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Antimetastatic effects of synthetic peptides containing the core sequence of the type III connecting segment domain (IIICS) of fibronectin

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The antimetastatic activities of synthetic peptides corresponding to fragments of the adhesion-related molecules, such as fibronectin and laminin, were examined. We prepared three peptides derived from the type III connecting segment domain (IIICS) of fibronectin: Glu-Ile-Leu-Asp-Val (EILDV), Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr (EILDVPST), Arg-Glu-Asp-Val (REDV), and a laminin-related peptide, Tyr-Ile-Gly-Ser-Arg (YIGSR). Each peptide inhibited the experimental tumor metastasis of B16-BL6 melanoma, while EILDV had the strongest effect. The peptides conjugated with poly(ethylene glycol) (PEG) were more effective than the unmodified peptides in molar ratio terms. A mixture composed of PEG hybrids with EILDV, REDV and YIGSR significantly inhibited tumor metastasis.

Key words: Fibronectin, laminin, metastasis, peptide–poly(ethylene glycol) hybrid.

Introduction

Several studies have suggested that some synthetic peptides corresponding to fragments of the adhesion-related molecules, such as fibronectin and laminin, could modulate the function of tumor cell metastasis. These peptides have been found to promote tumor cell attachment after surface immobilization and to inhibit tumor metastasis when co-injected with tumor cells. The Arg-Gly-Asp (RGD) core sequence in the cell-binding domain of fibronectin and other adhesive proteins have been shown to contribute to cell functions[1,2] (adhesion, spreading and migration) and the RGD-containing peptide Gly-Arg-Gly-Asp-Ser (GRGDS) was able to inhibit experimental metastasis of murine melanoma.[3] Similarly, Tyr-Ile-Gly-Ser-Arg (YIGSR) derived from laminin has been shown to inhibit experimental metastasis.[4]

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Recently several analogs of RGD- or YIGSR-related peptides which have a stronger inhibitory effect on tumor metastasis have been reported: the cyclic peptides,[5,6] polymeric peptides[7–9] and recombinant fusion peptides.[10,11] We previously reported that hybrids of amino-poly(ethylene glycol) (aPEG) and Tyr-Ile-Gly-Ser-Arg-Gly (YIGSRG) or RGD inhibited the experimental metastasis effectively.[12,13]

A 33 kDa heparin binding fragment of fibronectin could promote tumor cell adhesion[14] as well as the RGD- or YIGSR-related peptides. CS1 and CS5 peptides which were present within the type III connecting segment domain (IIICS) of 33 kDa heparin binding fragment have been shown to promote B16-F10 melanoma cell adhesion through an RGD-independent mechanism.[15,16] The minimal active sequence within CS1 and CS5 were Leu-Asp-Val (LDV)[17] and Arg-Glu-Asp-Val (REDV), respectively. In the present study, two CS1- related peptides: Glu-Ile-Leu-Asp-Val (EILDV) and Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr (EILDVPST), CS5-related peptide (REDV) and laminin-related peptide (YIGSR) were synthesized. Furthermore, the hybrids of each peptide and PEG were prepared. The antimetastatic effects of all these peptides were examined.

Materials and methods

Cell and cell culture

Highly metastatic B16-BL6 cells were kindly provided by Dr M Sano (School of Pharmaceutical Sciences, University of Shizuoka, Japan). Melanoma cells were maintained as monolayer cultures in Eagle's minimal essential medium supplemented with 10% fetal calf serum and L-glutamine.

Synthetic PEG hybrid peptides and reagents

Two types of PEG derivatives were synthesized to prepare the peptide–PEG hybrid. PEG #6000 (MW 7300–9000) was converted to aPEG which was acylated with a peptide to form a hybrid. PEG monomethylether (average MW 5000, Aldrich) was oxidized to carboxyl-PEG monomethylether (cPEG) and a peptide was acylated with cPEG to form a hybrid. REDV–aPEG was synthesized by the solution method. First Boc-Arg (Tos)-Glu (OcHx)-Asp (OcHx)-Val-OH was prepared and then coupled with aPEG as shown in Figure 1. aPEG was prepared from PEG #6000 according to the procedure reported by Pillari and Mutter.[18] aPEG was purified by Dowex 50 (H^+) column chromatography using 2% ammonia as an eluent. Amino acid contents of aPEG were 0.14–0.27 meq/g. All coupling reactions for peptide synthesis were performed by the mixed anhydride method using isobutyl chloroformate.[19] Boc-Asp (OcHx)-OH and H-Val-OBzl were coupled to give a protected dipeptide, followed by trifluoroacetic acid (TFA) treatment to remove the Boc group. Boc-Glu (OcHx)-OH was reacted with the dipeptide to give a protected tripeptide, followed by TFA treatment. Boc-Arg (Tos)-OH was coupled with tripeptide 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 with aPEG by the dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HoBt) method,[20] reportedly a racemization-free method. The protected hybrid was treated with hydrogen fluoride (HF) to remove all protecting groups and the product was purified by high performance liquid chromatography (HPLC).

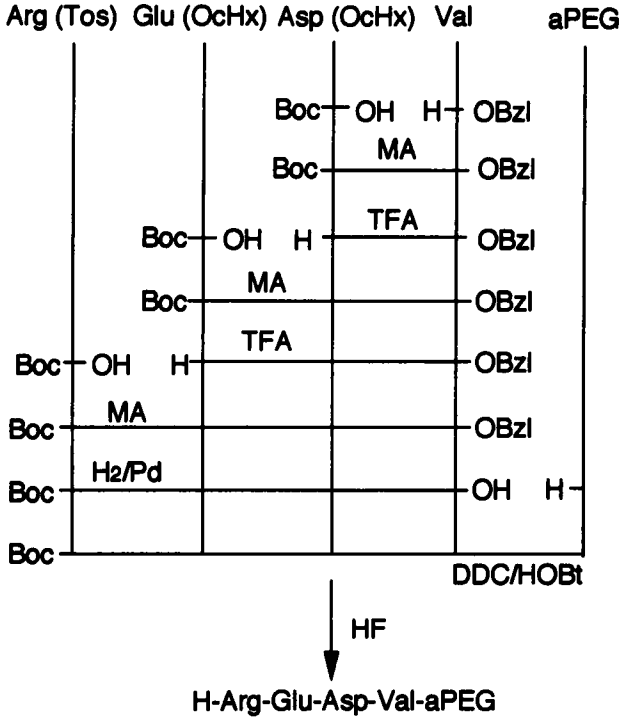

Figure 1. Synthetic scheme for the REDV–aPEG hybrid. MA, mixed anhydride method; TFA, trifluoroacetic acid; DCC/HOBt, dicyclohexylcarbodiimide/1-hydroxybenztriazole.

Other peptides and hybrids were prepared by the solid-phase method. Methylbenzhydrylamine resin (0.64 meq/g) was used for a solid support. PEG monomethylether (average MW 5000, Aldrich) was oxidized to cPEG with potassium permanganate according to the procedure of Ueyama *et al.*[21] cPEG was purified by Diaion WA21 using 2% AcOH as an eluent. The carboxyl content of cPEG was 0.16–0.22 meq/g. To form PEG hybrids with EILDV, EILDVPST or YIGSR, the peptides were acylated with cPEG. Since acylation with cPEG was very slow, the acylation was repeated three times using a 5-fold excess of cPEG in molar ratio. Final deprotection of the solid-phase syntheses was performed by HF treatment[22] and products were purified by HPLC. All synthetic peptides and hybrids showed correct
amino acid ratios and gave homogeneous spots on thin-layer chromatography. Details of synthesis will be reported elsewhere.

Experimental metastasis assay

The inhibitory effect of each synthetic peptide on experimental metastasis of B16-BL6 melanoma was examined in mice according to the method reported previously with slight modification.[12,13] Tumor cells (1×10^5) were admixed with various concentrations of peptides in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline and immediately 0.1 ml of these suspensions was injected into the tail vein of C57BL/6 mice. Two weeks later the animals were killed, and then the lungs were excised and fixed in 10% formaldehyde. The number of surface melanoma colonies were counted macroscopically.

Results

We first examined the effect of the YIGSR-related peptides on experimental metastasis in mice (Figure 2). YIGSR and CDPGYIGSR inhibited the tumor colonization of B16 melanoma as reported.[4] Since

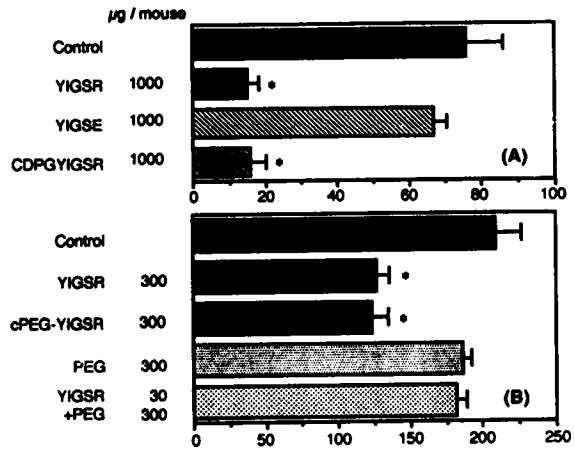

Figure 2. Inhibitory effects of YIGSR-related peptides on the formation of lung metastasis. (A) Effect of YIGSR-related peptides on lung colonization. B16-BL6 cells (1×10^5) were injected i.v. with or without admixing with 1 mg of peptides into five mice per group. Lung tumor colonies were examined 14 days later. Values were the mean ± SE. $^*p < 0.001$ compared with untreated controls by Student's *t*-test. (B) Inhibition of lung colonization by cPEG–YIGSR. B16-BL6 cells (1×10^5) were injected i.v. with or without admixing with 300 μg of peptides into five mice per group. Lung tumor colonies were examined 14 days later. Values are the mean ± SE. $^*p < 0.05$ compared with untreated controls by Student's *t*-test.

YIGSE did not show the inhibitory effect, the antimetastatic effects of YIGSR-related peptides were specific for YIGSR pentapeptide (Figure 2A). The hybrid cPEG–YIGSR, also caused a significant reduction of tumor colonies of B16-BL6 melanoma. PEG did not show the inhibitory effect. The effect of the mixture of YIGSR (30 μg) and PEG (300 μg) was also examined; 300 μg of cPEG–YIGSR contained 30 μg of YIGSR. Although the mixture did not inhibit tumor colonization, the presence of the covalent bond between the YIGSR and PEG was necessary to exhibit the antimetastatic effect.

Next, the inhibitory effects of the synthetic peptides on experimental metastasis were studied in detail. As shown in Figure 3, cPEG–YIGSR inhibited B16 melanoma lung colonization dose dependently. Since the inhibitory effect of 600 μg of cPEG–YIGSR (containing about 0.09 μmol YIGSR) was nearly equal to that of 1000 μg of YIGSR (1.58 μmol), the inhibitory effect of the PEG hybrid was about 15 times as potent as that of YIGSR in molar ratio terms. Compared with YIGSR, the CS1-related peptide, EILDV, had a stronger inhibitory effect. Only 300 μg of the peptide inhibited about 95% B16 melanoma metastasis. In our previous experiments, YIGSR and RGD needed more than 2 mg of peptide to inhibit over 90% of tumor metastasis. The

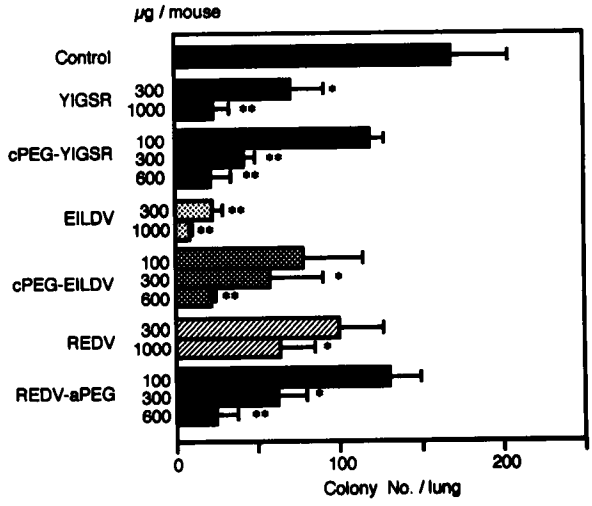

Figure 3. Inhibitory effects of synthetic peptides on the formation of lung metastasis. B16-BL6 cells (1×10^5) were injected i.v. with or without admixing with various concentrations of peptides into five mice per group. Lung tumor colonies were examined 14 days later. Values are the mean ± SE. $^*p < 0.005$, ** $p < 0.001$ compared with untreated controls by Student's *t*-test.

antimetastatic effect of 600 μg cPEG–EILDV (0.11 μmol) was as potent as 300 μg of EILDV (0.48 μmol). Thus the inhibitory effect of the hybrid was four times as potent as that of EILDV. From this point of view, EILDV and its hybrid with cPEG, cPEG–EILDV, could be the favorite candidates for a tumor metastasis inhibitor. The REDV peptide was less active than the other peptides. After modification with PEG, its hybrid peptide, REDV–aPEG, had a higher effect than REDV tetrapeptide.

To examine the effect of the CS1-related peptide in more detail, we studied the antimetastatic effect of C-terminal chain-extended peptide EILDVPST. As shown in Figure 4, the inhibitory effect of EILDVPST was nearly equal to that of EILDV and its hybrid

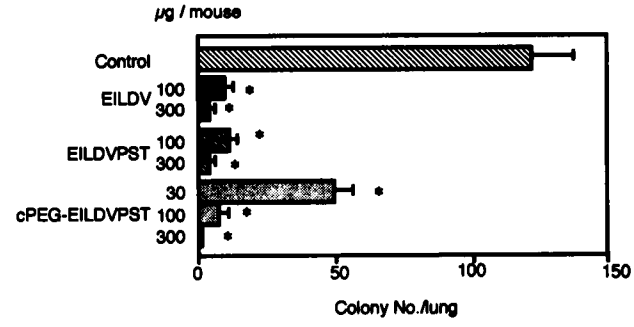

Figure 4. Inhibitory effects of EILDV-related peptides on the formation of lung metastasis. The tumor colonized assay was carried out as described in Figure 3. Values are the mean ± SE. $^*p < 0.001$ compared with untreated controls by Student's *t*-test.

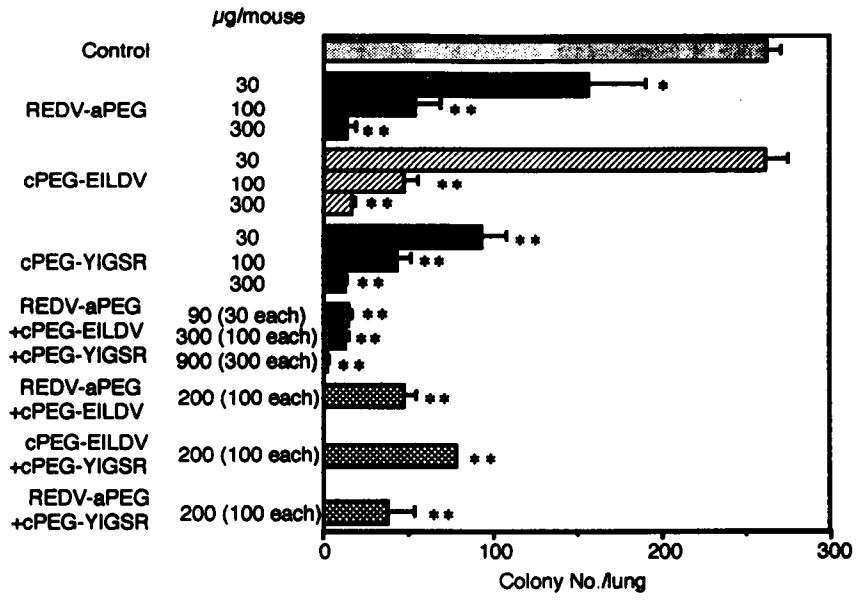

Figure 5. Inhibition of lung colonization by the mixture of three peptide–PEG hybrids, i.e. REDV–aPEG, cPEG–EILDV and cPEG–YIGSR. The tumor colonization assay was carried out as described in Figure 3. Values are the mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with untreated controls by Student's *t*-test.

peptide, cPEG–EILDVPST showed markedly strong activity.

Next the combined effects of antimetastatic peptides were studied. We examined various combinations of our peptide–PEG hybrids, i.e. cPEG–YIGSR, cPEG–EILDV and REDV–aPEG, which are the potent inhibitor of experimental murine metastasis. We found that the mixture consisting of cPEG–YIGSR, cPEG–EILDV and REDV–aPEG was the most effective. As shown in Figure 5, only 90 μg (30 μg from each peptide) of the mixture of peptides was sufficient to inhibit tumor metastasis by 95% and at 900 μg the mixture of peptides inhibited lung metastasis completely.

Discussion

In this paper, we reported the possibility of PEG hybrids with several cell-binding peptides, including the peptide with the core sequence of the type III connecting segment domain (IIICS) of fibronectin, as candidates for antimetastatic agents. In our experiments, we found that peptides conjugated with PEG were more effective than the unmodified peptides in molar ratio terms. Although it is still unclear, this high inhibitory effect of PEG–hybrid peptides could be explained by the hypothesis that the bulky PEG moiety might prevent enzymatic hydrolysis of peptides and stabilize the binding between peptides and the cell surface receptor. Peptide was easily hydrolyzed but its hybrid was hydrolyzed very slowly by serum peptidase (data not shown).

To obtain a more potent inhibitor, various combinations of PEG–hybrid peptides were considered. A mixture composed of PEG hybrids with EILDV, REDV and YIGSR significantly inhibited tumor metastasis. These findings indicated that the mixture of fibronectin IIICS-related peptide and laminin-related peptide might inhibit the interaction of metastatic tumor cells with the extracellular matrix and basement membrane more effectively than the fibronectin IIICS-related peptide or laminin-related peptide alone. This effect required three peptides because none of the mixtures consisting of any two peptides had any obvious synergistic effects.

In conclusion, CS1-related peptides, EILDV and EILDVPST were more potent inhibitors of B16 lung metastasis than the well known antimetastatic peptides, YIGSR-related peptide or RGD-related peptide. Furthermore, the peptides conjugated with PEG were more effective than the unmodified peptides in molar ratio terms. These hybrids inhibited B16 melanoma metastasis not only in the experimental metastasis model but also in a spontaneous model (unpublished data). The mixture, composed of PEG hybrids with EILDV, REDV and YIGSR, inhibited tumor metastasis completely. Our findings suggested that the hybrids of cell attaching peptides and PEG are the favorite candidates for a tumor metastasis inhibitor.

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