was added. The resulting precipitate was collected by filtration and washed with ether. Yield 8.8 g (85%).

Ac–YIGSR–NH₂·HCl Synthetic H–Tyr(Bu)c–Ile–Gly–Ser(Bu)c–Arg(Pmc)–Rink amide resin (1.50 g) was treated with acetic anhydride and pyridine in DMF. After the reaction, the resin was washed with DMF and CHCl₃, and dried. The resin was treated with a mixture (TFA/anisole/thioanisole, 96/2/2, v/v/v) for 1.5 h. TFA was removed in vacuo and the residue was washed with ether repeatedly. The peptide was extracted from the residue with 5% AcOH and purified by HPLC. The product was lyophilized from water containing HCl. Yield 127 mg (45%, calculated from the Rink amide resin).

Ac–YIGSR–NH₂ Synthetic H–Tyr(Bu)c–Ile–Gly–Ser(Bu)c–Arg(Pmc)–Rink amide resin (300 mg, 0.12 meq) was used in a reaction with cPEG (740 mg, 0.36 mmol) by the DIC/HOBt method. The reaction was repeated 3 times and the resulting resin was treated with the mixture of TFA/anisole/thioanisole in the same manner described above. The hybrid was extracted with water and purified by HPLC. The purified product was treated with the mixture of TFA/anisole/thioanisole (96/2/2, v/v/v) in the same manner described above. The product was purified by HPLC and converted to its hydrochloride by lyophilization from water containing HCl. Yield 75 mg (49%, calculated the Tentagel resin). [α]D₅₂⁰ = −6.6° (c = 1.0, H₂O). Average molecular weight measured by TOF-MS, 6120. Amino acid ratios in an acid hydrolysate: Tyr 0.98; Ile 1.00; Gly 0.97; Ser 1.00; Arg 1.03. Peptide content calculated from amino acid analysis of the acid hydrolysate: 0.14 mmol/g.

Viability of B16-BL6 Melanoma Admixed with PEG Hybrids Cells in minimum essential medium (MEM) containing 0.1% bovine serum albumin (BSA) (2×10⁶/ml) and samples (10 mg/ml) were mixed in the ratio of 1 to 1, and incubated at room temperature. After 60 min, the cells were seeded onto culture dishes, and colonies were counted after 1 week. Each value represents the mean ± S.E.

Metastasis Assay B16-BL6 melanoma cells (1×10⁶/0.1 ml) and a synthetic PEG hybrid (1 mg/0.1 ml/mouse) were intravenously injected separately into C57BL/6 mice. The mice were killed at 14 d after tumor inoculation, and the lungs were removed. The number of surface melanoma colonies on the lungs was counted under a stereoscopic microscope.

References