Preparation and Biological Activities of a Bivalent Poly(Ethylene Glycol) Hybrid Containing an Active Site and Its Synergistic Site of Fibronectin[1)]

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A bivalent poly(ethylene glycol) or PEG hybrid of fibronectin-related peptides was prepared. An active site peptide (RGD) and its synergistic site peptide (PHSRN) of fibronectin were conjugated with an amino acid-type PEG (aaPEG) to form PHSRN–aaPEG–RGD. A moderate spatial array between RGD and PHSRN in fibronectin may be required for synergic activity. The bivalent hybrid exhibited potent cell spreading activity and exhibited potent anti-metastatic activity in a model of experimental metastasis with B16–BL6 cells in mice. PEG may serve as a spacer for maintaining the desired spatial array.

Key words poly(ethylene glycol); fibronectin; hybrid; spreading activity; anti-metastatic activity

Bioactive peptides and proteins are the focus of many medical applications, but they have some disadvantages as drugs. They are rapidly hydrolyzed with loss of biological activities. Furthermore, proteins derived from non-human species are often antigenic. To overcome these disadvantages, protein hybrids with natural and artificial polymers have been studied. Among various polymers, poly(ethylene glycol) (PEG) has been used for preparation of hybrids with biologically-active proteins, since PEG has low toxicity, low immunogenicity and good solubility in both aqueous and organic solvents. Pegylation (hybrid formation with PEG) of proteins has been well documented and pegylated adenosine deamidase[2)] and interferon[3)] are used in clinical therapeutics. In contrast, relatively few studies have focused on the pegylation of small peptides. This may be based upon the assumption that modification of a small bioactive peptide with a large molecule such as PEG would result in a loss of activity. However, we prepared PEG hybrids of laminin- and fibronectin-related peptides and proved that PEG is also a promising drug carrier for bioactive small peptides.[4,5)] These PEG hybrids were pegylated at the carboxyl terminal of the peptides. Site-specific pegylation of large proteins is difficult but site-specific pegylation of relatively small peptides is possible by careful selection of synthetic strategies. Lu and Felix[6)] succeeded in the site-specific pegylation of an interleukin-related peptide. Furthermore, PEG can function as a flexible spacer, linking 2 functional groups that allow a structural conformation for optimal biological activity. We designed an amino acid type PEG (aaPEG) as a novel peptide carrier to prepare multivalent PEG-peptide hybrids. We were successful in forming bivalent PEG hybrids of fibronectin- and laminin-related peptides (Arg–Gly–Asp–aaPEG–Glu–Ile–Leu–Asp–Val and Pro–Asp–Ser–Gly–Arg–aaPEG–Tyr–Ile–Gly–Ser–Arg)[7,8)] which have two active sites in each protein. Here, we report the preparation and biological activity of a bivalent poly(ethylene glycol) hybrid containing an active site (Arg–Gly–Asp, RGD) and its synergistic site (Pro–His–Ser–Arg–Asn, PHSRN) of fibronectin.

Fibronectin, a glycoprotein, is a multifunctional extracellular matrix protein. It plays an important biological role in many cell-surface interactions, mediating cell adhesion, embryonic cell migration, and wound healing.[9,10)] Among the many active sites in extracellular matrix proteins, the cell-adhesive domain of fibronectin has been well studied.[11)] An RGD sequence located in the 10th type III repeating unit is a critical cell-adhesive site for the cell-surface receptor integrin,[11,12)] and a PHSRN sequence in the 9th type III repeating unit, although itself not biologically active, enhances the cell-adhesive activity of RGD.[13—15)] However, the cell-adhesive activity of a recombinant protein corresponding to the 10th type III repeating unit of fibronectin is not stimulated by a PHSRN peptide. Based upon this evidence, the spatial array between PHSRN and RGD is important for a synergistic interaction.[15)]

We designed a bivalent PEG hybrid, PHSRN–aaPEG–RGD, in which aaPEG functions as a spacer between two functional peptides. aaPEG [$H_2NCH_2CH_2NHCOCH_2$-$(OCH_2CH_2)_nOCH_2COOH$] was prepared from commercial poly(oxyethylene)diglycolic acid 3000 (carboxymethylated PEG, cmPEG. average MW 2500—3400).[16)] The flexible conformation of PEG may allow the two functional groups to bind properly. As shown in Fig. 2, the PEG hybrid, PHSRN–aaPEG–RGD, was prepared on a Rink amide resin[17)] using a Fmoc-based solid-phase strategy.[18)] The Fmoc groups were removed by treatment with 20% piperidine/dimethylformamide (DMF) for 20 min. Coupling reactions were performed by the diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) method.[19)] The side chains of amino acids were protected as follows: His and Asn by a trityl (Trt) group; Ser

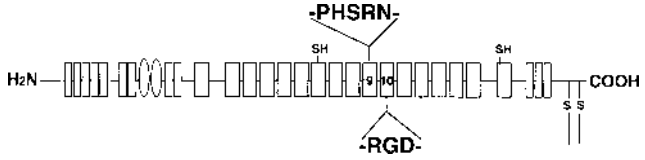

Fig. 1. Structure of Fibronectin

□, Type I module; ○, type II module; ▒, type III module.

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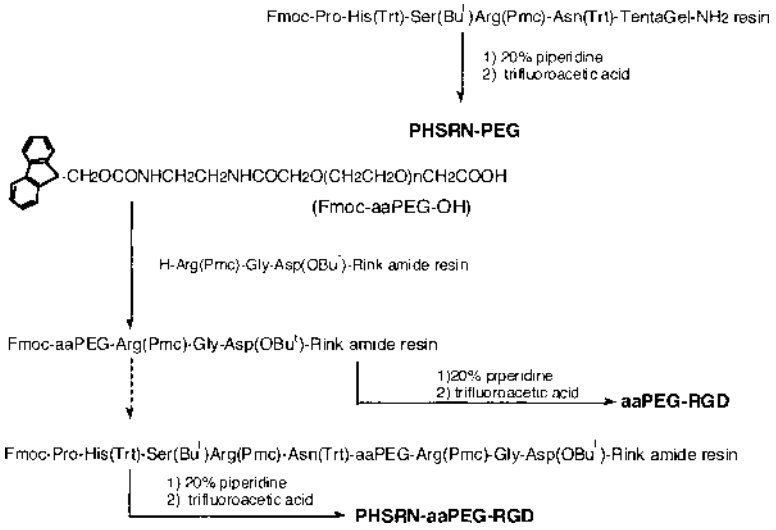

Fig. 2. Synthetic Scheme for PHSRN–aaPEG–RGD

and Asp by a *tert*-butyl (Bu^t) group; and Arg by a 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group.[20] Since the coupling reaction of Fmoc–aaPEG–OH was slow, it was pre-incubated with DIC and HOBt at 0 °C for 1 h and allowed to be used in a coupling reaction. The reaction was repeated once to completion. Fmoc–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin (prepared by the manual method) was treated with 20% piperidine/DMF to form the H–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin, followed by treatment with trifluoroacetic acid (TFA)/thioanisole/ethanedithiol (94/3/3). The resulting aaPEG–RGD was purified by HPLC on a DAISOPAK SP-120-5-ODS-B column (DAISO Co., Ltd.).

After introduction of Fmoc–aaPEG–OH on the resin, the deprotection reaction of the Fmoc group with piperidine became slow and quantitative measurement of deprotection could not be determined by the Kaiser test (ninhydrin test).[21] Therefore, synthesis of PHSRN–aaPEG–RGD was performed by an automatic peptide synthesizer (ABI 433A) which facilitated monitoring the deprotection reaction after introduction of Fmoc–aaPEG on the resin. The deprotection reaction could be monitored by the peptide synthesizer, measuring the conductivity of the solvent from the deprotection reaction mixture. Synthetic Fmoc–Pro–His(Trt)–Ser(Bu^t)–Arg(Pmc)–Asn(Trt)–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin was treated first with 20% piperidine/DMF and then with TFA/thioanisole/ethanedithiol (94/3/3) at room temperature for 2 h. The resulting product was purified by HPLC using a DAISOPAK SP-120-5-ODS-B column followed by an ASAHIPAK GS-320P (ASAHI CHEMICAL INDUSTRY Co., Ltd.) column. The average molecular weight of the purified PHSRN–aaPEG–RGD (measured by time of flight mass spectrometer (TOF-MS)) was 4007. PHSRN–PEG was prepared on TentaGel–NH_2 resin (RAPP POLYMER GmbH). The TentaGel–NH_2 resin contains PEG and liberates a peptide–PEG hybrid by TFA treatment after the completion of peptide synthesis. According to the manufacturer, the average molecular weight of the PEG portion of TentaGel–NH_2 is approximately 3000. Synthetic Fmoc–Pro–His(Trt)–Ser(Bu^t)–Arg(Pmc)–Asn(Trt)–TentaGel–NH_2 resin was treated first with 20% piperidine/DMF and then with TFA/thioanisole/ethanedithiol (94/3/3). The crude PHSRN–PEG was purified by HPLC on a DAISOPAK SP-120-5-ODS-B column. Molecular weight of the product (measured by TOF-MS) was 4127. Since molecular weight of PHSRN portion is 593, the average molecular weight of PEG portion of TentaGel NH_2 was approximately 3500. The molecular weight of the PEG portion in the resin was larger than what the manufacturer specified.

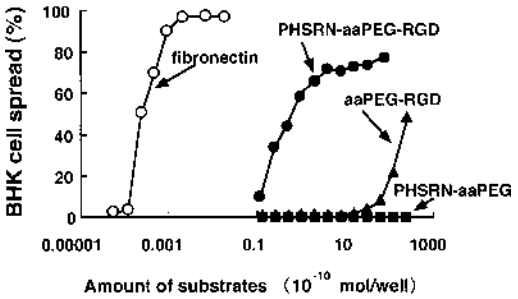

Fig. 3. Cell Spreading Activity of Synthetic Fibronectin-Related Peptide–PEG Hybrids

Various amounts of synthetic PEG hybrids and fibronectin were coated on 96-well dishes and dried overnight. After blocking with BSA, BHK cells (5×10^3/well) were added and incubated for 45 min. The percentage of cells that had spread (%) was counted under a microscope. Each value represents the mean of five separate determinations. Triplicate experiments gave similar results.

The cell spreading activity of the PEG hybrids (aaPEG–RGD, PHSRN–PEG, and PHSRN–aaPEG–RGD) was examined, using Baby Hamster Kidney (BHK) cells as described previously.[13] Fibronectin was used as a positive control.

Fibronectin was most effective in terms of cell spreading activity (Fig. 3). The aaPEG–RGD hybrid displayed cell spreading activity but PHSRN–PEG lacked significant activity. These findings are comparable to previous results obtained using synthetic peptides.[15] The PHSRN–aaPEG–RGD peptide had a greater cell spreading activity than aaPEG–RGD, suggesting that PHSRN synergistically enhances the cellular activity of RGD in the PEG–bridged compounds. These observations are comparable to previous results obtained using recombinant proteins.[15] Thus, aaPEG is potentially useful as a spacer and support matrix that allows the binding of functional peptides.

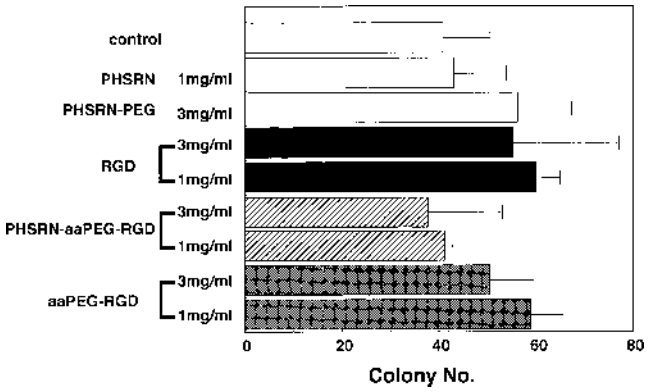

Fig. 4. Viability of B16—BL6 Melanoma with PEG Hybrids

Cells in MEM(−) containing 0.1% BSA (2×10^4/ml) and samples (10 mg/ml) were admixed at 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.

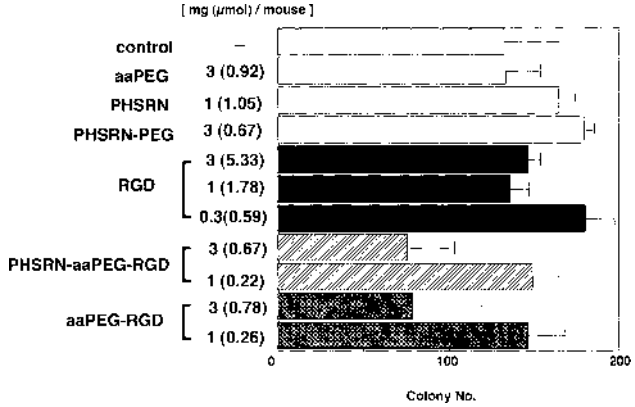

Fig. 5. *In Vivo* Anti-metastatic Activity of PEG Hybrids

B16–BL6 melanoma cells (1×10^5/0.1 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. Each value represents the mean±S.E.

Recently many biologically active sequences have been identified in extra-cellular matrix proteins.[22] Some of the active peptides may synergistically interact with cellular receptors,[23] thus, the bivalent aaPEG hybrid method may be useful for enhancing the synergistic interactions of these peptides.

Since peptides containing the RGD sequence were reported to be inhibitors of experimental metastasis,[12] RGD–peptides are a focus for development of anti-cancer drugs. The anti-metastatic effect of PHSRN–aaPEG–RGD on experimental metastasis was examined in mice. Prior to the metastasis assay, the viability of B16–BL6 incubated with synthetic hybrids was examined and the results are shown in Fig. 4. Previous studies indicated that aaPEG was not cytotoxic[8] and the data in Fig. 4 indicates that the synthetic hybrids are not cytotoxic.

The inhibitory effect of the hybrids on experimental metastasis in mice was examined and the results are shown in Fig. 5. 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 anti-metastatic effect of the sample, avoiding other anti-metastatic factors. The mice were sacrificed 14 d 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. 5, when compared to control values, hybrids appear to have a dose-dependent inhibitory effect. Although RGD, which is an inhibitor of experimental metastasis, did not show an inhibitory effect at a dose of 3 mg(5.33 μmol)/mouse, PHSRN–aaPEG–RGD and aaPEG–RGD did show a similar inhibitory effect at 3 mg/mouse. Since each 3 mg of PHSRN–aaPEG–RGD and aaPEG–RGD corresponds to 0.67 μmol and 0.78 μmol, the inhibitory effect of RGD is potentiated by the PEG-hybrid formation. These results are comparable to previous studies of PEG-hybrid formation with RGD.[5] Although significant difference in cell spreading activity was observed, a significant difference in the anti-metastatic effect between PHSRN–aaPEG–RGD and aaPEG–RGD was not observed. The relationship between the anti-metastatic effect and cell spreading activity of RGD is not explained by these results. The potentiation of anti-metastatic effect of RGD by PEG-hybrid formation may be in part due to a longer half-life in blood.[24] Stability of RGD to enzymatic degradation in blood may be increased by PEG-hybrid formation.

Based upon these studies, aaPEG will be a useful material not only to prepare a bivalent PEG-hybrid, but also to prepare a multivalent PEG-hybrid and a multifunctional PEG-hybrid of biological peptides.

Experimental

Acid hydrolyses 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 reversed phase (RP)-HPLC was performed using a Waters 600 with a DAISOPAK column and gradient systems of CH_3CN/water containing 0.05% TFA. TOF-mass spectra were obtained from a SHIMADZU/KRATOS KOMPACT MALDI IV spectrometer. ^{1}H-NMR spectra were obtained on a Brucker DPX-400 (400 MHz) spectrometer. Rink amide resin, protected amino acids and coupling reagents were purchased from Watanabe Chemical Industries, Ltd. TentaGel–NH_2 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–NH_2 is approximately 3000.

General Procedure for Peptide Synthesis by the Manual Solid Phase Method Peptides and hybrids were synthesized on Rink amide or TentaGel–NH_2 resin by a manual method according to the procedure shown below. Coupling reactions were checked by the Kaiser test (ninhydrin test).[21]

Step	Reagents	Reaction time
1	Fmoc–amino acid (3 eq) in DMF	1 h
	1 M DIC/DMF (3 eq) in DMF	
	1 M HOBt (3 eq) in DMF	
2	DMF	2 min×7
3	20% piperidine/DMF	3 min×3
		20 min×1
4	DMF	2 min×7

H–aaPEG–Arg–Gly–Asp–NH_2 Fmoc–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin was prepared from 330 mg of Rink amide resin (amino content 0.11 mmol) following the above procedure (manual method). Fmoc–aaPEG–OH was preincubated with DIC and HOBt at 0 °C for 1 h and allowed to be used in a coupling reaction. Since reaction of Fmoc–aaPEG–OH (3 eq) was slow, it was reacted with H–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin for 10 h and the reaction was repeated once. Then the Fmoc group was removed with 20% piperidine/DMF and the resulting resin (580 mg) was treated with a mixture of TFA/thioanisole/ethanedithiol (94/3/3) at room temperature for 2 h. The resin was removed by filtration and the TFA was removed by evaporation. The residue was washed with ether (3 times) and ethyl acetate (3 times), and dried. The mate-

rial was purified by HPLC on a DAISOPAK SP-120-ODS column. Yield 123 mg (33%, calculated from NH_2 content of the used resin). Amino acid ratios in an acid hydrolysate: Arg 0.99, Gly 1.00, Asp 1.06 (average recovery 98%). TOF-MS *m/z* 3355(average).

H–Pro–His–Ser–Arg–Asn–PEG Peptide was constructed on TentaGel–NH_2 resin (500 mg, amino content 0.11 mmol) according to the above procedure (manual method). The synthetic Fmoc–Pro–His(Trt)–Ser(Bu^t)–Arg(Pmc)–Asn(Trt)–TentaGel–NH_2 resin (610 mg) was treated with 20% piperidine/DMF, followed by treatment with a mixture of TFA/thioanisole/ethanedithiol (94/3/3). The resulting crude H–Pro–His–Ser–Arg–Asn–PEG was purified by HPLC on a DAISOPAK SP-120-ODS column. Yield 220 mg (45% , calculated from NH_2 content of the used resin). Amino acid ratios in an acid hydrolysate: Pro 1.00, His 1.01, Ser 0.87, Arg 0.93, Asp 1.00 (average recovery 96%). TOF-MS *m/z* 4127 (average).

H–Pro–His–Ser–Arg–Asn–aaPEG–Arg–Gly–Asp–NH_2 After synthesis of Fmoc–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin [220 mg, prepared from 125 mg (amino content 0.078 mmol) of Rink amide resin] by the manual method, the desired material [H–Pro–His(Trt)–Ser(Bu^t)–Arg(Pms)–Asn(Trt)–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin] was prepared with an ABI 433A automatic peptide synthesizer which facilitates monitoring the deprotection reaction of the Fmoc group. Synthetic H–Pro–His(Trt)–Ser(Bu^t)–Arg(Pmc)–Asn(Trt)–aaPEG–Arg(Pmc)–Gly–Asp(OBu^t)–Rink amide resin (240 mg) was treated with TFA/thioanisole/ethanedithiol (94/3/3, 20 ml) for 2 h at room temperature. The TFA was removed by evaporation and the residue was dissolved in water. The solution was washed with ether and lyophilized. The resulting crude H–Pro–His–Ser–Arg–Asn–aaPEG–Arg–Gly–Asp–NH_2 (83 mg) was purified by HPLC using a DAISOPAK SP-120-ODS column (20×250 mm. Eluent; gradient of CH_3CN/water containing 0.05% TFA, 10/90→50/50). The material (36 mg) was purified again by HPLC using an ASAHIPAK GS-320P column (21.5×500 mm. Eluent; gradient of CH_3CN/water containing 0.05% TFA, 10/90→50/50). Yield 21 mg (6%, calculated from NH_2 content of the used resin). Amino acid ratios in an acid hydrolysate: Pro 0.92, His 0.98, Ser 0.83, Arg 2.03, Asp 2.00, Gly 1.00 (average recovery 92%). TOF-MS *m/z* 4007 (average).

The Cell Spreading Activity The activity was examined using Baby Hamster Kidney (BHK) cells as described previously.[13,14] Ninety-six well plastic tissue culture plates were coated with various amounts of PEG-peptides or fibronectin, and dried overnight. The plates were subsequently blocked by 3% bovine serum albumin (BSA, Sigma) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) for 1 h at room temperature. BHK cells were detached with trypsin/EDTA and recovered in the presence of 10% fetal bovine serum (Life Technologies, Inc.) for 20 min at 37 °C. After washing twice with 0.1% BSA in DMEM, cells were placed in the coated wells at 5×10^3 cells/well. After a 45 min incubation at 37 °C, the cells were fixed with 3% formaldehyde and the percentage of spreading BHK cells was counted under a phase contrast microscopy.

Viability of B16-BL6 Melanoma Admixed with PEG Hybrids Cells in MEM(−) containing 0.1% BSA (2×10^4/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 (1×10^5/0.1 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.

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