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Antimetastatic effect of synthetic Glu–Ile–Leu–Asp–Val peptide derivatives containing D-amino acids

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The aim of this study was to increase the antimetastatic potency of the fibronectin-related peptide, Glu–Ile–Leu–Asp–Val (EILDV), and to determine the minimal core sequence of EILDV required to inhibit tumor metastasis *in vivo*. The EILDV subpeptide analog, ILDV, markedly inhibited the adhesion of B16-BL6 melanoma cells to fibronectin. EILD and ILD were only slightly inhibitory, and the smaller overlapping tripeptide, LDV, was inactive. The inhibitory activities of ILDV and LDV on the migration of B16-BL6 melanoma cells were as potent as those of EILDV, whereas ILD did not inhibit cell migration. These results suggested that the minimal sequences essential for cell adhesion and migration are ILD and LDV, respectively. However, the antimetastatic effects of all subpeptide analogs were lower than that of EILDV. In order to improve the stability *in vivo*, we synthesized various EILDV-related peptides substituted with a D-amino acid. EILDV containing D-Glu or D-Ile inhibited cell adhesion and migration as potent as EILDV, whereas replacing Leu, Asp or Val with the corresponding D-isomer reduced the antiadhesive activities. The inhibitory effect of EILDV-related peptides containing D-Leu, D-Asp or D-Val on migration was also lower than that of EILDV. All synthetic EILDV-related peptides containing D-amino acids inhibited metastasis by B16-BL6 melanoma cells to the same extent as EILDV, whereas the specific activity of EILDV was decreased by the D-amino acid substitution. These results indicated that the balance of stability *in vivo* and biological activity *in vitro* is important in inhibiting tumor metastasis.

Key words: D-Amino acid, cell adhesion, EILDV, fibronectin, migration, tumor metastasis.

Introduction

The dissemination and metastasis of tumor cells is initiated when cells detach from the primary tumor and intravasate through the basement membrane and extracellular matrix (ECM).[1] After attachment to endothelial cells, tumor cells extravasate and invade surrounding tissue.[2] The adhesive interaction of tumor cells with components of ECM are regulated by various adhesion molecules.[3] Therefore, blocking these interactions should prevent tumor metastasis.

Fibronectin (FN) is a major constituent of ECM and is a multifunctional glycoprotein that promotes the attachment, spreading and migration of various cells.[4] FN contains two major domains that play an important role in cell adhesion.[5] One site is the central cell-binding domain that contains the Arg–Gly–Asp–Ser (RGDS) sequence. The other is the alternatively spliced type III connecting segment (IIICS), the N-terminal end of which consists of Glu–Ile–Leu–Asp–Val (EILDV). Synthetic peptides containing these sequences are competitive inhibitors of cell adhesion to FN.[6,7] We reported that the synthetic RGD peptide inhibited the experimental metastasis of B16-BL6 melanoma cells.[8] However, to effectively inhibit metastasis, large amounts of peptide were required. Thus increased antimetastatic activity was needed to facilitate the effectiveness of cell adhesive peptides.

To enhance the antimetastatic potency of cell adhesive peptides, various strategies have been adopted.[9–11] Hybrid proteins with various polymers such as polyethylene glycol (PEG), albumin and dextran increase their biological activity *in vivo*.[12] We reported that chemical modification of tumor necrosis factor (TNF)-α with PEG increased its antitumor potency.[13,14] We synthesized more hybrid cell-adhesive peptides with PEG to increase their plasma half-lives. PEG–peptides inhibited the experimental metastasis of B16-BL6 melanoma cells more effectively than native peptides. We found that PEGylation of peptides increased their resistance against various peptidases, which improved their antimetastatic activity due to a longer half-life in blood.[15] However, the increase in the molecular weight of the peptides caused by PEGylation restricted their transport from blood to target organs and the steric hindrance caused by PEG inhibited peptide binding to receptors.

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We reported that the conformational modification of cell adhesive peptides, such as cyclization or substitution with another amino acid, increases their antimetastatic potency.[16] However, it was attempted to enhance peptide stability *in vivo* based on substitution with D-amino acids.[17]

In the present study, we synthesized EILDV derivatives substituted with a D-amino acid, and examined their inhibitory effect on cell adhesion, cell migration and tumor metastasis. We synthesized EILDV subpeptides to identify the minimal core sequence of EILDV required for antimetastatic activity.

Materials and methods

Mice

Six-week-old specific-pathogen-free male C57BL/6 mice were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan).

Cells and culture

Highly metastatic and invasive B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion, were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum, sodium bicarbonate and L-glutamine.[18]

Synthesis of EILDV-related peptide

All peptides were prepared by the solid-phase method on methylbenzhydrylamine resin, which renders the C-terminal of all synthetic peptides amides.[19] N^{α}-amimo groups were protected with a *t*-butyloxycarbonyl (Boc) group. Side-chain carboxyl groups of Asp and Glu were protected with cyclohexly groups. Coupling reactions proceeded using dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) by the same manner as our previous report.[20] Peptides were finally deprotected using HF. All synthetic peptides were detected by TLC on Silica gel G (Type 60). Solvent systems for ascending TLC were indicated as follows: Rf^1=*n*-BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^2=BuOH–pyridine–AcOH–H_2O (4:1:1:2). After purification by reverse-phase high performance liquid chromatography (RP-HPLC), all synthetic peptides were converted to dydrochlorides by lyophilization from water containing HCl. Synthetic peptides were hydrolyzed in 6 N HCl at 110°C for 20 h. The purity and identity of the synthetic peptides were confirmed by RP-HPLC and amino acid analysis. Amino acid compositions of acid hydrolyzates were determined with a Hitachi 835 amino acid analyzer. FAB-MS were measured on a VG Analytical ZAV-SE spectrometer. Physical parameters of all synthetic peptides in this study were as follows: EILDV; hygroscopic powder, Rf^1 0.32, Rf^2 0.26, $[\alpha]_D^{21}$ −160.2° (*c*=1.0, H_2O). FAB-MS *m/z* 588(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 1.06, Ile 0.99, Leu 1.00, Asp 1.09, Val 1.03 (average recovery 88%). EILD; fluffy powder, Rf^1 0.27, Rf^2 0.48, $[\alpha]_D^{23}$ −39.7° (*c*=1.0, H_2O). FAB-MS *m/z* 488(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 1.01, Ile 1.00, Leu 1.00, Asp 0.98 (average recovery 75%). EIL; fluffy powder, Rf^1 0.48, Rf^2 0.53, $[\alpha]_D^{23}$ −30.3° (*c*=1.0, H_2O). FAB-MS *m/z* 373(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 1.06, Ile 1.00, Leu 1.03 (average recovery 79%). ILDV; fluffy powder, Rf^1 0.36, Rf^2 0.38, $[\alpha]_D^{23}$ −51.5° (*c*=1.0, H_2O). FAB-MS *m/z* 458(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Ile 1.00, Leu 1.00, Asp 0.98, Val 0.98 (average recovery 75%). LDV: fluffy powder, Rf^1 0.35, Rf^2 0.55, $[\alpha]_D^{23}$ −27.3° (*c*=1.0, H_2O). FAB-MS *m/z* 488 (M+1)$^+$. Amino acid ratios in an acid hydrolyzate Leu 1.00, Asp 1.01, Val 1.00 (average recovery 79%). ILD; fluffy powder, Rf^1 0.43, Rf^2 0.63, $[\alpha]_D^{23}$ −21.8° (*c*=1.0, H_2O). FAB-MS *m/z* 488(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Ile 0.99, Leu 1.00, Asp 1.06 (average recovery 79%). DEILDV; fluffy powder, Rf^1 0.32, Rf^2 0.56, $[\alpha]_D^{23}$ −91.4° (*c*=1.0, H_2O). FAB-MS *m/z* 588(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 1.00, Ile 0.98, Leu 1.01, Asp 1.01, Val 0.97 (average recovery 89%). EDILDV; fluffy powder, Rf^1 0.32, Rf^2 0.56, $[\alpha]_D^{23}$ −37.3° (*c*=1.0, H_2O). FAB-MS *m/z* 588(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 1.00, Ile 0.98, Leu 1.01, Asp 1.01, Val 0.97 (average recovery 89%). EIDLDV; fluffy powder, Rf^1 0.32, Rf^2 0.56, $[\alpha]_D^{23}$ −2.2° (*c*=1.0, H_2O). FAB-MS *m/z* 588(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 1.01, Ile 1.00, Leu 1.01, Asp 1.02, Val 0.95 (average recovery 78%). EILDDV; fluffy powder, Rf^1 0.32, Rf^2 0.56, $[\alpha]_D^{23}$ −16.4° (*c*=1.0, H_2O). FAB-MS *m/z* 588(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 0.97, Ile 1.00, Leu 1.01, Asp 1.02, Val 0.96 (average recovery 84%). EILDDV; fluffy powser, Rf^1 0.32, Rf^2 0.56, $[\alpha]_D^{23}$ −36.6° (*c*=1.0, H_2O). FAB-MS *m/z* 588(M+1)$^+$. Amino acid ratios in an acid hydrolyzate Glu 0.99, Ile 1.00, Leu 1.01, Asp 0.99, Val 0.96 (average recovery 83%).

Cell adhesion assay

Plates (96-well) were coated with FN in PBS(−) (3 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, pre-diluted samples in MEM containing 0.05% BSA (50 μl/well) were added to a 96-well microplate and 3-fold serial dilutions were made with the medium in the wells. This assay was studied in tetraplicate wells. B16-BL6 melanoma cells (1×10^5/50 μl/well) were admixed in wells and incubated at 37°C for 60 min under 5% CO_2/air. Thereafter, adherent cells were fixed with 10% formalin in a neutral buffer. The fixed cells were stained with 0.05% methylene blue in PBS(−). Following washing with water, 200 μl of 0.3 N HCl was added to each well and the absorbance at 655 nm was measured.

Cell migration assay

The chemotactic migration of tumor cells was measured using Boyden chambers.[21] Polyvinylpyrrolidone-free polycarbonate filters (pore diameter, 8 μm) coated with 0.0003% gelatin separated the upper and lower compartments of the chamber. FN in MEM containing 0.1% BSA (2 μg/100 ml) was added to the lower compartment and 100 μl of peptides at a dose of 200 μg/ml was added to the upper compartment. Cell suspensions (1.5×10^5/100 μl) were applied to the upper compartment and the chamber was incubated at 37°C in a 5% CO_2 atmosphere for 5 h. The filters were fixed with methanol and the migrated cells were stained with Giemsa solution, then counted under a microscope at ×400.

Experimental pulmonary metastasis assay

B16-BL6 melanoma cells detached with 1 mM EDTA in PBS(−) were resuspended to a concentration of 5×10^5/ml in MEM containing 0.1% BSA. C57BL/6 mice were i.v. injected with B16-BL6 cells (1×10^5/200 μl) admixed with or without peptides (300 μg). Groups of five male C57BL/6 mice were used in this assay. The mice were sacrificed 2 weeks after tumor inoculation and the lungs were stained with Bouin's solution. Colonies were counted under a stereoscopic microscope.

Statistical analysis

Statistical evaluations of colony number on tumor metastasis, migrated cell number and adherent cell number were analyzed by Student's *t*-test.

Results

Inhibitory effect of EILDV-related peptides on adhesion of B16-BL6 melanoma to FN

We examined the inhibitory effect of EILDV-related peptides on B16-BL6 melanoma adhesion to FN. EILDV substituted with a D-amino acid for Glu or Ile inhibited cell adhesion as potently as EILDV peptide (Figure 1). However, substitution of a D-amino acid for Leu, Asp or Val reduced the inhibitory activity compared with native EILDV. The inhibitory effect of ILDV on the adhesion of B16-BL6 to FN was as potent as EILDV peptide. ILD inhibited adhesion of B16-BL6 melanoma to FN in a dose-dependent manner and EILD slightly inhibited. EILDV subpeptide analogs, EIL and LDV, did not have the inhibitory activity (Figure 2).

Inhibitory effect of EILDV-related peptides on migration of B16-BL6 melanoma

The inhibitory activity of EILDV derivatives on cell migration was evaluated using Boyden chamber.

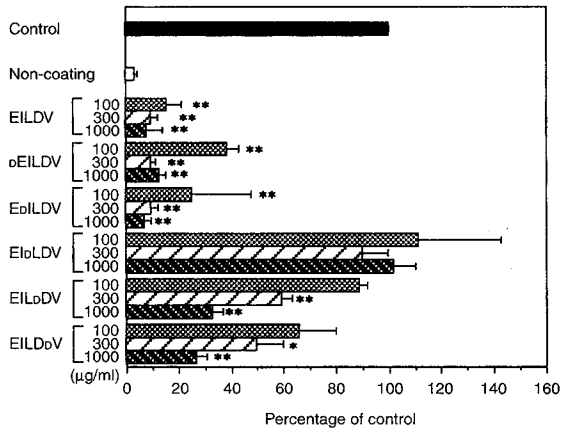

Figure 1. Inhibitory effect of EILDV derivatives substituted with a D-amino acid on the adhesion of B16-BL6 melanoma cells to FN. B16-BL6 melanoma cells (1×10^5/50 μl/well) and peptides (50 μl/well) were admixed in FN-coated wells and incubated at 37°C for 60 min under 5% CO_2/air. The adherent cells were fixed and stained with 0.05% methylene blue in PBS(−). After dissolving the stained cells with 0.3 N HCl, the absorbance of each well at 655 nm was measured. The assay was studied in tetraplicate wells. Each value represents mean ± SE. *$p < 0.005$; **$p < 0.001$ significantly different from control.

Figure 3 shows the inhibitory effect of EILDV-related peptides substituted with a D-amino acid. The EILDV derivative substituted with a D-isomer for Glu or Ile inhibited cell migration. However, substitution with D-Leu, D-Asp or D-Val almost totally abrogated the inhibitory activity on cell migration. On the other hand, the EILDV subpeptide analogs, ILDV and LDV, inhibited the migration of B16-BL6 melanoma cells, but to a lesser extent than EILDV (Figure 4). The EILDV subpeptides EILD, EIL and ILD had little inhibitory effect as compared with EILDV.

Inhibitory effect of EILDV-related peptides on experimental metastasis of B16-BL6 melanoma

B16-BL6 melanoma cells admixed with each peptide were injected i.v. into C57BL/6 mice. Figure 5 shows the antimetastatic effect of EILDV-related peptides containing a D-amino acid. EILDV reduced the numbers of B16-BL6 colonies by up to 40% of control. EILDV containing D-Glu inhibited tumor metastasis as potently as EILDV. Although substitution with D-Leu, D-Asp or D-Val decreased the *in vitro* bioactivity (antiadhesion and antimigration) of B16-BL6 melanoma cells towards FN, their inhibitory effect upon experimental metastasis was comparable to that of EILDV. None of the EILDV subpeptide analogs were antimetastatic (Figure 6).

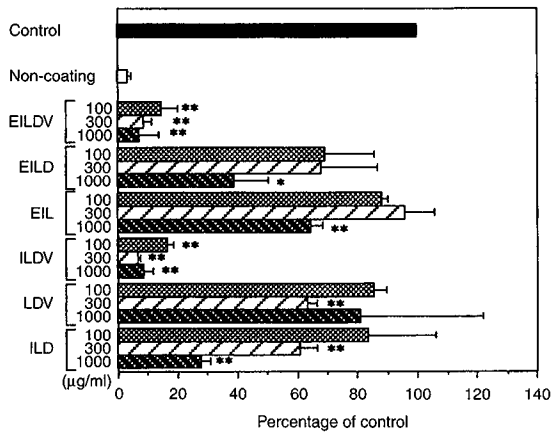

Figure 2. Inhibitory effect of EILDV-related peptides on the adhesion of B16-BL6 melanoma cells to FN. B16-BL6 melanoma cells (1×10^5/50 μl/well) with or without peptides (50 μl/well) were incubated at 37°C for 60 min under 5% CO_2/air and the adherent cells were counted. Each value represents mean ± SE. *$p<0.005$; **$p<0.001$ significantly different from control.

Discussion

We described that the chemical modification of cell adhesive peptides, such as YIGSR and RGD, with PEG

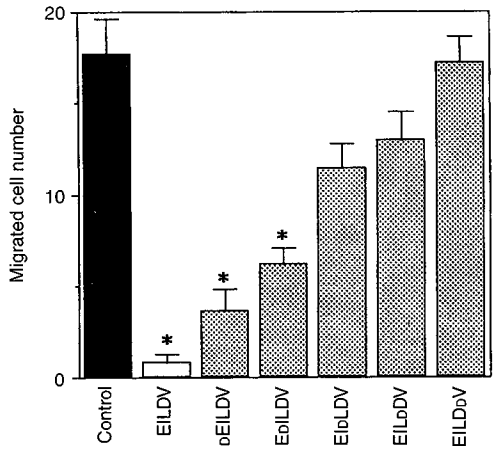

Figure 3. Inhibitory effect of EILDV derivatives substituted with a D-amino acid on the migration of B16-BL6 melanoma cells to FN. FN in MEM (2 μg/100 ml) containing 0.1% BSA was added to the lower compartment of Boyden chambers and 100 μl of peptides at a dose of 200 μg/ml was added to the upper compartment. Cell suspensions (1.5×10^5/100 μl) were applied to the upper compartment and the chamber was incubated at 37°C in a 5% CO_2 atmosphere for 5 h. The migrated cells were counted under a microscope at ×400. Each value represents mean ± SE. *$p<0.001$ significantly different from control.

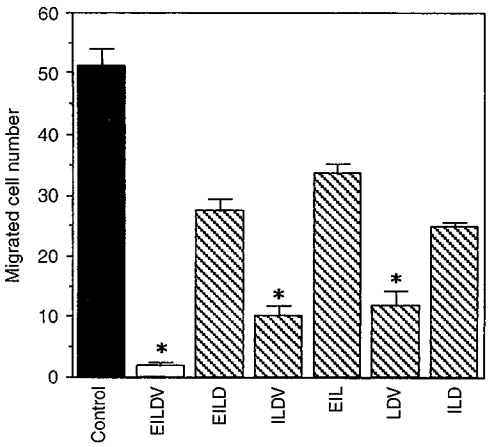

Figure 4. Inhibitory effect of EILDV-related peptides on the migration of B16-BL6 melanoma to FN. FN in MEM (2 μg/100 ml) containing 0.1% BSA was added to the lower compartment of Boyden chambers. B16-BL6 melanoma cells (1.5×10^5/100 μl) and 100 μl of peptides at a dose of 200 μg/ml was added to the upper compartment. After incubation at 37°C in a 5% CO_2 atmosphere for 5 h, the migrated cells were counted under a microscope at ×400. Each value represents mean ± SE. *$p<0.001$ significantly different from control.

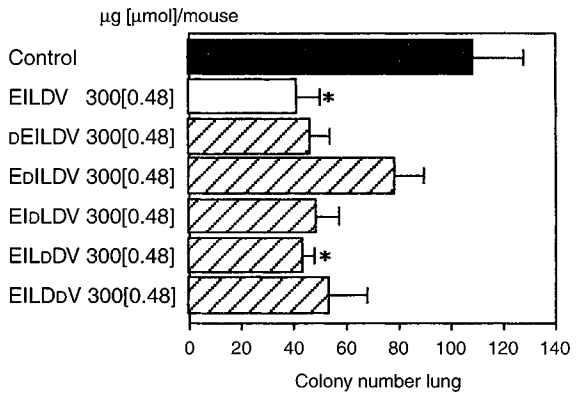

Figure 5. Antimetastatic effect of EILDV derivatives substituted with a D-amino acid on experimental metastasis of B16-BL6 melanoma. B16-BL6 melanoma cells and peptides (300 μg) were injected into the tail vein of syngenic mice. Groups of five male C57BL/6 mice were used. Each value represents mean ± SE. *$p < 0.001$ significantly different from control.

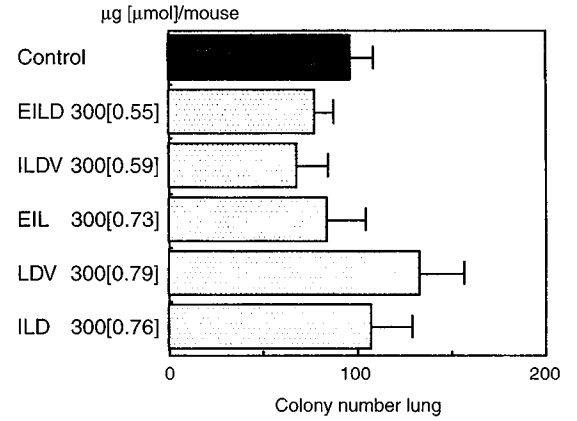

Figure 6. Antimetastatic effect of EILDV-related peptides on experimental metastasis of B16-BL6 melanoma. B16-BL6 melanoma cells and peptides (300 μg) were administrated i.v. to male C57BL/6 mice. The colony number was counted at 2 weeks after tumor inoculation. Groups of five mice were used.

increased their inhibitory effect on experimental metastasis.[22] PEG-modified peptides were more stable in serum than native peptides. As a result, the half-lives of cell-adhesive peptides were effectively prolonged in the circulation and the biological activity was dramatically enhanced *in vivo*. The chemical modification of TNF-α with PEG greatly prolonged its plasma half-life, which augmented its antitumor potency. These results indicate that PEGylation of various peptides and proteins is a useful strategy for markedly increasing their therapeutic efficacy. However, the clinical application of PEGylated proteins and peptides has been limited, because of the following reasons. (i) The steric hindrance that arises when PEG is attached to proteins and peptides inhibits their receptor binding, while protecting them from attack by proteinases. (ii) The increase in molecular weight caused by PEGylation not only increases their plasma half-lives, but also restricts their transport from the blood to the target tissues. Optimal modified proteins and peptides could achieve well-balanced tissue transport, receptor binding and plasma clearance. Many conformational modifications of peptides, including peptide cyclization and amino acid substitution, enhance their biological activities. Substitution with a D-amino acid inhibits the degradaion of peptide analogs by peptidases *in vivo*. Furthermore, the biological activities of peptide analogs substituted with D-amino acids may be enhanced as a consequence of conformational alteration.[23] In this study, we attempted to increase the antimetastatic potency of the FN-related peptide, EILDV, by D-amino acid substitution. We also investigated the minimal core sequence of EILDV required for antimetastatic activity *in vivo*.

We examined the inhibitory effects of D-amino acid substituted EILDV peptides on cell adhesion and migration (Figures 1 and 3). D-Glu- or D-Ile-substituted EILDV markedly inhibited the adhesion and migration of B16-BL6 melanoma cells, but the effects of these two peptides were slightly less potent than that of native EILDV. EILDV substituted for D-Leu or D-Val had weak activity. However, the antimetastatic activities of all EILDV substituted with a D-amino acid were as potent as native EILDV, as shown in Figure 5. We speculated that the stability of EILDV in the blood is enchanced by D-amino substitution, which augments *in vivo* activity. In other words, these apparent discrepanices may be due to a balance between the reduced specific activity of the D-amino acid substituent and resistance to degradation by various peptidases *in vivo*. The EILDV subpeptide analogs, ILDV and ILD, inhibited the cell adhesion of B16-BL6 (Figure 2). However, the inhibitory effect of the smaller overlapping peptide analog, LDV, on cell adhesion was reduced. These results indicated that the minimal essential sequence for cell adhesion is ILD.

Figure 4 shows the inhibitory effect of EILDV subpeptides on cell migration. EILDV-related peptides containing LDV and ILDV inhibited cell migration towards FN. However, the specific activity of several peptide residues lacking the LDV sequence was reduced. These results implied that the critical conformational sequence for receptor recognition is LDV. However, all EILDV subpeptides scarcely affected the metastasis of B16-BL6 melanoma (Figure 6).

We found that the minimal core sequences of EILDV require to inhibit cell adhesion and migration were ILD and LDV, respectively. Thus, ILDV had two distinct functions, i.e. in antiadhesion and antimigration. ILDV was not antimetastatic *in vivo*, whereas EILDV markedly inhibited tumor metastasis. We predicted that Glu plays an important role *in vivo*. However, we have to inquire into these differences between cell adhesion and migration more closely by physicochemical analysis. In addition, the stabilities of peptides *in vivo* must be examined to estimate the antimetastatic potency. These basic studies to solve the relation between the chemical structures of cell adhesion peptides and the biological activity *in vivo* and *in vitro* may enable us to understand the mechanism of tumor metastasis.

The results of this study suggest that D-amino acid substituents of cell-adhesive peptides will provide novel therapeutic drugs to prevent tumor metastasis and that the minimal core sequence of EILDV required for *in vivo* antimetastatic activity is EILDV itself.

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