# Amino Acids and Peptides. XXIX.[1] Synthesis and Antimetastatic Effects of Peptides and Peptide–Poly(Ethylene Glycol) Hybrids Related to the Core Sequence of the Type III Connecting Segment Domain of Fibronectin

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**Peptides (H-Glu-Ile-Leu-Asp-Val-$NH_2$, H-Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr-$NH_2$, H-Arg-Glu-Asp-Val-$NH_2$) and their poly(ethylene glycol) (PEG) hybrids related to the core sequence of the type III connecting segment domain of fibronectin A chain were prepared by the solution method or the solid phase method. Their inhibitory effects on the adhesion and migration of B16-BL6 melanoma cells to fibronectin were assessed *in vitro*, and their therapeutic potency against tumor metastasis were also examined. Anti-adhesive and anti-migrative effects of the synthetic fibronectin-related peptids were superior to those of their PEG hybrids, so we found that the *in vitro* bioactivity of peptides decreased by PEGylation. In the *in vivo* assay, we found that the synthetic peptides containing Glu-Ile-Leu-Asp-Val and Arg-Glu-Asp-Val sequences exhibited an inhibitory effect on the experimental metastasis of B16-BL6 melanoma. Of the synthetic peptides, H-Glu-Ile-Leu-Asp-Val-$NH_2$ exhibited the most potent inhibitory effect. Hybrid formation of Arg-Glu-Asp-Val with poly(ethylene glycol) resulted in potentiation of the inhibitory effect of the parent peptides. A mixture composed of PEG hybrids of Glu-Ile-Leu-Asp-Val, Arg-Glu-Asp-Val and Tyr-Ile-Gly-Ser-Arg dramatically inhibited tumor metastasis.**

**Key words** fibronectin; metastasis inhibitor; poly(ethylene glycol); peptide hybrid; poly(ethylene glycol) hybrid

Modification of proteins and oligopeptides with poly(ethylene glycol) (PEG) is a focus of study for drug-delivery systems. Because PEG is low toxic, low immunogenic and has good solubility in both aqueous and organic solvents, it seems to be a promising canditate 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 been published, and the hybrid formations have been reported to be effective in enhancing and prolonging activities of the parent proteins. Furthermore, some enzyme–PEG hybrids have been reported to be soluble and active in organic solvents. But few PEG hybrids of oligopeptides have been reported because modification of a small bioactive peptide with such a big molecule like PEG was suspected to result in a loss of activity of the parent peptide. However, we speculated that the hybrid formation of an oligopeptide with a PEG may potentiate and prolong the activity of the parent peptide since the hybrid may have a prolonged plasma half-life similar to the PEG–protein hybrid. We also speculated that a flexible conformation of PEG may not prevent the binding of an oligopeptide portion of a PEG–peptide hybrid to its receptor.

Fibronectin[5] is a glycoprotein which is classified as a cell adhesion protein consisting of 2 (A and B) peptide chains. Each chain contains an Arg–Gly–Asp (RGD) sequence and the RGD sequence was found to be minimum sequence for cell attachment activity.[6] Furthermore, peptides containing a RGD sequence were reported to be inhibitors of experimental metastasis in mice.[7] In addition to RGD, Glu–Ile–Leu–Asp–Val (EILDV) and Arg–Glu–Asp–Val (REDV) sequences were found in the IIICS domain of the A chain as important sequences for cell attachment.[8] Synthetic REDV was reported to be inhibitory *in vitro* for melanoma cell adhesion to fibronectin, but was inactive for fibroblastic cell adhesion.[8b]

We expected that these peptides might also have an inhibitory effect on experimental metastasis *in vivo*, as RGD peptides did. We already reported that hybrid formations of RGD[9] and Tyr–Ile–Gly–Ser–Arg (YIGSR, laminin related peptide)[10] with PEG were effective in potentiating the anti-metastatic effects of the parent peptides. Following our report, hybrid formation of Arg–Gly–Asp–Thr (RGDT)[11] with PEG was also reported to be effective. Here, we describe in detail the preparation and activities [*in vivo* activity (anti-metastatic effect) and *in vitro* activity (effect on cell adhesion and migration)] of EILDV, REDV and their PEG hybrids.

## MATERIALS AND METHODS

Melting points of synthetic materials are uncorrected. Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, E. Merck) are indicated as follows: $Rf^1$ = BuOH–AcOH–$H_2O$ (4 : 1 : 5, upper phase), $Rf^2$ = BuOH–AcOH–pyridine–$H_2O$ (4 : 1 : 1 : 2), $Rf^3$ = $CHCl_3$–MeOH–$H_2O$ (8 : 3 : 1, lower phase), $Rf^5$ = $CHCl_3$–MeOH–

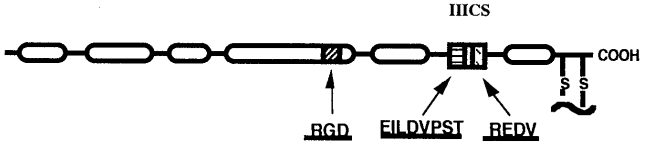


Fig. 1. Structure of Fibronectin A Chain

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AcOH (90:8:2). Synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 20 h and PEG–peptide hybrids were hydrolyzed for 48 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. The peptide content of PEG–peptide hybrid was calculated from the recovery of amino acids in an acid hydrolysate. RP-HPLC was conducted with a Waters 600 on a YMC Pack AQ-ODS-5 column using a mixture of 0.1% trifluoroacetic acid (TFA)–containing $CH_3CN$/$H_2O$ as an eluent. *p*-Methylbenzhydrylamine resin (amino content 0.64 meq/g) was purchased from Peptide Institute Inc. FAB-MS were measured on a VG Analytical ZAV-SE spectrometer. PEGs were purchased from Nacalai Tesque Inc.

**Amino-Poly(Ethylene Glycol) ♯6000 (aPEG ♯6000)** PEG ♯6000 (M.W. 6700—9000) was converted to aPEG according to the procedure reported by Pillai and Mutter.[12] aPEG was purified by Dowex 50 ($H^+$) column chromatography using 1% ammonia as an eluent. The amino group content of PEG was determined by titration. aPEGs which have 0.21—0.27 meq/g of an amino group were obtained.

**Carboxyl-Poly(Ethylene Glycol) Methyl Ether (cPEG)** PEG monomethyl ether (average M.W. 5000) was oxidized according to the procedure reported by Ueyama *et al.*[13] cPEG was purified by Diaion WA21using 2% AcOH as an eluent. cPEGs which have 0.18—0.20 meq/g of a carboxyl group were obtained.

**Boc–Asp(OcHx)–Val–OBzl** To a solution of $N^{\alpha}$-*tert*-butoxycarbonyl-$\beta$-cyclohexyl aspartate [Boc–Asp(OcHx)–OH] (2 g, 6.34 mmol) and *N*-methylmorpholine (NMM) (0.70 ml, 6.34 mmol) in dimethylformamide (DMF) (15 ml), isobutyl chloroformate (0.82 ml, 6.34 mmol) was added at −10 °C, and the mixture was stirred for 10 min. A DMF (20 ml) solution of H–Val–OBzl [prepared from its tosylate (2.41 g, 6.34 mmol) and triethylamine (0.88 ml, 6.34 mmol)] was combined with the mixture, and the whole was stirred for 4 h. The solvent was removed *in vacuo* and the residue was extracted with ethyl acetate (AcOEt), followed by washing with 5% citric acid, 3% $Na_2CO_3$ and water. After drying with $Na_2SO_4$, the solvent was removed *in vacuo* and the residue was purified by silica gel column chromatography. The desired material was obtained in 3% MeOH/$CHCl_3$ eluate. Yield 2.14 g (67%), oily material, $Rf^3$ 0.89, $[\alpha]_D^{22}$ −31.2° ($c$=1.0, MeOH), MS $m/z$ 505 $(M+1)^+$.

**Boc–Glu(OcHx)–Asp(OcHx)–Val–OBzl** Boc–Asp(OcHx)–Val–OBzl (2 g, 3.96 mmol) was treated with 5% anisole/TFA at 0 °C for 1 h, followed by the addition of petroleum ether. The resulting precipitate was washed with petroleum ether and dried. The material was dissolved in DMF (15 ml) and neutralized with NMM. A mixed anhydride solution [prepared from Boc–Glu(OcHx)–OH (1.30 g, 3.96 mmol), NMM (0.44 ml, 3.96 mmol) and isobutyl chloroformate (0.51 ml, 3.96 mmol) in DMF (12 ml)] was added to the solution at 0 °C, and the whole was stirred for 5 h. The solvent was removed *in vacuo* and the residue was extracted with AcOEt, followed by washing with 5% citric acid, 3% $Na_2CO_3$ and water. After being dried with $Na_2SO_4$, the solvent was removed *in vacuo* and the residue was precipitated from AcOEt/petroleum ether. Yield 1.87 g (66%), mp 75—77 °C, $Rf^3$ 0.88, $Rf^5$ 0.81, $[\alpha]_D^{25}$ −35.3° ($c$=1.0, MeOH). *Anal.* Calcd for $C_{38}H_{57}N_3O_{10}$: C, 63.75; H, 8.02; N, 5.86. Found: C, 63.55; H, 8.19; N, 5.60. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 h): Glu 1.00, Asp 0.98, Val 0.94 (average recovery 88%).

**Boc–Arg(Tos)–Glu(OcHx)–Asp(OcHx)–Val–OBzl** Boc–Glu(OcHx)–Asp(OcHx)–Val–OBzl (1 g, 1.4 mmol) was treated with 5% anisole/TFA to remove the Boc group and coupled with $N^{\alpha}$-Boc-$N^{G}$-tosyl-arginine [Boc–Arg(Tos)–OH (857 mg, 2 mmol)] by the mixed anhydride method in the same manner described above. Yield 858 mg (59%), amorphous powder, $Rf^3$ 0.90, $[\alpha]_D^{22}$ −26.9° ($c$=1.0, MeOH). *Anal.* Calcd for $C_{52}H_{75}N_7O_{13}S$: C, 60.15; H, 7.28; N, 9.44. Found: C, 60.01; H, 7.49; N, 9.08. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 h): Arg 0.93, Glu 1.00, Asp 0.96, Val 1.02 (average recovery 91%).

**Boc–Arg(Tos)–Glu(OcHx)–Asp(OcHx)–Val–OH** Boc–Arg(Tos)–Glu(OcHx)–Asp(OcHx)–Val–OBzl (500 mg, 0.48 mmol) was hydrogenated with Pd catalyst in MeOH (10 ml). After removal of the solvent, the product was precipitated from MeOH/petroleum ether. Yield 321 mg (71%), amorphous powder, $Rf^3$ 0.84, $[\alpha]_D^{24}$ −17.4° ($c$=1.0, MeOH), *Anal.* Calcd for $C_{44}H_{69}N_7O_{13}S$: C, 56.45; H, 7.43; N, 10.47. Found: C, 56.42; H, 7.66; N, 10.19. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 h): Arg 0.92, Glu 1.00, Asp 0.99, Val 0.98 (average recovery 89%).

**H–Arg–Glu–Asp–Val–aPEG** To a solution of Boc–Arg(Tos)–Glu(OcHx)–Asp(OcHx)–Val–OH (474 mg, 0.5 mmol) and aPEG ♯6000 ($NH_2$-content: 0.27 meq/g) (555 mg, 0.15 meq) in DMF, DCC (124 mg, 0.6 mmol) and HOBt·$H_2O$ (81 mg, 0.6 mmol) were added at −10 °C and the mixture was stirred for 20 h. The solvent was removed *in vacuo* and the residue was dissolved in a mixture of MeOH and dichloromethane (DCM) (1/1). The solution was applied on a Sephadex LH-20 column (3.1 × 135 cm) and 15 g fractions were collected. Fractions 28—32 were pooled and the solvent was evaporated. The residue (810 mg) was treated with 10% anisole/HF at 0 °C for 45 min. The HF was removed and the residue was washed with ether. The residue was extracted with water, lyophilized and purified by RP-HPLC. The purified material was converted to its hydrochloride by lyophilization from HCl-containing $H_2O$. Yield 188 mg, fluffy powder, $Rf^1$ 0.07, $Rf^2$ 0.57, $Rf^3$ 0.05. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 h): Arg 0.91, Glu 1.00, Asp 0.98, Val 0.96. The peptide-content of the hybrid: 0.25 mmol/g.

**General Procedure for the Solid Phase Peptide Synthesis** *p*-Methylbenzhydrylamine resin ($NH_2$-content: 0.64 meq/g) was used for the preparation of peptides. The following amino acid derivatives were used: Boc–Tyr(Bzl)–OH, Boc–Arg(Tos)–OH, Boc–Glu(OcHx)–OH, Boc–Asp(OcHx)–OH, Boc–Val–OH, Boc–Ile–OH, Boc–Gly–OH, Boc–Ser(Bzl)–OH. The synthetic protocol for the solid-phase peptide synthesis is shown below.

| step | reagent | reaction time (min) | times |
|---|---|---|---|
| 1 | NMM/DCM | 5 | ×2 |

| | | | |
|---|---|---|---|
| 2 | DCM | 3 | ×4 |
| 3 | Boc-amino acid or cPEG (2 eq) in DMF (or DCM) 1 M DCC/DCM (2 eq)[a] | 120 | |
| 4 | 50% MeOH/DCM | 5 | ×3 |
| 5 | DCM | 2 | ×2 |
| 6 | 50% TFA/DCM | 2 | ×1 |
| | | 45 | ×1 |
| 7 | DCM | 3 | ×5 |

*a*) 1 M HOBt/DMF (2 eq) was added when Boc–Arg(Tos)–OH was reacted.

**HF Treatment** The peptide–resins (or the hybrid resins) were treated with HF (20 ml/g of the peptide or the hybrid resins) containing 5% anisole for 45 min at 0 °C. The HF was removed *in vacuo* and the residue was washed with ether 5 times. The peptides (or the hybrids) were extracted with water and lyophilized. The materials were purified by RP-HPLC. The desired products were converted to their hydrochloride by lyophilization from HCl-containing water.

The following peptides were prepared by the solid phase method, followed by HF treatment and RP-HPLC.

**H–Arg–Glu–Asp–Val–$NH_2$·2HCl** Hygroscopic powder, $Rf^2$ 0.13, $[\alpha]_D^{25}$ −25.8° ($c$=1.0, $H_2O$), MS $m/z$ 517 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Arg 0.95, Glu 1.03, Asp 1.00, Val 1.06 (average recovery 86%).

**H–Glu–Ile–Leu–Asp–Val–$NH_2$·HCl** Hygroscopic powder, $Rf^2$ 0.26, $[\alpha]_D^{25}$ −160.2° ($c$=1.0, $H_2O$), MS $m/z$ 588 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Glu 1.06, Ile 0.99, Leu 1.00, Asp 1.09, Val 1.03 (average recovery 88%).

**cPEG–Glu–Ile–Leu–Asp–Val–$NH_2$** Fluffy powder, $Rf^3$ 0.38. Amino acid ratios in an acid hydrolysate: Glu 0.97, Ile 1.03, Leu 1.00, Asp 1.01, Val 1.03. Peptide content: 0.15 mmol/g.

**H–Glu–Ile–Leu–Asp–Val–Pro–Ser–Thr–$NH_2$·HCl** Fluffy powder, $Rf^2$ 0.19, $[\alpha]_D^{21}$ −103.0° ($c$=1.0, $H_2O$), MS $m/z$ 873 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Glu 1.03, Ile 1.02, Leu 1.00, Asp 1.04, Val 1.01, Pro 1.06, Ser 0.91, Thr 0.94 (average recovery 86%).

**cPEG–Glu–Ile–Leu–Asp–Val–Pro–Ser–Thr–$NH_2$** Fluffy powder, $Rf^3$ 0.26. Amino acid ratios in an acid hydrolysate: Glu 1.03, Ile 0.96, Leu 1.00, Asp 1.06, Val 1.01, Pro 1.08, Ser 0.90, Thr 0.94 (peptide content 0.15 mmol/g).

**Experimental Metastasis Assay** Inhibitory effects of the synthetic peptides and the hybrids on experimental metastasis were examined in mice as reported.[1] Briefly, B16-BL6 melanoma cells ($1\times10^5$/0.2 ml) admixed with or without peptides were intravenously injected into C57BL/6 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 were counted under a stereoscopic microscope.

**Cell Spreading Assay** A 48-well plate was coated with fibronectin in PBS(−) (1 μg/ml) for 120 min at room temperature. After blocking with PBS(−) supplemented with 1.0% heat-denatured (80 °C for 5 min) BSA for 60 min, peptide solutions in MEM containing 0.05% BSA (50 μl/well) were added. B16-BL6 melanoma cells ($1\times10^5$/50 μl/well) were applied into wells and incubated at 37 °C for 60 min. After an incubation period, the adherent cells were fixed with a 10% formalin neutral buffer solution and the spreading cell number was counted.

**Cell Migration Assay** The chemotactic migration of tumor cells was measured by the Boyden chamber method.[14] Polyvinylpyrrolidone-free polycarbonate filters (pore diameter, 8 μm) coated with $3\times10^{-4}$% gelatin solution were used to separate the upper and lower compartments of the chamber. Fibronectin in MEM, supplemented with 0.1% BSA (0.3 μg/ml), was added to the lower compartment, and the peptide solution was added to the upper compartment. Cell suspensions ($1.5\times10^5$/100 μl) were applied to the upper compartment, and the chamber was incubated at 37 °C in a 5% $CO_2$ atmosphere. After incubation for 5 h, the filters were fixed with 100% methanol solution. The migrated cells were stained with Giemsa solution and counted under a microscope at ×150.

## RESULTS AND DISCUSSION

Two types of PEG were used for the preparation of hybrids. PEG #6000 (M.W. 6700—9000) was converted to aPEG *via* tosylate and phthalimidate according to Pillai's method.[12] PEG monomethylether (average M.W. 5000) was oxidized to carboxyl-PEG monomethylether (cPEG) according to the method reported by Ueyama *et al.*[13] Arg–Glu–Asp–Val–aPEG(REDV–aPEG) was synthesized by the solution method, as shown in Fig. 2.

First, Boc–Arg(Tos)–Glu(OcHx)–Asp(OcHx)–Val–OH was prepared and then coupled with aPEG. All coupling reactions for peptide synthesis were performed by the mixed anhydride method using isobutyl chloroformate.[15] Boc–Asp(OcHx)–OH and H–Val–OBzl were coupled to give a protected dipeptide, followed by TFA treatment to remove the Boc group. The resulting dipeptide was reacted with Boc–Glu(OcHx)–OH to give a protected tripeptide, followed by TFA treatment. The resulting tripeptide was coupled with Boc–Arg(Tos)–OH to give a protected tetrapeptide, followed by hydrogenation to remove the benzyl group on Val. The resulting tetrapeptide, Boc–Arg(Tos)–Glu(OcHx)–Asp(OcHx)–Val–OH, was reacted

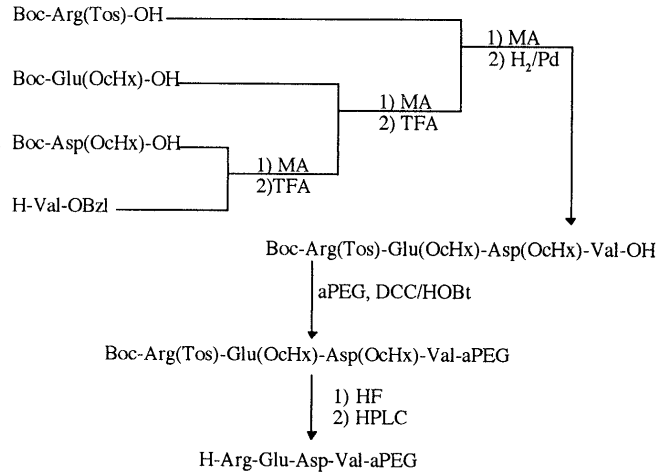


Fig. 2. Synthetic Scheme for H–Arg–Glu–Asp–Val–aPEG

MA, mixed anhydride method. TFA, trifluoroacetic acid. OcHx, cyclohexyl ester. DCC/HOBt, dicyclohexylcarbodiimide/1-hydroxybenzotriazole.

with aPEG by the DCC/HOBt method,[16] which was reported as a racemization-free method. The protected hybrid was treated with HF[17] to remove all protecting groups and the product was purified by HPLC. Other peptides and hybrids were prepared by the solid phase method on *p*-methyl-benzhydrylamine resin. Dicyclohexylcarbodiimide (DCC) was used as a coupling reagent and Boc groups were removed by TFA treatment.

Since the coupling reaction of cPEG was slow, the reaction was repeated 3 times using 5-fold excess cPEG in a molar ratio. Final deprotection of the synthetic protected peptide-resins was performed by HF treatment, and the products were purified by HPLC. All synthetic peptides and hybrids showed correct amino acid ratios and gave a homogenous spot on thin-layer chromatography and HPLC. The HPLC profile of cPEG–EILDV is shown in Fig. 3. The inhibitory effects of fibronectin-related peptides (EILDV, REDV) and their hybrids on experimental metastasis are shown in Fig. 4. The inhibitory effects of a laminin-related peptide (YIGSR) and its PEG hybrid (cPEG–YIGSR)[18] were also examined for comparison with those of the fibronectin-related peptides and the hybrids. PEG itself did not show any inhibitory effect, as we reported.[19] cPEG–YIGSR inhibited metastasis dose dependently. Since 600 $\mu$g of cPEG–YIGSR (containing about 0.1 $\mu$mol YIGSR) was nearly equal to that of 1000 $\mu$g of YIGSR (1.58 $\mu$mol), the inhibitory effect of the PEG hybrid was about 15 times as potent as that of YIGSR in terms of molar ratio. EILDV and REDV exhibited an antimetastatic effect, as we expected. Compared with YIGSR, EILDV had a more potent inhibitory effect. Only 300 $\mu$g of the peptide inhibited about 95% melanoma metastasis. In our previous experiments, YIGSR and RGD needed more than 2 mg of peptide to inhibit over 90% of tumor metastasis. The antimetastatic effect of 600 $\mu$g cPEG–EILDV (0.11 $\mu$mol) was as potent as 300 $\mu$g of EILDV (0.48 $\mu$mol). Thus, the inhibitory effect of the hybrid was 4 times as potent as that of EILDV. From this point of view, EILDV and its cPEG hybrid could be favorite canditates as a tumor metastasis inhibitor. The REDV peptide was less active than the other peptides. After hybrid formation with aPEG, the hybrid, REDV–aPEG, had a higher effect than REDV itself. To examine the effect of EILDV in more detail, we studied the antimetastatic effect of the C-terminal chain-extended peptide EILDVPST. As shown in Fig. 5, the inhibitory effect of EILDVPST was nearly equal to that of EILDV, and its hybrid, cPEG–EILDVPST, showed markedly strong activity.

Next, the combined effects of antimetastatic peptides were studied. We examined various combinations of our

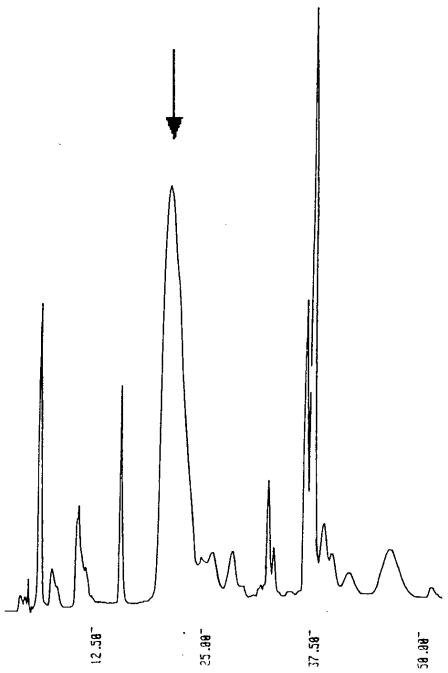


Fig. 3. Preparative HPLC Profile of cPEG–Glu–Ile–Leu–Asp–Val–$NH_2$

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, A/B: 80/20→40/60 (0→60 min). cPEG–EILDV was obtained in the peak indicated by the arrow.

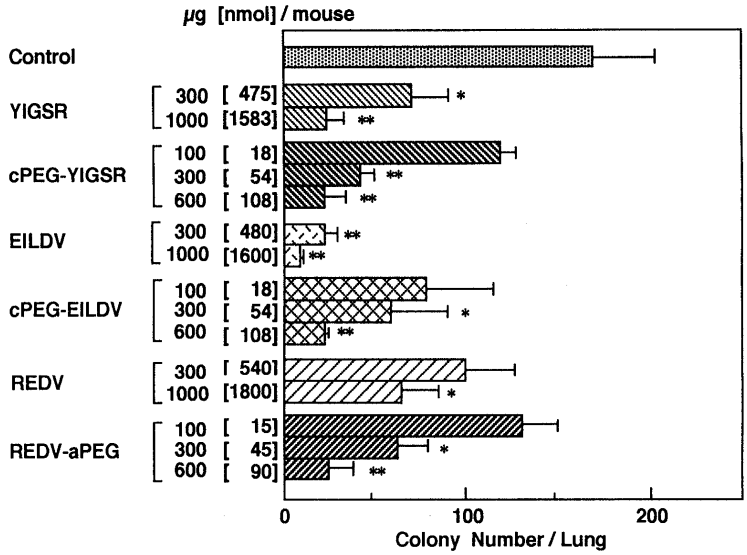


Fig. 4. Inhibitory Effects of Synthetic Peptides on the Formation of Lung Metastasis

B16-BL6 cells ($1 \times 10^5$) were injected i.v. with or without admixing with various concentrations of peptides into five mice per group. Lung tumor colonies were examined 2 weeks later. Values were the mean ± S.E. $*p<0.005$; $**p<0.001$ compared with the untreated control by Student's *t*-test.

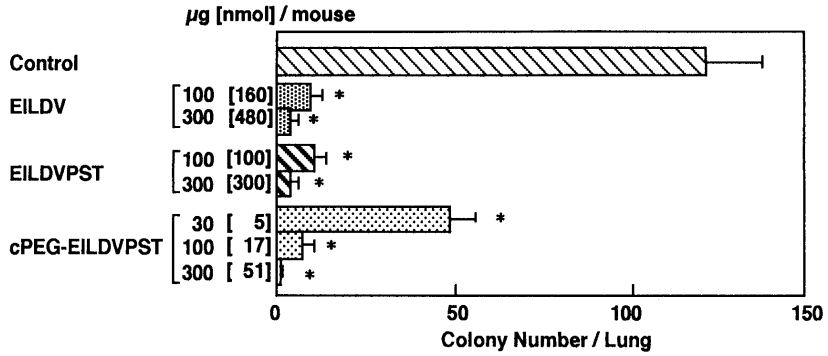


Fig. 5. Inhibitiory Effects of EILDV-Related Peptides on the Formation of Lung Metastasis

The tumor colonized assay was carried out as described in Fig. 4. Values were the mean ± S.E. $*p<0.001$ compared with the untreated control by Student's $t$-test.

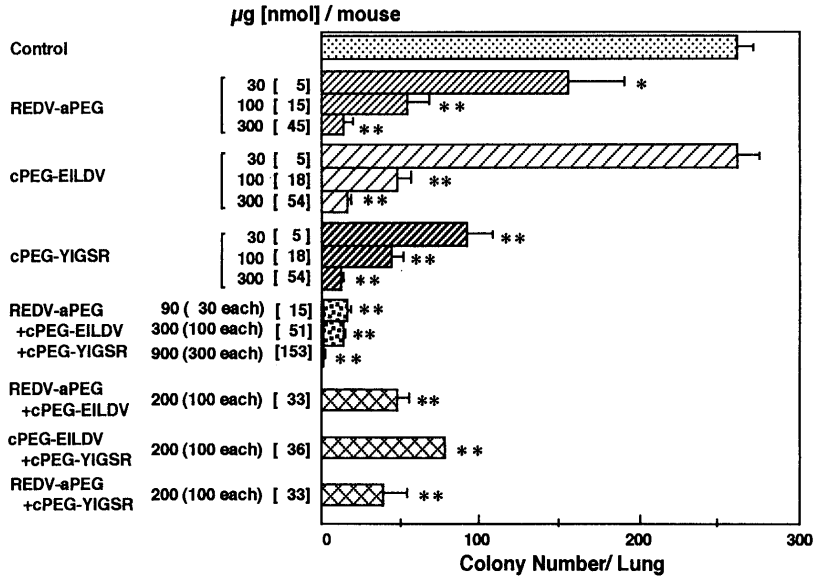


Fig. 6. Inhibition of Lung Colonization by the Mixture of Three Peptide–PEG Hybrids, REDV–aPEG, cPEG–EILDV, and cPEG–YIGSR

The tumor colonization assay was carried out as described in Fig. 3. Values were the mean ± S.E. $*p<0.05$; $**p<0.01$ compared with the untreated control by Student's $t$-test.

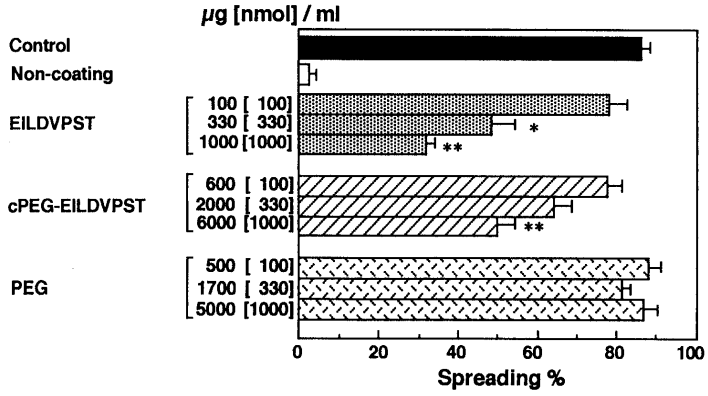


Fig. 7. Inhibitory Effect of EILDVPST and Its PEG Hybrid on Cell Spreading

Each value represents the mean ± S.E. $*p<0.005$, $**p<0.001$ compared with the untreated control by Student's $t$-test.

PEG hybrids, *i.e.* cPEG–YIGSR, cPEG–EILDV and REDV–aPEG, which are potent metastasis inhibitors. We found that the mixture consisting of cPEG–YIGSR, cPEG–EILDV and REDV–aPEG was the most effective. As shown in Fig. 6, only 90 μg (30 μg from each peptide) of the mixture of the hybrids was sufficient to inhibit tumor metastasis by 95%, and at 900 μg the mixture inhibited lung metastasis almost completely.

The inhibitory effects of PEG hybrids on the adhesion and migration of B16-BL6 melanoma cells to fibronectin were studied. EILDVPST inhibited the adhesion and spreading of B16-BL6 in the dose dependent manner. However, cPEG–EILDVPST reduced the inhibitory effects compared to native EILDVPST in terms of weight

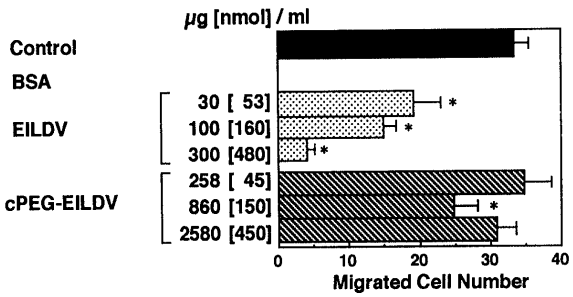


Fig. 8. Inhibitory Effect of EILDV and Its PEG Hybrid on Cell Migration

Each value represents the mean ± S.E. $*p<0.001$ compared with the untreated control by Student's *t*-test.

ratio. As shown in Fig. 8, EILDV markedly inhibited the migration of B16-BL6 to fibronectin. But, the inhibitory effect of cPEG–EILDV was lower than that of EILDV. These results suggested that chemical modification of cell adhesive peptides with PEG decreased their inhibitory effect on adhesion and spreading migration, whereas the *in vivo* anti-metastatic effects of PEG hybrids were significantly higher than those of the parent peptides. Since hybrid formation was not effective in the spreading and migration assays, the bulky PEG moiety might prevent the function of the peptide portion.

In conclusion, we found that EILDV, EILDVPST and REDV were inhibitors of experimental metastasis of B16-BL6 melanoma in mice.

Of the synthetic peptides, EILDV exhibited the most potent inhibitory effect. The inhibitory effect of the parent peptides on metastasis was potentiated by hybrid formation with PEG either at the N-terminal or C-terminal of the parent peptides. Hybrid formation with PEG will be an interesting field of study regarding the drug-delivery system of bioactive oligopeptides. The reason the inhibitory effects of parent peptides on metastasis were potentiated by the hybrid formation with PEG is not yet exactly understood. The stability of the parent peptides in blood may be increased by hybrid formation with PEG. We reported that the hydrolysis of YIGSRG–aPEG hybrid by aminopeptidase M and chymotrypsin was slower than that of the peptide itself.[19] Quite recently, a detailed study on the stability of YIGSR and its PEG hybrid *in vivo* was done, and the results are published elsewhere.[20] A mixture of hybrids also exhibited potent and synergistic effect. For clinical use as a metastasis inhibitor, a combination of different types of inhibitors is essential. From this point of view, the inhibitory effect of the mixture is noteworthy.

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