Amino Acids and Peptides. XXI. 1) Laminin-Related Peptide Analogs Including Poly(Ethylene Glycol) Hybrids and Their Inhibitory Effect on Experimental Metastasis

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Laminin-related peptides, Tyr—Ile—Gly—Ser—Arg analogs, were prepared and their inhibitory effects on experimental metastasis were examined. Of the amino acids in the Tyr—Ile—Gly—Ser—Arg sequence, Ile was very important and Ile was not essential for the inhibitory effect. To obtain a potent inhibitor of metastasis, hybrids of Tyr—Ile—Gly—Ser—Arg—Gly and 2 types of poly(ethylene glycol) were prepared. The inhibitory effects of the hybrids were more potent than that of Tyr—Ile—Gly—Ser—Arg—Gly.

Keywords laminin; metastasis inhibitor; poly(ethylene glycol); hybrid; poly(ethylene glycol) hybrid; polymer hybrid

Laminin is a glycoprotein which is classified as a cell adhesion protein. It promotes the adhesion and growth of epithelial and tumor cells. It consists of three peptide chains (A, B1 and B2) and partial sequences of the B1 chain, Tyr—Ile—Gly—Ser—Arg (YIGSR) and Cys—Asp—Pro—Gly—Tyr—Ile—Gly—Ser—Arg (CDPGYIGSR), were found to be inhibitors of experimental metastasis in mice by Iwamoto et al. 2) We prepared YIGSR analogs and examined their inhibitory effect on experimental metastasis in mice. In the preceding communication, 3) we reported that the inhibitory effect of the hybrid of poly(ethylene glycol) (PEG) and YIGSRG was more potent than that of YIGSRG. Here we present full details of that work, and additional studies on the preparation and the inhibitory effect of YIGSR analogs. First, YIGSR, CDPGYIGSR, (CDPGYIGSR)2, (the disulfide-bonded dimer of CDPGYIGSR), 2) YIGS, IGSR, YIGSRC, and YIGSRDC, and YIGSE 2) were prepared by the solid phase method. 3) The x-amino group was protected with a tert-butyloxy carbonyl (Boc) group or p-methoxybenzylxy carbonyl group, which was removable by trifluoroacetic acid (TFA) treatment. The following groups were used for side chain protection: p-methylbenzyl group for Cys, benzyl group for Tyr and Ser, tosyl or nitro group for Arg, and cyclohexyl group for Asp and Glu. Final deprotection was performed by HF treatment 6) and products were purified by reverse phase high performance liquid chromatography (RP-HPLC). CDPGYIGSR 2) was prepared by air oxidation of CDPGYIGSR in aqueous solution at pH 8 and its molecular weight was examined by mass spectroscopy. The products were converted to the hydrochlorides and their inhibitory effects were examined. Each synthetic peptide (1 mg) was mixed with B16-F10 melanoma cells and the mixture was injected into the tail vein of mice. Three weeks later, the mice were killed and the numbers of surface melanoma colonies on the lungs were counted macroscopically. The results are shown in Fig. 2. YIGSR and CDPGYIGSR exhibited an inhibitory effect as reported. 2) The inhibitory effects of YIGSRC and VCDPGYIGSRC were expected to be more potent than those of YIGSR and CDPGYIGSR, but in fact these chain-extended peptides were less active than YIGSR and CDPGYIGSR. The reason is not clear, but the extension of the peptide chain might change the conformation and ionic charge and these changes might result in decreased activity. The dimer of CDPGYIGSR was less active than the monomer. IGSR and YIGSE did not show any inhibitory effect but YIGS showed a weak effect. Tyr may have a more important role in the inhibitory effect compared with Arg.

Next, YIGSdr (dR = dextro Arg), YCGSR, (YCGSR)2 (the disulfide-bonded dimer of YCGSR), and (YIGSR)2K were similarly prepared by the solid phase method. B16-BL6 melanoma cells were used to examine the

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Fig. 1. Amino Acid Sequence of Mouse Laminin B1 Chain 921—936

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Fig. 2. Inhibitory Effect of Synthetic Peptides on Experimental Metastasis in Mice

B16-F10 melanoma cells (1 × 10⁶/0.2 ml) were injected i.v. with or without admixing with 1 mg of peptide into five mice per group. Lung tumor colonies were examined 21 d later. Values are the mean ± S.D. a) p < 0.01, b) p < 0.001.
inhibitory effect of these synthetic peptides on experimental metastasis since they were more metastatic than B16-F10. The inhibitory effects of the synthetic peptides are shown in Fig. 3.

YIGSR also did not show an inhibitory effect on B16-BL6 melanoma cells at 1 mg dose. YIGSR, CDPGYIGSR, (CDPGYIGSR), were inhibitory and (CDPGYIGSR)2 was less effective than CDPGYIGSR. YIGSR was not effective. This result suggested a very important role of L-Arg for the inhibitory effect. YCGSR and (YCGSR)2 were inhibitory, indicating that Ile was not essential for the inhibitory effect. As with CDPGYIGSR, YCGSR was more effective than its dimer, (YCGSR)2. Recently it was reported that a thiol group might exist in \( \mu \)-opioid receptor and enkephalin analogs containing an activated thiol group bind to the receptor through a disulfide bond. Similarly, a functional group which reacts with the thiol group of CDPGYIGSR and YCGSR may exist in the receptor of YIGSR analogs. The dimer through the Lys residue, (YIGSR)2K, was not effective.

To obtain a more potent inhibitor, other types of inhibitor were considered. Two types of potent inhibitors have been reported: cyclic YIGSR[8] and polymers of YIGSR and RGD[9]. Since these peptides have specifically cyclic and polymeric forms, we prepared a cyclic YIGSR analog and polymer-bound YIGSR analogs. Since we found that Ile was not essential for the inhibitory effect, YCGSR was prepared by the solid phase method and oxidized it to form a cyclic peptide through an intramolecular disulfide bond. PEG is a polymer which has many advantages as a drug carrier. It is stable, weakly toxic, soluble in aqueous and organic solvents, weakly immunogenic, and has little effect on the conformation of peptides. Though many PEG hybrids of proteins have been studied to improve the stability and the activity of the proteins[10], small-peptide hybrids have not been investigated. Therefore, we prepared PEG hybrids of YIGSRG and examined their inhibitory effect on metastasis. PEG44000 (4K, M.W. 3000–3700) and PEG46000 (6K, M.W. 6700–9000) were converted to amino-poly-(ethylene glycol) [aPEG, \( H_2N-CH_2CH_2OCH_2CH_2\)]=NH\(_3\) according to the procedure reported by Pillai and Mutter. aPEGs were purified by Dowex 50 column chromatography. YIGSRG–aPEG was prepared as shown in Fig. 3. Gly[8] was introduced as a spacer. Fmoc-YIGSRG–OH was prepared by the solid phase method starting from chloromethyl resin. For protection of \( \alpha \)-amino groups, a Boc group was used for Ile, Gly, Ser and Arg. Fmoc–Tyr(OBz)–OH was used at the final coupling on resin and Fmoc–Tyr(Bzl)–Ile–Gly–Ser(Bzl)–Arg(NO\(_2\))–Gly–resin was treated with HF to give Fmoc–YIGSRG–OH. The Fmoc hexapeptide was purified by HPLC and converted to its hydrochloride. Then the peptide was coupled with aPEG (4K and 6K) by the diphenylphosphoryl azide (DPPA) method. The Fmoc group on Tyr was removed by piperidine treatment to give YIGSRG–aPEG. The product was purified by HPLC. Peptide content of each hybrid was calculated from the amino acid content in an acid hydrolysate. The peptide contents of hybrids 4K and 6K were 0.34 and 0.16 mmol/g respectively. Prior to the examination of the inhibitory effect of hybrids 4K and 6K, the inhibitory effect of PEG itself was examined and the result is shown in Fig. 5. PEG 4K and PEG 6K (2 mg) did not show any inhibitory effect on metastasis. The inhibitory effects of the cyclic peptide and hybrid 4K are shown in Fig. 6. The effects of YIGSR
and YIGSRG were not distinctive. The cyclic peptide was less active than expected, but the hybrid 4K exhibited potent inhibitory effect. The effect of 0.3 mg of hybrid 4K was comparable to that of 1.0 mg of YIGSR or CDPGYIGSR.

The effects of hybrids 4K and 6K were compared and the results are shown in Fig. 7. The inhibitory effects of hybrids 4K and 6K were almost equal, and the effects of 300 µg of hybrids 4K and 6K were equivalent to that of 600 µg of YIGSRG. Considering the molecular weight of the hybrids, the inhibitory effect of hybrid 6K was about twice as potent as that of hybrid 4K in term of molecular ratio. The peptide content of the hybrid 6K is 0.16 mmol/g, so 300 µg of the hybrid 6K contains 0.048 µmol of YIGSRG. Since 600 µg of YIGSR-2HCl is 0.829 µmol, it can be said that the inhibitory effect of YIGSRG is potentiated about 17-fold by the hybrid formation. Why the inhibitory effect of the PEG hybrids was more potent than that of YIGSRG is not clear, but presumably one reason is slower enzymatic degradation of the YIGSRG portion. Enzymatic degradation of the YIGSRG moiety may be prevented by PEG. YIGSRG was easily hydrolyzed by aminopeptidase M but hydrolysis of the YIGSR portion of the PEG hybrid 4K by the enzyme was very slow. Hydrolysis of the hybrid 6K by z-chymotrypsin was also slower than that of YIGSRG. PEG with its flexible conformation does not prevent the binding of the hybrid to the receptor, and its bulk in the hybrid can stabilize the binding between the YIGSRG portion and the receptor.

**Experimental**

Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, Merck) are indicated as follows: $R_f^1 = \text{BuOH-} \text{AcOH-} \text{H}_2\text{O}$ (4:1:5, upper phase), $R_f^2 = \text{BuOH-} \text{pyridine-} \text{AcOH-} \text{H}_2\text{O}$ (4:1:1:2), $R_f^3 = \text{CHCl}_3-\text{MeOH-} \text{H}_2\text{O}$ (90:8:3, lower phase). Synthetic peptides were hydrolyzed in 6N HCl at 110°C for 24h and PEG-peptide hybrids were hydrolyzed for 48h. Amino acid compositions of acid hydrolyzates were determined with a Hitachi 835 amino acid analyzer. RP-HPLC was conducted with a Waters 6000A YMC Pack AQC-ODS-5 column using gradient systems of CH$_3$CN/H$_2$O containing 0.1% TFA. FAB-MS was measured on a VG Analytical ZAV-SE spectrometer. PEG was purchased from Nacalai Tesque, Inc.

A) Peptide Synthesis by the Solid Phase Method. General Procedure; $p$-Methylbenzhydrolamine resin and chloromethylated resin were purchased from Peptide Institute, Inc. The following amino acid derivatives were used: Z(OMe)-Gly-OH, Boc-Tyr(Bzl)-OH, Boc-Ser(Bzl)-OH, Boc-Arg(Tos)-OH, Boc-Ile-OH, Boc-Glu(OBzl)-OH. The synthetic protocol for solid-phase peptide synthesis is shown below. Reactions were checked by using the ninhydrin test. (13)

**Fig. 5. Effect of PEG on B16 Melanoma BL6 Cell Lung Metastasis**

B16 BL6 cells ($1 \times 10^7/0.2 ml$) were injected i.v. with or without admixing with 2 mg of PEG into five mice per group. Lung tumor colonies were examined 21d later. Values are the mean±S.D. a) $p<0.05$, b) $p<0.01$ compared with untreated control (MEM) by Student's t test.

**Fig. 6. Inhibitory Effect of Synthetic Peptides on Experimental Metastasis in Mice**

B16 BL6 cells ($1 \times 10^7/0.2 ml$) were injected i.v. with or without admixing with 0.3 mg ( ), 1.0 mg ( ), 2.0 mg ( ) of peptide into five mice per group. Lung tumor colonies were examined 21d later. Values are the mean±S.D. a) $p<0.05$, b) $p<0.01$ compared with untreated control (MEM) by Student's t test.

**Fig. 7. Inhibitory Effect of YIGSRG-aPEG Conjugates on the Formation of Lung Tumors**

B16 BL6 cells ($1 \times 10^7/0.2 ml$) were injected i.v. with or without admixing with various concentrations of peptide into five mice per group. Lung tumor colonies were examined 21d later. Values are the mean±S.D. a) $p<0.005$, b) $p<0.001$ compared with untreated control by Student's t test.
A 1m HOB/DMF solution (2 eq) was added when Boc-Arg(Tos)-OH was activated. Final deprotection was performed by HF treatment. The product was purified by RP-HPLC. Yields were calculated from crude debrominated material. The peptides purified by HPLC were converted to their hydrochlorides by lyophilization from HCl-containing water.

H-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 26%, hygroscopic powder, Rf 0.05, Rf 0.12, [x]D 25° 18.0'(c = 1.0, H₂O). MS m/z: 594 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ile 0.97, Gly 1.00, Ser 0.88, Arg 0.94 (average recovery 80.3%).

H-Tyr-Ile-Gly-Ser-NH₂ Yield 24%, hygroscopic powder, Rf 0.10, Rf 0.27, [x]D 25° 3.8'(c = 1.0, H₂O). MS m/z: 438 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.97, Ile 0.94, Gly 1.00, Ser 0.91 (average recovery 83%).

H-Tyr-Gly-Ser-AcNH₂ Yield 28%, hygroscopic powder, Rf 0.02, Rf 0.65, [x]D 25° 8.0'(c = 1.0, H₂O). MS m/z: 431 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Ile 0.96, Gly 1.00, Ser 0.91, Arg 0.99 (average recovery 92%).

H-Tyr-Ile-Gly-Ser-Arg-Cys-Asp-NH₂ Yield 10%, hygroscopic powder, Rf 0.05, [x]D 25° 60.4'(c = 1.0, H₂O). MS m/z: 813 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.92, Ile 0.91, Gly 1.00, Ser 0.97, Arg 0.99, Cys 0.91, Asp 1.01 (average recovery 84%).

H-Tyr-Ile-Gly-Ser-Glu-NH₂ Yield 24%, hygroscopic powder, Rf 0.02, [x]D 25° 19.4'(c = 1.0, H₂O). MS m/z: 567 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 1.04, Ile 0.95, Gly 1.00, Ser 0.95, Glu 1.07 (average recovery 85%).

H-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 26%, hygroscopic powder, Rf 0.03, [x]D 25° 14.9'(c = 1.0, H₂O). MS m/z: 584 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.97, Cys 0.83, Gly 1.00, Ser 1.02, Arg 1.04 (average recovery 85%).

H-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 10%, hygroscopic powder, Rf 0.12, [x]D 25° 8.0'(c = 1.0, H₂O). MS m/z: 594 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.95, Ile 0.96, Gly 1.00, Ser 1.00, Arg 1.02 (average recovery 76%).

H-Tyr-Ile-Gly-Arg-Lys-NH₂ Yield 14%, Rf 0.05, [x]D 25° −28.5'(c = 1.0, H₂O). MS m/z: 1166 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.86, Cys 0.86, Gly 1.00, Ser 0.96, Arg 1.03, Lys 1.04 (average recovery 84%).

H-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 10%, hygroscopic powder, Rf 0.12, [x]D 25° 8.0'(c = 1.0, H₂O). MS m/z: 594 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Tyr 0.95, Ile 0.96, Gly 1.00, Ser 1.00, Arg 1.02 (average recovery 76%).

H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 11%, hygroscopic powder, Rf 0.02, [x]D 25° −53.3'(c = 1.0, H₂O). MS m/z: 967 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Cys 0.89, Asp 0.94, Pro 0.98, Gly 2.00, Tyr 0.95, Ile 0.98, Ser 0.95, Arg 0.97 (average recovery 81%).

H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 11%, hygroscopic powder, Rf 0.02, [x]D 25° −61.3'(c = 1.0, H₂O). MS m/z: 1931 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Cys 0.91, Asp 0.95, Pro 1.02, Gly 2.00, Tyr 0.94, Ile 0.95, Ser 0.98, Arg 1.00 (average recovery 81%).

H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ Yield 83% (12mg), hygroscopic powder, Rf 0.02, [x]D 25° −57.4'(c = 1.0, H₂O). MS m/z: 1283 (M⁺ + 1). Amino acid ratios in an acid hydrolysate: Val 0.96, Cys 1.67, Asp 1.98, Pro 1.06, Gly 2.00, Tyr 0.85, Ile 1.03, Ser 0.89, Arg 0.99 (average recovery 77%).

D) Peptide Synthesis by the Solution Method. H-Tyr-Ile-Gly-Ser-Arg-Gly-α-Peptide (PPA 32 µl, 0.15 mol%) and 10% Et₃N in DMF (20 µl, 0.15 mol%) were added to a DMF solution (7 ml) of FMoc-Tyr(Ile)-Gly-Ser-Arg-Gly-α-OBu (OMe)-Gly-OBu was introduced on chloromethyl resin by the cesium salt method. The peptide-resin (4.4g) was treated with HF (44 ml) containing 2.5% m-cresol and 2.5% anisole at 0°C for 90 min. The HF was removed in vacuo and the product was extracted with 50% dioxane. The resin was removed by filtration and the filtrate was lyophilized. The residue was washed with MeOH and collected by centrifugation.

The residue was purified by LH20 column (3 x 160 cm) chromatography using DMF as an eluent. The DMF was removed in vacuo and the residue was further purified by HPLC. Yield 290 (mg) (19%), amorphous powder, Rf 0.55, [x]D 25° 14.9'(c = 1.0, MeOH). MS m/z: 875 (M⁺ + 1). Anal. Caled. for C₂₂H₃₀N₄O₂: C, 52.77; H, 5.92; N, 12.31. Found: C, 52.84; H, 5.63; N, 12.21. Amino acid ratios in an acid hydrolysate: Tyr 0.95, Ile 0.99, Gly 2.00, Ser 0.96, Arg 0.06 (average recovery 94%). For the next coupling reaction, the peptide was converted to its hydrochloride by lyophilization from dioxane:H₂O containing HCl.

E) Preparation of a Peptide (PEG 56000) and PEG 66000 were added to the corresponding α-peptide according to the procedure reported by Pilall and Mutter. The α-peptide (10g) was dissolved in H₂O (100ml) and the solution was passed through a Dowex 50 (H⁺) column (6 x 30 cm). After washing with H₂O, the column was washed with 50% MeOH and H₂O. Finally the α-peptide was eluted with 3% NH₄OH. The eluate was evaporated to give a white solid. Both α-peptide 4K and 6K gave the same Rf value: Rf 0.02. Each α-peptide was treated with 0.1 N HCl using methyl red as an indicator. Amino content: α-peptide 4K 0.56 mmol/g, α-peptide 6K 0.27 mmol/g.

D) Enzymatic Hydrolysis of YIGSRG and Its α-Peptide Hybrids. The peptide (or hybrid) and the enzyme (10 ml) were dissolved in 0.2 M Tris buffer (pH 7.9, 0.1 ml) and the solution was shaken at 37°C for 24h. Then 0.1N HCl (1 ml) was added and the solution was filtered with a Chromatodisk 13A (0.45 µm, Kurabo). An aliquot of the filtrate was examined with an amino acid analyzer. The following enzyme solutions were used: α-aminopeptidase M (Boehringer Mannheim GmbH, 5 mg/ml) and α-chymotrypsin (Sigma, 10 units/ml).

YIGSRG: YIGSRG (485 µg and 241 µg) was digested by α-aminopeptidase M and α-chymotrypsin, respectively. Amino acid ratios in an α-aminopeptidase M digestion: Tyr 1.37, Ile 1.68, Gly 2.00, Ser 1.72, Arg 1.36 (average recovery 82%). α-Chymotrypsin digest: recovery of Tyr 17.8%.

YIGSRG-α-peptide: The hybrid 4K (1.048 mg) was digested by α-aminopeptidase M. The peptide portion was almost unaffected by the enzyme. Amino acid ratios in an α-aminopeptidase M hydrolysatase: Tyr 5.44, Ile 1.50, Gly 2.00, Ser 5.18, Arg 2.40 (average recovery 15%). Traces of some other amino acids (Ala, Cys, Leu, Orn etc.) were also detected. These were derived from the enzyme.
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References and Notes
1) Standard abbreviations are used for amino acids, protecting groups, and peptides [Eur. J. Biochem., 138, 9 (1984)]. Other abbreviations include: Mts = mesitylenesulfonyl, DMF = dimethylformamide, TFA = trifluoroacetic acid, DCM = dichloromethane, NMM = N-methylmorpholine.