Amino Acids and Peptides. XXX.[1] Preparation of Arg–Gly–Asp (RGD) Hybrids with Poly(Ethylene Glycol) Analogs and Their Antimetastatic Effect

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Received May 16, 1997; accepted July 11, 1997

Hybrids of a fibronectin-related peptide[Arg–Gly–Asp (RGD)] with poly(ethylene glycol) (PEG) analogs were prepared by a simple and easy procedure. Two amino-PEG analogs were used as carriers for hybrid formation of the RGD. One was poly(oxyethylene)dipropylamine and the other was Jeffamine ED type, which has branched chains. RGD peptides were formed stepwise on PEG analogs by the diisopropylcarbodiimide method. The synthetic intermediates were easily purified by molecular-sieve gel chromatography and the final products were purified by molecular-sieve gel chromatography, followed by HPLC. This simple and easy preparation procedure using molecular-sieve gel chromatography for purification of synthetic intermediates is advantageous for the preparation of peptide-polymer hybrids. We found that PEG is stable to HF treatment at 0 °C for 1 h. The inhibitory effect of the RGD hybrids on experimental metastasis of B16–BL6 was examined in mice. The Jeffamine type hybrid showed no inhibitory effect at the dose of 1 mg/mouse, but poly(oxyethylene)dipropylamine type hybrid was inhibitory at the same dose. The effect of the latter hybrid was about the same as that of 1 mg of RGD. One mg of the hybrid contains 0.18 μmol of RGD and 1 mg of RGD is 2.38 μmol. Thus it can be said that the inhibitory effect of RGD was potentiated by hybrid formation with poly(oxyethylene)diisopropylamine.

Key words fibronectin; poly(ethylene glycol); poly(ethylene glycol) hybrid; metastasis; metastasis inhibitor; Arg–Gly–Asp; RGD

Since poly(ethylene glycol) (PEG) has low toxicity, low immunogenicity, and good solubility in both aqueous and organic solvents, it seems to be a promising candidate as a drug-carrier. Many reports of studies on protein-PEG hybrids (such as PEG-insulin,[2] PEG-asparginase,[3] and PEG-urokinase[4] hybrids) have appeared, and the term "PEGylation" has been introduced. Hybrid formation of proteins with PEG has been reported to be effective in enhancing and prolonging the activities of the parent proteins. However, "over-PEGylation" of proteins results in loss of the activity.[5] Few PEG hybrids of oligopeptides have been reported, because modification of a small bioactive peptide with such a large molecule as PEG was considered likely to result in loss of activity of the parent peptide. However, we speculated that the hybrid formation of an oligopeptide with PEG may potentiate and prolong the activity of the parent oligopeptide since the hybrid may have a prolonged plasma half-life, similar to a PEG-protein hybrid. We also speculated that the conformation of PEG is sufficiently flexible that hybrid formation would not prevent binding of the oligopeptide portion of a PEG-peptide hybrid to its receptor. We found that hybrid formation of Tyr–Ile–Gly–Ser–Arg (YIGSR, a partial sequence of laminin-related peptide) with PEG was effective in potentiating the anti-metastatic effect of the parent peptide.[6]

Arg–Gly–Asp (RGD) is a sequence which is found in cell adhesive proteins, such as fibronectin, vitronectin, fibrinogen, laminin, and von Willebrand factor. Since peptides containing the RGD sequence were reported to be inhibitors of experimental metastasis[7] and platelet aggregation,[8] RGD analogs are of interest. We have already reported that hybrid formation of RGD and RGDS with amino-PEG (aPEG) was effective in potentiating the antimetastatic effect of the parent peptides.[9] Following our report, hybrid formation of Arg–Gly–Asp–Thr (RGDT)[10] with PEG was also reported to be effective to enhance the biological effect of the peptide. Lu and Felix reported preparation of PEG hybrids (PEGylated peptides) by the solid-phase method.[11] In our preceding study,[9] we converted PEG to aPEG according to the method reported by Pillai and Mutter,[12] and coupled it with RGD (or RGDS) prepared by the solution method. Since the preparations of aPEG and RGD (or RGDS) were not easy, a simple and easier procedure to prepare PEG hybrid analogs was needed. Since PEGs are large molecules, we considered the use of molecular-sieve gel filtration for purification. Two kinds of PEG analogs are commercially available. One is the Jeffamine polyoxyalkyleneamines,[13] which have methyl side chains, and the other is poly(oxyethylene)dipropylamine,[14] which has no side-chain. We were interested in Jeffamine polyoxyalkyleneamines because they are more hydrophobic and have a bulkier chain than PEGs (Fig. 1). These features may confer a different character (*e.g.* hydrophobic affinity to various tissues, steric effect) when these molecules are used as drug-carriers.

Among the Jeffamine polyalkyleneamines, Jeffamine ED-6000 (Jfa, approximate molecular weight 6000) was used because PEG 6000 was more effective than PEG 4000 when they were used as carriers for RGD hybrid.[9] The benzyloxycarbonyl (Z) group was used as an α-amino-

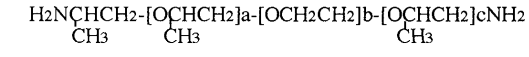

Fig. 1. Jeffamine ED Type General Structure

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protecting group of Asp and Gly. The guanidino group of Arg was protected with a 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group[15] and the β-carboxyl group of Asp was protected with a *tert*-butyl (Bu^t) group. The fluorenylmethyloxycarbonyl (Fmoc) group was used as an α-amino-protecting group of Arg. Arg–Gly–Asp–Jfa was prepared as shown in Fig. 2.

Z–Asp(OBu^t)–OH and Jfa were coupled by the diisopropylcarbodiimide (DIC) method in dichloromethane (DCM) and the product was purified by Sephadex LH-20 column chromatography using a mixture of MeOH/DCM (1/1) as an eluent. The Z group of the product [Z–Asp(OBu^t)–Jfa] was removed by catalytic hydrogenation. Z–Gly–OH and Fmoc–Arg(Pmc)–OH were introduced by the stepwise method on H–Asp(OcHx)–Jfa in the same way as described above. Thus synthesized Fmoc–Arg(Pmc)–Gly–Asp(OBu^t)–Jfa was treated with trifluoroacetic acid (TFA) to remove Bu^t and Pmc groups and then treated with 20% piperidine/dimethylformamide (DMF) to give H–Arg–Gly–Asp–Jfa(RGD–Jfa). The final product was purified by Sephadex LH-20 column chromatography and HPLC. The preparative HPLC profile of the product is shown in Fig. 3. The synthetic hybrid showed a broad peak on HPLC, probably reflecting heterogeneous molecular weight of Jfa.

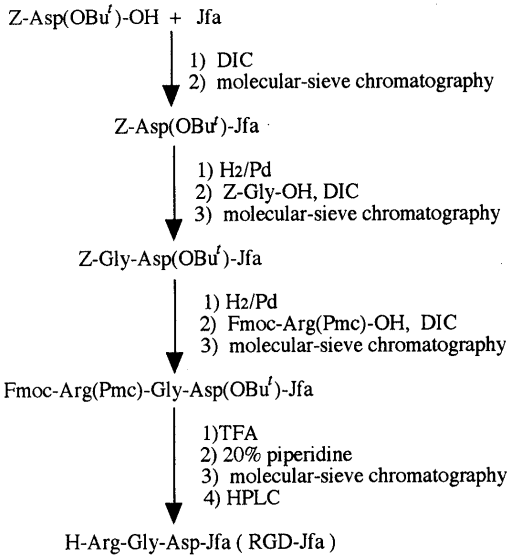

Fig. 2. Preparation of RGD–Jfa

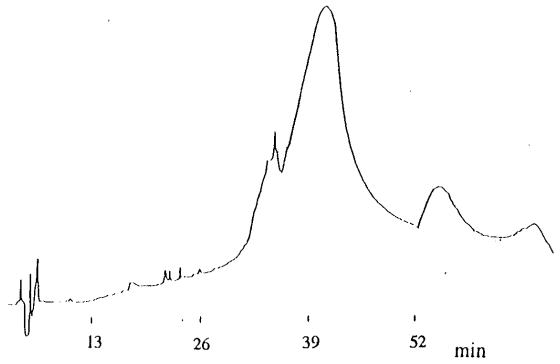

Fig. 3. Preparative HPLC Profile of RGD–Jfa

Column: Cosmosil 5C 18R (20 × 250 mm). Flow rate, 10 ml/min. Eluent, A) 0.1% TFA/water; B) 0.1% TFA/CH_3CN, Gradient:

	0	5 min	35 min	45 min	60 min	75 min
A	80	80	50	50	20	50
B	20	20	50	50	80	50

The peptide content of the RGD–Jfa was 256 μmol/g. Inhibitory effect of the RGD–Jfa on experimental metastasis was examined in mice. B16–BL6 cells and the hybrid were intravenously injected separately into mice. The mice were sacrificed 14 d after tumor inoculation, and the lungs were taken out. The number of surface melanoma colonies on the lungs was counted under a stereoscopic microscope. Prior to the assay, viabilities of B16–BL6 admixed with RGD, Jfa and RGD–Jfa were examined and the results are shown in Fig. 4.

The results indicated that Jfa and its hybrid were not cytotoxic. The results of metastasis assay are shown in Fig. 5.

RGD–aPEG6000 was prepared by the solution method as reported[9a] and its antimetastatic effect was also

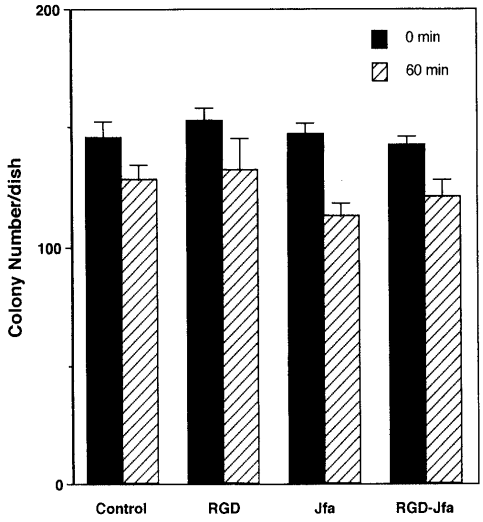

Fig. 4. Viability of B16–BL6 Melanoma Admixed with Jfa and RGD–Jfa

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 0 or 60 min, the cells were seeded onto culture dishes, and colonies were counted after 1 week. Each value represents the mean ± S.E.

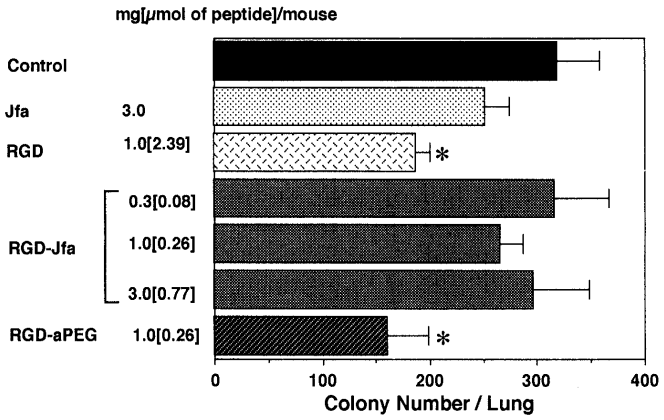

Fig. 5. Inhibitory Effect of RGD–Jfa on Experimental Lung Metastasis of B16–BL6 Melanoma

B16–BL6 cells (1×10^5) were injected into five mice per group and then various concentrations of peptides were injected. Lung tumor colonies were examined 2 weeks later. Each value represents the mean ± S.E. $*p < 0.05$ significantly different from control.

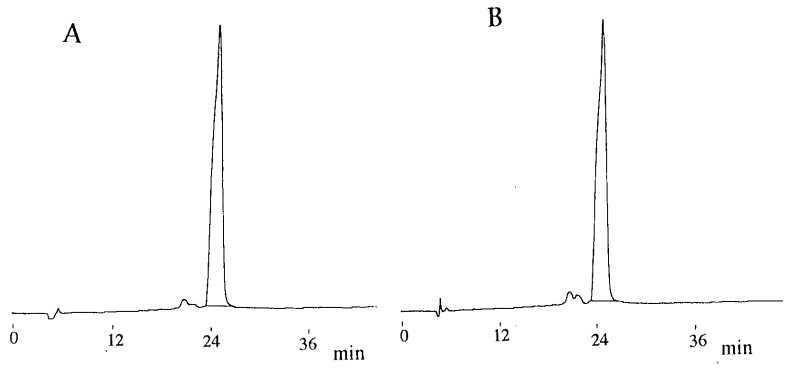

Fig. 6. HPLC Profiles of *p*NB–apPEG

*p*NB–apPEG was treated with HF at 0 °C for 1 h. A: before HF treatment. B: after HF treatment. Optical density at 220 nm.

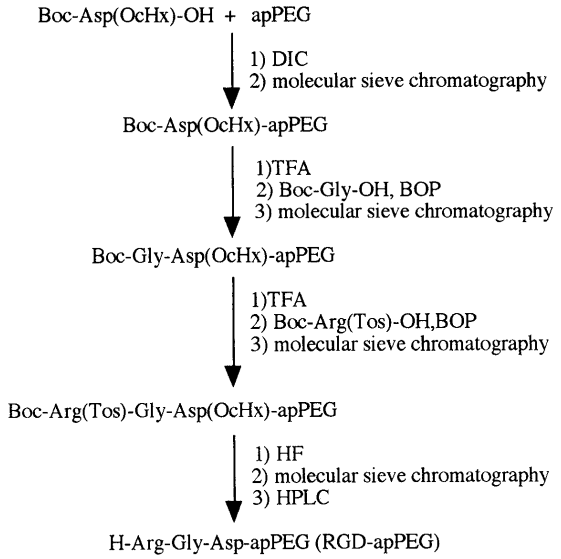

Fig. 7. Preparation of RGD–apPEG

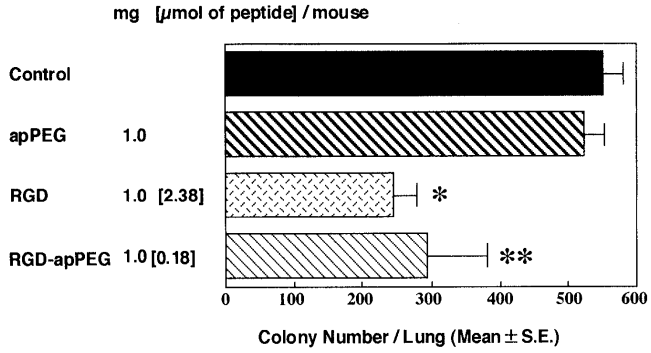

Fig. 8. Antimetastatic Effect of RGD–apPEG

The tumor colonization assay was carried out as described in the legend to Fig. 5.

examined for comparison, because the molecular weight of Jfa (average molecular weight, 6000) is similar to that of aPEG6000. Jfa did not show an appreciable inhibitory effect at a dose of 3 mg (0.5 μmol)/mouse. No appreciable inhibitory effect of RGD–Jfa was found up to a dose of 3 mg (RGD content: 0.77 μmol)/mouse, although RGD and RGD–aPEG6000 were inhibitory at a dose of 1 mg/mouse. One mg/mouse of RGD moderately suppressed metastasis.[9] One mg of RGD is 2.39 μmol and 1 mg of RGD–Jfa contains 0.26 μmol of RGD. One mg of RGD–Jfa and 1 mg of RGD–aPEG6000 contain equal amount of RGD, but the Jfa hybrid did not show an inhibitory effect. The structural difference between Jfa and aPEG is that Jfa has many methyl side chains, whereas aPEG has none. Jfa is more hydrophobic and bulkier than aPEG and these properties may be responsible for the lack of inhibitory effect of the Jfa hybrid.

Next, the RGD hybrid of poly(oxyethylene)dipropylamine (apPEG) was prepared. apPEG was not cytotoxic, like Jfa. The *tert*-butyloxycarbonyl (Boc) group was used as an α-amino-protecting groups for Asp, Gly and Arg. The guanidino group of Arg was protected with a tosyl (Tos) group and the β-carboxyl group of Asp was protected with cyclohexyl (cHx) group. Tos and cHx groups are removable by HF treatment, but Lu and Felix[11a] suggested that this might cause partial degradation of PEG. We therefore examined the stability of apPEG to HF treatment. Since PEGs do not show UV absorption, *p*-nitrobenzoyl (*p*NB)–apPEG was prepared and its stability to HF treatment was examined. *p*NBchloride was reacted with apPEG to give *p*NB–apPEG. The product was purified by HPLC and then treated with HF at 0 °C for 1 h. The resulting material was examined by HPLC and no change was observed (Fig. 6).

This result indicates that PEG is stable to HF treatment at 0 °C for 1 h. The synthetic scheme for RGD–apPEG is shown in Fig. 7. Boc–Asp(OcHx)–OH and apPEG were coupled by the DIC method in DCM and the product (Boc–Asp(OcHx)–apPEG) was purified by Sephadex LH-20 column chromatography. The Boc group was removed by TFA treatment and Boc–Gly–OH and Boc–Arg(Tos)–OH were introduced stepwise method manner by the benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) method.[16] The synthesized Boc–Arg(Tos)–Gly–Asp(OcHx)–apPEG was treated with HF and the product (RGD–apPEG) was purified by Sephadex LH-20 column chromatography and HPLC successively. Prior to metastasis assay, the viability of B16–BL6 admixed with RGD, apPEG and RGD–apPEG was examined. These products were not cytotoxic. The inhibitory effect of RGD–apPEG on experimental metastasis of B16–BL6 was examined and the results are shown in Fig. 8.

RGD–apPEG was inhibitory at a dose of 1 mg. RGD was also inhibitory at the same dose. One mg of RGD is 2.39 μmol and 1 mg of the hybrid contains 0.18 μmol of RGD. Thus, it can be said that the inhibitory effect of

RGD was potentiated more than 10 times by the hybrid formation. We reported that potentiation of the effect of a parent peptide by hybrid formation with PEG was due to prolongation of the plasma half-life of the hybrid.[17] It may be necessary to use PEG molecule that are sufficiently large to inhibit enzymatic hydrolysis of the peptide portion without disturbing the binding of the peptide portion to its receptor, which would result in loss of the activity. Here we proved that linear PEGs (without a side chain) with a molecular weight up to 10000 did not inactivate RGD's antimetastatic effect.

In the preceding study,[9a] we constructed a protected peptide by the solution method and then coupled it with aPEG. The present simple procedure for preparation of PEG hybrids using molecular-sieve gel chromatography for purification of synthetic intermediates is easier and faster than the procedure reported in the preceding paper. PEG hybrids are of interest as drug-delivery systems for peptides, and a simple procedure for preparation of PEG hybrids will be useful.

Experimental

Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, Merck) are indicated as follows: Rf^3 = $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase), Rf^5 = $CHCl_3$–MeOH–AcOH (90 : 8 : 2). Synthetic hybrids were hydrolyzed in 6 N HCl at 110 °C for 48 h. Amino acid compositions of acid hydrolysates were determined with a Kyowa K-202SN amino acid analyzer. HPLC was conducted with a Waters 600 on a YMC Pack AQ-ODS-5 column using gradient systems of CH_3CN/H_2O containing 0.1% TFA. FAB-MS were measured on a VG Analytical ZAV-SE spectrometer. Platinum chloride test of eluates of LH-20 column chromatography was performed as follows: an aliquot of each fraction was spotted on TLC and a mixture of 4% $H_2PtCl_6 \cdot 6H_2O/H_2O$ and 6% KI/H_2O (1/1) was sprayed on it. apPEG was purchased from Wako Pure Chemical Industries, Ltd. Jfa was kindly supplied by Texaco Chemical Company.

RGD–Jfa Jfa (300 mg, amino group content 0.1 meq/g) and Z–Asp(OBu^t)–OH (323 mg, 1 mmol) were dissolved in DCM (5 ml) and 1 M DIC/DCM (1 ml) was added to the solution. The mixture was stirred overnight and became ninhydrin test negative. It was washed with water, 5% Na_2CO_3, and water successively and the solvent was removed *in vacuo*. The residue was purified by Sephadex LH-20 column (3 × 150 cm) chromatography using 50% MeOH/DCM as an eluent. Each fraction (17 g) was checked by the ninhydrin test and platinum chloride test. Fractions 19—24, which were ninhydrin test negative and platinum chloride test positive, were pooled and the solvent was evaporated off. Yield 305 mg, wax-like material, Rf^3 0.67. The material was dissolved in MeOH (10 ml) and hydrogenated over a Pd catalyst for 5 h. The solvent was removed *in vacuo* and the residue was lyophilized. 280 mg, wax-like material, Rf^3 0.56. Z–Gly–OH (209 mg, 1 mmol) was reacted with the H–Asp(OBu^t)–Jfa and the product was purified by Sephadex LH-20 column chromatography followed by hydrogenation according to the procedure described above. 255 mg, Rf^3 0.56. Next, Fmoc–Arg(Pmc)–OH (736 mg, 1 mmol) was reacted with the H–Gly–Asp(OBu^t)–Jfa in the same manner as described above and the product was purified in the same manner: 238 mg. Rf^3 0.56.

The material (Fmoc–Arg(Pmc)–Gly–Asp(OBu^t)–Jfa) was treated with TFA (5 ml) containing 5% anisole for 1 h and the TFA was removed *in vacuo*. The residue was purified by LH-20 column chromatography in the same way. 220 mg, Rf^3 0.50. This material was dissolved in 20% piperidine/MeOH (4 ml) and the solution was stirred for 20 min. The product was purified by Sephadex LH-20 column chromatography in the same manner as described above and purified again by HPLC as shown in Fig. 3. Yield 87 mg, Amino acid ratios in an acid hydrolysate: Arg 0.96, Gly 1.00, Asp 1.05. Peptide content calculated from average recovery of each amino acid: 256 μmol/g.

***p*NB–apPEG** apPEG (1 g, 0.1 mmol), *p*NB–Cl (186 mg, 1 mmol) and triethylamine (0.5 ml, 34.6 mmol) were dissolved in DCM (5 ml) and the reaction mixture was stirred at 40 °C for 2 h. The mixture became the ninhydrin test negative. The solvent was removed and the residue was dissolved in water (20 ml). The solution was washed with ether 4 times and lyophilized. The residue was purified by Sephadex G-25 column (3 × 150) chromatography using water as an eluent. 995 mg. The product was purified again by HPLC on a Cosmosil 5C18-AR column (20 × 250 mm) using 0.1% TFA CH_3CN/H_2O and lyophilized. Pale yellow fluffy powder, 714 mg, Rf^3 0.65.

HF Treatment of *p*NB–apPEG *p*NB–apPEG (100 mg) was treated with HF at 0 °C for 1 h and the HF was removed *in vacuo*. The residue was dissolved in water and lyophilized. The material was compared with original *p*NB–apPEG by HPLC as shown in Fig. 6.

RGD–apPEG Boc–Asp(OcHx)–OH (757 mg, 2.4 mmol) was dissolved in DMF (8 ml) and apPEG (2 g, 0.2 mmol) was dissolved in DCM (10 ml). The solutions were combined and cooled to −10 °C. One M DIC/DMF (2.4 ml) was added and the reaction mixture was stirred for 4 h. The solvent was removed *in vacuo* and the product was purified on a Sephadex LH-20 column (3 × 150 cm) chromatography with MeOH as an eluent. Fractions (15 g each) were checked by mean of the platinum chloride test on TLC. Fractions 18—26 which showed Rf^5 0.31 were pooled and the solvent was evaporated off. Boc–Asp(OcHx)–apPEG (1.50 g) thus obtained was treated with 50% TFA/DCM (15 ml) for 40 min and the solvent was removed *in vacuo* at room temperature. The residue was dried *in vacuo*, followed by lyophilization. The material was dissolved in DMF/DCM (1/1, 16 ml) and the solution was neutralized with diisopropylethylamine (DIEA). Boc–Gly–OH (299 mg, 1.7 mmol) and DIEA (0.6 ml, 3.4 mmol) were dissolved in the solution and BOP (752 mg, 1.7 mmol) was added. The reaction mixture was stirred overnight and the solvent was removed *in vacuo*. The residue was purified by Sephadex LH-20 column chromatography with MeOH as described above. Yield 1.18 g. Rf^3 0.45. The product [Boc–Gly–Asp(OcHx)–apPEG] was treated with TFA and coupled with Boc–Arg(Tos)–OH (566 mg, 1.3 mmol) by the BOP method as described above. The product was purified by Sephadex LH-20 column chromatography. Yield 787 mg. Rf^3 0.50. Amino acid ratios in an acid hydrolysate: Arg 0.90, Gly 1.00, Asp 0.93. The product [Boc–Arg(Tos)–Gly–Asp(OcHx)–apPEG, 278 mg] was treated with 5% anisole/HF (20 ml) at 0 °C for 1 h and the HF was removed *in vacuo*. The residue was washed with ether, extracted with water and lyophilized. The product was purified by Sephadex LH-20 column chromatography and HPLC in the same manner as described for RGD–Jfa. After HPLC purification, 120 mg of fluffy powder was obtained, but its HPLC profile had a small shoulder. Therefore the product was purified again by HPLC (Asahipack ODP-90 column, 30 × 300 mm). Yield 50 mg from fraction 1, 25 mg from fraction 2 and 14 mg from fraction 3 (fluffy powder in each case). Amino acid ratios in acid hydrolysates are shown below.

	Arg	Gly	Asp	Peptide content
Fraction 1	0.93	1.00	0.99	185 μmol/g
Fraction 2	0.87	1.00	1.01	128 μmol/g
Fraction 3	0.83	1.00	1.07	92 μmol/g

Judging from the amino acid recoveries of these samples, fraction 1 is the desired material, which contains 2 mol peptide/1 mol apPEG, and fraction 3 is the hybrid which contains 1 mol peptide/1 mol apPEG. The *Rf* values of these fractions were almost the same; Rf^3 0.48.

Metastasis Assay Inhibitory effects of the synthetic hybrids on experimental metastasis were examined in mice as reported.[8] Briefly, B16–BL6 melanoma cells (1 × 10^5/0.2 ml) were intravenously injected into C57BL/6 mice and a hybrid was injected into the mice in the same way. The mice were killed 14 d after tumor inoculation, and the lungs were excised. The number of surface melanoma colonies on the lungs was counted under a stereoscopic microscope.

Colony Formation Assay The assay was performed as described in the legend to Fig. 4. Details will be published elsewhere.

Acknowledgements This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Science and Culture and by a grant from The Health Science Fund of Kobe Gakuin University. The authors thank Texaco Chemical Company for providing Jfa.

References and Notes

1) *a*) IUPAC standard abbreviations for amino acids, protecting groups, and peptides are used; *b*) A part of this paper was reported in the preceding communication [Yamamoto S., Kaneda Y., Okada N., Nakagawa S., Kubo K., Inoue S., Maeda M., Yamashiro Y., Kawasaki K., Mayumi T., *Anti-Cancer Drugs*, **5**, 424—428 (1994)];

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