Inhibition of Lung Metastasis by Synthetic and Recombinant Fragments of Human Fibronectin with Functional Domains

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We have investigated the antimetastatic effect of synthetic or recombinant peptides containing the functional domains of fibronectin on experimental and spontaneous lung metastases of murine tumor cells. CS1 peptide which is present within type III homology connecting segment (HICS) as well as C-274 (cell-binding domain) were able to inhibit experimental lung metastasis when co-injected intravenously (iv) with B16-BL6 melanoma cells, while H-271 (heparin-binding domain) could not. In the spontaneous metastasis model, multiple iv administrations of CS1 or C-274 after surgical excision of primary tumors caused a significant reduction of metastatic colonies in the lung. Both CS1 and C-274 significantly inhibited cell adhesion and migration to fibronectin-coated substrates when added freely in solution. CS1 peptide also inhibited the cell adhesion and migration to laminin-coated substrates, but C-274 did not. H-271 did not have any inhibitory effect on cell adhesion or migration to either of the substrates. Similarly, CS1 inhibited tumor invasion to both Matrigel/fibronectin- and Matrigel/laminin-coated filters, whereas C-274 inhibited the invasion to only Matrigel/fibronectin-coated filter. These results indicate that CS1 peptide of fibronectin, lacking the Arg-Gly-Asp-containing domain, actively inhibits tumor metastases in spontaneous and experimental metastasis models. The use of such a peptide might offer a promising therapeutic approach for combatting or preventing cancer metastasis.

Key words: Recombinant fibronectin fragment — Metastasis — Haptotactic migration — Cell adhesion — Invasion

During the sequential steps of metastasis, metastasizing tumor cells interact with various host cells (platelets, lymphocytes or endothelial cells) and/or extracellular matrix and basement membrane components (fibronectin and laminin).1–4) This encounter may lead to enhancement of the survival, arrest, or invasiveness of tumor cells.2,4–7) Such specific interaction is therefore a fundamental event in the metastatic process.

DNA technology has resulted in the identification of the primary structures of some cell adhesion proteins such as fibronectin,8) vitronectin9) and laminin,10,11) and the receptors for some adhesive molecules on the cell surface. A common and characteristic Arg-Gly-Asp (RGD) core sequence in cell-binding domain of fibronectin and other related adhesion molecules has been shown to contribute to the cell functions including adhesion, spreading and migration of cells.12–14) Several studies have suggested that some synthetic peptides based on the adhesion molecules that are present in cell matrices, basement membranes, or plasma can modulate the mechanism involved in the metastasizing function of tumor cells. A proteolytic fragment of laminin has been used to inhibit experimental metastasis of murine melanoma and it has been shown to bind to a high-affinity 70 kDa glycoprotein receptor on the surface of cells.15,16) Humphries et al.17,18) have shown that treatment of tumor cells ex vivo with GRGDS peptide, which is present in the cell-binding domain of fibronectin, was able to inhibit experimental metastasis of a murine melanoma. We have recently reported that poly(RGD), which consists of repeated RGD sequences, inhibited experimental and spontaneous lung metastases of tumor cells, as well as cell-adhesive properties, more effectively than RGD-containing oligopeptides.19–21) On the other hand, McCarthy et al.16) have shown that the ex vivo pretreatment of tumor cells with a purified 33-kDa heparin-binding fragment of fibronectin, which promotes tumor cell adhesion by an RGDS-independent mechanism,22) effectively inhibited experimental pulmonary metastases of melanoma or fibrosarcoma. The CS1 peptide, which is present within a 33-kDa fibronectin fragment22) and lies between the carboxyl-terminal heparin- and fibrin-binding domains, has been shown to promote cell adhesion and spreading,23–26) but its inhibitory effect on tumor metastasis has not previously been examined.

In the present study, we examined the effect of synthetic and recombinant human fibronectin fragments containing functional domains on the lung metastases of
murine melanoma cells, and studied their biological characteristics in relation to the metastatic cascade in order to gain insight into the action of the polypeptides.

MATERIALS AND METHODS

Recombinant fibronectin polypeptides and other reagents

We prepared two kinds of recombinant fibronectin fragment (C-274 and H-271) by expressing human fibronectin cDNA in *E. coli*, using an expression vector pUC118N/119N described by Maki et al.\(^\text{26}\) C-274 and H-271 correspond to the cell- and heparin-binding domains of fibronectin, respectively (Fig. 1). Two plasmids, pLF5 and pLF2435, were used as a source of the cDNA.\(^\text{27}\) The cell-binding polypeptide C-274 was expressed through a recombinant plasmid pTF7221 which had been constructed mainly from pLF5 and pUC119N. The plasmid pTF7221 was derived from pTF7121, which expresses a cell-binding polypeptide C-279, with five additional amino acids at the carboxy-terminal of C-274. The heparin-binding polypeptide H-271 was expressed by used of a recombinant plasmid pHDtol; this had been constructed from pLF2435 and pUC118N. Detailed accounts for these constructions and expressions will be presented elsewhere (Kimizuka et al., manuscript in preparation). The recombinant fragment C-274 expressed in *E. coli* was purified from the cell extract by DEAE ion exchange chromatography, followed by gel filtration. H-271 was purified by CM ion exchange chromatography, followed by affinity chromatography using heparin as a ligand. The polypeptides thus prepared were analyzed on a 15% SDS-polyacrylamide gel under reducing conditions and visualized by CBB staining to verify their purity. CS1 peptide (DELPQLVTLPNLHGPILDVPS) which is present within type III connecting segment (IIICS) of human fibronectin,\(^\text{23, 24}\) was synthesized at Takara Shuzo Co., Ltd. using an LKB Biolynx 4170 peptide synthesizer with solid-phase Fmoc chemistry. The peptide was purified by preparative reverse-phase HPLC on a C-18 column with a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. The amino-terminal sequence was checked with an automated peptide sequencer 477A (Applied Biosystems Inc., Foster City, CA). These polypeptides were dissolved in Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline (PBS) before use. Purified human fibronectin was purchased from Biomedical Technologies Inc., MA. Basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) and rabbit anti mouse laminin IgG were obtained from Collaborative Research Inc., MA. All the reagents and media in this study were endotoxin-free (<0.1 ng/ml), as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co. Ltd., Tokyo).

Animals Specific pathogen-free mice of C57BL/6 strain, 8–13 weeks old, were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. Mice were maintained in the Laboratory of Animal Experiment, Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. All mice used in this study were sex-matched.

Cells Highly metastatic B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion,\(^\text{28}\) were kindly provided by Dr. I. J. Fidler, M.D. Anderson

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Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open, dotted and closed bars. The cell-binding polypeptide C-274 covers three units of type III homology in a cell-binding domain. The heparin-binding polypeptide H-271 covers the complete region of a heparin-binding domain. CS1 peptide is present within the IIICS region. The boxes at the top represent the locations of the type I, II and III homology repeats. The vertical arrows indicate the GRGDS, CS1, CS5,\(^\text{23, 25}\) and peptide I and II sites.\(^\text{29}\) ED-A and ED-B indicate that extra domains arise from alternative splicing, respectively.
Cancer Center, Houston, TX. B16-BL6 cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, non-essential amino acids and l-glutamine.

**Microassay for cell adhesion** The cell attachment assay was carried out by the method described.

10 B16-BL6 melanoma cells in an exponential growth phase were incubated for 24 h in MEM containing 5% FBS supplemented with 0.3 μCi/ml [125I]iododeoxyuridine ([125I]IUdR) (specific activity, 200 mCi/mmol, New England Nuclear, Boston, MA). The cells were washed twice in warm PBS to remove unbound radiolables, harvested by adding 0.02% EDTA for 1 min at 37°C, and resuspended in cold serum-free MEM to form a single-cell suspension. [125I]IUdR-labeled tumor cells (2 × 10^6) in a volume of 0.05 ml/well were added to microculture wells precoated with fibronectin or laminin. The cultures were incubated at 37°C for 30 min and then the wells were washed four times with PBS to remove unattached cells. The remaining substrate-bound tumor cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed on cotton swabs and monitored for radioactivity by gamma counting. The binding capacity (No. of cells bound/substrate) was expressed as follows;

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\text{Binding capacity} = \frac{\text{cpm of targets bound to substrate}}{\text{cpm of total tumor cells added}} \times \text{total number of tumor cells added}
\]

**Haptotactic migration assay** Tumor cell migration along a gradient of substrate-bound fibronectin or laminin was assayed in a Transwell cell culture chamber (Costar No. 3422, Cambridge, MA) according to the methods reported by McCarthy et al. with some modifications.

Polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μm pore size (Nucleopore, Pleasanton, CA) were precoated with either 5 μg of fibronectin or laminin in a volume of 50 μl on their lower surfaces, and dried overnight at room temperature. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 1 mM EDTA in PBS, washed three times with serum-free MEM, and resuspended to a final concentration of 2 × 10^6/ml in MEM with 0.1% bovine serum albumin (BSA). Cell suspensions (100 μl) with or without agents were added to the upper compartment, and incubated for the appropriate number of hours at 37°C in a 5% CO₂ atmosphere. The filters were fixed with methanol, and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping them with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope in 5 predetermined fields at a magnification of 400, and each assay was performed in triplicate.

**Invasion assay** The invasive activity of tumor cells was assayed according to the method reported by Albini et al. with some modifications. Briefly, the lower surface of the filters was precoated with fibronectin or laminin, as described above. The Matrigel was diluted to 100 μg/ml with cold PBS, applied to the upper surfaces of the filters (5 μg/filter), and dried at room temperature under a hood. The filters thus prepared were designated Matrigel/fibronectin- or Matrigel/laminin-coated filters, respectively. The subsequent procedures were the same as those of the haptotactic migration assay.

**Experimental and spontaneous lung metastases** C57BL/6 mice were given iv injections (0.2 ml/mouse) of B16-BL6 melanoma (3 × 10^6) admixed with various concentrations of the recombinant fragments in PBS. Fourteen days after the inoculation of tumors, the mice were killed and the number of lung tumor colonies was recorded (experimental lung metastasis). In a spontaneous pulmonary metastasis assay, mice were given sc injections of B16-BL6 melanoma cells (5 × 10^5) into the right hind footpad. The primary tumor were surgically removed on day 21 after tumor inoculation. The recombinant polypeptides were administered iv on various days before or after the amputation. Mice were killed 14 days after the amputation. The lungs were fixed in Bouin's solution and the number of lung tumor colonies was counted under a dissecting microscope.

**Statistical analysis** The statistical significance of the differences between groups was determined by applying Student's two-tailed t test.

**RESULTS**

**Inhibition of experimental lung metastasis by synthetic and recombinant domain polypeptides** We investigated the effect of domain polypeptides on experimental lung metastasis caused by intravenous co-injection with B16-BL6 melanoma cells (Table I). Increasing doses of CS1 or C-274 led to significant reductions in the number of lung tumor nodules (P<0.001). In contrast, H-271 achieved no reduction of lung tumor colonies at any of the doses used in this study.

**Inhibition of spontaneous lung metastases by systemic administration of synthetic and recombinant polypeptides** We first examined the effect on spontaneous lung metastasis by multiple systemic administrations of C-274, which contains the cell-binding RGD sequence of fibronectin (Expt. I of Table II). Seven or five intermittent iv injections of 100 μg of C-274 before or after surgical excision of primary tumors achieved a statistically significant reduction of lung tumor colonies, while three intermittent treatment had no effect. In this exper-
Table I. Effect of Recombinant Fibronectin Fragments on Experimental Lung Metastases by Intravenous Injection of B16-BL6 Melanoma Cells

| Administered iv with | Dose (μg/mouse) | No. of metastases on day 14 mean ± SD (range) |
|----------------------|-----------------|---------------------------------------------|
|                      |                 | Expt. I                                      | Expt. II                                   |
| Untreated            | —               | 45 ± 10 (32–55)                              | 87 ± 11 (72–98)                            |
| C-274                | 40              | 30 ± 6 (26–39)                               |                                           |
|                      | 100             |                                             |                                           |
|                      | 200             | 42 ± 10 (25–51)                              | 90 ± 20 (69–113)                           |
|                      | 500             |                                             |                                           |
|                      | 1000            | 13 ± 16 (3–32)*                              | 28 ± 14 (14–48)**                          |
| H-271                | 40              | 51 ± 6 (44–55)                               |                                           |
|                      | 100             | 32 ± 14 (19–48)                              | 66 ± 20 (44–90)                            |
|                      | 200             | 34 ± 4 (29–40)                               | 105 ± 16 (81–124)                          |
|                      | 500             |                                             |                                           |
|                      | 1000            |                                             |                                           |
| CS1                  | 40              | 30 ± 6 (25–40)                               |                                           |
|                      | 200             | 12 ± 6 (6–21)**                              | 23 ± 2 (22–25)**                           |
|                      | 1000            | 8 ± 6 (4–18)**                               |                                           |

Five C57BL/6 mice per group were inoculated iv with B16-BL6 cells (3 × 10⁶) admixed with or without recombinant fragments of fibronectin. Mice were killed 2 weeks after tumor inoculation and tumor colonies in the lungs were counted.

*; P<0.02, **; P<0.001

Table II. Therapeutic Effect of Synthetic and Recombinant Domain Polypeptides of Fibronectin on Spontaneous Lung Metastases by Intrafootpad Injection of B16-BL6 Melanoma Cells

| Administered iv with | Dose (μg/mouse) | Primary tumor size on day 21 (mm ± SD) | No. of lung metastases on day 35 mean ± SD (range) | P*      |
|----------------------|-----------------|---------------------------------------|---------------------------------------------------|---------|
|                      |                 |                                       |                                                   |         |
| Expt. I              |                 |                                       |                                                   |         |
| Untreated            | —               | 10 ± 3                                | 65 ± 17 (41–90)                                   |         |
| C-274                | Days 7, 9, 11, 13, 15, 17, 19 | 100 × 7                              | 11 ± 3                                            | 21 ± 11 (13–37) | < 0.01  |
|                      | Days 7, 10, 13, 16, 19 | 100 × 5                              | 10 ± 2                                            | 36 ± 10 (23–44) | < 0.02  |
|                      | Days 7, 13, 19   | 100 × 3                              | 10 ± 3                                            | 74 ± 23 (43–93) |         |
| Days 22, 24, 26, 28, 30, 32, 34 | 100 × 7          | 10 ± 3                              | 21 ± 13 (8–38)                                   | < 0.01  |
|                      | Days 22, 25, 28, 31, 34 | 100 × 5                              | 10 ± 2                                            | 29 ± 2 (26–31) | < 0.01  |
|                      | Days 22, 28, 34  | 100 × 3                              | 10 ± 2                                            | 57 ± 29 (31–98) |         |
| Expt. II             |                 |                                       |                                                   |         |
| Untreated            | —               | 10 ± 2                                | 86 ± 14 (70–101)                                  |         |
| C-274                | Days 22, 24, 26, 28, 30, 32, 34 | 50 × 7                              | 10 ± 3                                            | 44 ± 14 (23–63) | < 0.01  |
|                      | Days 22, 24, 26, 30, 32, 34 | 100 × 7                              | 10 ± 2                                            | 31 ± 15 (12–49) | < 0.001 |
| H-271                | Days 22, 24, 26, 28, 30, 32, 34 | 100 × 7                              | 11 ± 2                                            | 89 ± 24 (56–122) |         |
| CS1                  | Days 22, 24, 26, 28, 30, 32, 34 | 100 × 7                              | 10 ± 3                                            | 28 ± 15 (4–43) | < 0.001 |

Five C57BL/6 mice per group were given fibronectin polypeptides iv on the indicated days after tumor inoculation. Primary tumors were surgically removed on day 21 and mice were killed 2 weeks after tumor excision.

*a*; Compared with the untreated control by Student's two-tailed t test.
Effect of Polypeptides on the Growth of B16-BL6 Melanoma Cells

Table III. Effect of Synthetic and Recombinant Domain Polypeptides on the Growth of B16-BL6 Melanoma Cells in vitro

| Treatment | Concentration (µg/ml) | Incorporation of [3H]thymidine into the cells (cpm±SD) |
|-----------|-----------------------|-----------------------------------------------------|
| Untreated (medium) | —                     | 40637±2629                                          |
| C-274     | 5                     | 43859±2870                                          |
|           | 50                    | 37689±8404                                          |
|           | 500                   | 45075±3578                                          |
| H-271     | 5                     | 41100±3995                                          |
|           | 50                    | 41886±3838                                          |
|           | 500                   | 46198±1249                                          |
| CS1       | 5                     | 38117±10895                                         |
|           | 50                    | 42030±2710                                          |
|           | 500                   | 43268±3426                                          |
| TNF-α (2×10⁴ U/ml) |                       | 22110±5063                                          |

B16-BL6 cells (5×10⁵) were incubated with MEM medium containing 5% FBS, domain polypeptides, or recombinant TNF-α for 3 days at 37°C. The cultures were pulsed with 0.5 µCi of [3H]thymidine for the last 4 h before the termination.

Effect of the polypeptides on the migration of tumor cells

Table IV. Effect of Recombinant Fragments of Fibronectin on Tumor Cell Invasion to Matrigel/fibronectin- or Matrigel/laminin-coated Filters

| Treatment | Dose (µg/ml) | No. of invaded cells (mean±SD) |
|-----------|-------------|--------------------------------|
|           |             | Fibronectin | Laminin                    |
| None      |             | 44±3        | 59±6                        |
| C-274     | 100         | 26±4**      | 58±6                        |
|           | 500         | 10±2**      | 61±14                       |
| H-271     | 500         | 49±13       | 43±6*                       |
| CS1       | 100         | 21±2**      | 30±5**                      |
| Rabbit anti mouse laminin IgG | 10         | 32±5**       |

Filters were precoated with 5 µg of fibronectin or laminin on their lower surfaces, and with Matrigel (5 µg) on their upper surfaces. B16-BL6 melanoma cells (2×10⁴/well) in 0.1% BSA medium were seeded with or without fragments of fibronectin into the upper compartment of a Transwell cell culture chamber. Anti mouse laminin IgG was added to the lower compartment of the chamber. After an 11-h incubation, the invaded cells on the lower surfaces were counted visually.

*; P<0.01, **; P<0.001

iv administration of the polypeptide before the amputation did not affect the primary tumor size (growth) at the time of amputation (on day 21). We next examined the effect on spontaneous lung metastasis of B16-BL6 by seven systemic administrations of the domain polypeptides. The polypeptides were administered iv into the lateral tail vein of mice after the amputation of the primary tumors. As shown in Expt. II of Table II, iv administrations of C-274 after surgical excision of the primary tumors resulted in a reduction of lung tumor colonization. H-271, however, did not affect the inhibition of lung metastasis. We also examined the direct effect of synthetic and recombinant polypeptides on the growth of B16-BL6 melanoma cells in vitro. Table III shows that the incubation of tumor cells with various concentrations of polypeptides did not affect the incorporation of [3H]thymidine into tumor cells. Recombinant human tumor necrosis factor (TNF-α) as a positive control potently inhibited the cell growth in vitro.

Effect of the polypeptides on the invasion of tumor cells

Tumor cell invasion into extracellular matrices and basement membranes is a crucial step in the complex multistage process of metastasis. We therefore examined the effect of these antimetastatic polypeptides on tumor cell invasion of Matrigel (reconstituted basement membrane components) (Table IV). The invasion of tumor cells through the Matrigel/fibronectin-coated filters was significantly inhibited by the addition of 100 or 500 µg/ml of CS1 or C-274 into the upper compartment of the chamber, whereas H-271 showed no anti-invasive activity. In contrast, the invasion through the Matrigel/laminin-coated filters was significantly inhibited by C-274 peptide or rabbit anti mouse laminin IgG (10 µg/ml), but not by C-274 or H-271.

Effect of the polypeptides on tumor cell adhesion

Since metastatic cells presumably interact with extracellular matrix components (cell adhesion proteins) during the invasive process, we tested the effect of the polypeptides on tumor cell adhesion to fibronectin- or laminin-coated wells. Fibronectin and laminin promoted the adhesion of B16-BL6 melanoma cells when they were immobilized (coated) on the culture dish surface. However, few B16-BL6 cells in serum-free MEM attached themselves to BSA-coated or uncoated plastic substrates. Figure 2 shows that C-274 at various concentrations ranging from 20 to 500 µg/ml inhibited specifically the adhesion of tumor cells to fibronectin substrate but not to the laminin substrate. H-271 was unable to inhibit tumor cell adhesion to either fibronectin or laminin. In contrast, CS1 inhibited tumor cell adhesion to both fibronectin and laminin substrates in a dose-dependent manner.

Effect of the polypeptides on the migration of tumor cells

We also investigated the effect of the domain polypeptides on the haptotactic migration of tumor cells to filters precoated on their lower surfaces with 5 µg of...
Fig. 2. Inhibition of tumor cell adhesion to fibronectin or laminin by the domain polypeptides. $^{32}$P-labeled B16-BL6 cells ($2 \times 10^4$) were added to wells precoated with 1 µg of fibronectin or 1 µg of laminin in the presence or absence of C-274, H-271 or CS1 peptides. After a 30-min incubation, nonadherent cells were washed away and the adherent cells were counted. Percent inhibition of cell adhesion was quantitated as: $1 - \frac{\text{the number of cells bound in the presence of inhibitor}}{\text{the number of cells bound in the absence of the inhibitor}} \times 100$.

Table V. Inhibition of Haptotactic Migration of B16-BL6 Melanoma to Fibronectin- or Laminin-coated Filters by Fibronectin Fragments

| Addition          | Dose (µg/ml) | No. of migrated cells ± SD | Fibronectin | Laminin |
|-------------------|--------------|----------------------------|-------------|---------|
| None              | —            | 53 ± 5                     | 97 ± 5      |
| C-274             | 20           | 35 ± 6**                   | 96 ± 6      |
|                   | 100          | 29 ± 3**                   |             |
|                   | 500          | 22 ± 4**                   |             |
| H-271             | 20           | 54 ± 10                    | 96 ± 10     |
|                   | 100          | 48 ± 5                     |             |
|                   | 500          | 45 ± 10                    |             |
| CS1               | 20           | 42 ± 4*                    | 96 ± 6      |
|                   | 100          | 30 ± 4**                   | 75 ± 6**    |
|                   | 500          | 24 ± 4**                   | 60 ± 6**    |
| Rabbit anti mouse | 10           | 30 ± 3**                   |             |
| laminin IgG       |              |                            |             |

Fibronectin or laminin without the matrix barrier (Matrigel) on the upper surface (Table V). B16-BL6 cells were incubated for 4 h at 37°C with polypeptides in the upper compartment of a Transwell cell culture chamber. CS1 or C-274 inhibited tumor cell migration to the fibronectin-coated filter in a dose-dependent manner, whereas H-271 did not. CS1 or rabbit anti mouse laminin IgG inhibited cell migration to the laminin-coated filter, but C-274 and H-271 did not.

**DISCUSSION**

We have attempted to elucidate the regulatory mechanisms involved in cell functions such as adhesion and motility that are important during the metastatic process. Tumor cell adhesion to components of the extracellular matrix, in particular fibronectin, is an important aspect of several steps of the metastatic process. Previous studies have utilized cell-adhesion-promoting fragments of laminin or heparin-binding fibronectin fragments or the synthetic RGDS peptide of fibronectin to inhibit experimental metastasis of tumor cells in mice.\(^\text{xiii}\) Our current studies extend those earlier studies, clearly demonstrating that synthetic and recombinant polypeptides of the functional domains of fibronectin can be used to inhibit the experimental and spontaneous lung metastases of tumor cells in mice and the penetration of tumor cells through reconstituted basement membrane (Matrigel) in vitro.

CS1 peptide and C-274 (containing RGD sequence) were able to inhibit the experimental and spontaneous lung metastases of melanoma cells, but did not affect the primary tumor size at the time of amputation. We also observed that incubation of B16-BL6 melanoma cells with these polypeptides for 3 days did not affect the growth of tumor cells in vitro (Table III). Therefore, the inhibitory effect of these polypeptides on lung tumor metastasis may not be due to their direct cytotoxicity against tumor cells.

Although some studies of proteolytic fragments of laminin or fibronectin, or synthetic RGD-containing peptides of fibronectin have been performed with an experimental metastasis model, it is of particular interest that, in our experiments with a spontaneous metastasis model, CS1 and C-274 showed therapeutic potential against tumor metastasis following systemic administration.

The penetration of tumor cells into basement membranes involves such distinct events as the attachment of tumor cells, the secretion by the tumor cells of enzymes that cause the degradation of the adjacent basement membrane, and the migration of the cells into the target tissue. C-274 (containing the cell-binding RGD sequence) specifically inhibited tumor cell invasion through
a Matrigel/fibronectin filter, and adhesion and migration to fibronectin substrates, but not to Matrigel/laminin or laminin substrates. H-271, however, did not have any such effects. On the other hand, CS1 peptide was able to inhibit the adhesion, migration and invasion of tumor cells to both fibronectin- and laminin-coated substrates. These results suggest that the inhibitory effects of CS1 peptide depend on interference with the interaction between tumor cells and extracellular matrix molecules by an RGD-independent mechanism, while C-274 (i.e. possibly the RGD sequences in the molecule) may inhibit specifically the adhesion, migration and invasion through interference with the RGD/integrin interaction in fibronectin-mediated cell behavior. Further detailed study will be needed to determine the precise mechanism by which CS1 inhibits tumor metastasis and invasion.

The mechanism of the inhibition of tumor metastasis by the administration of C-274 or CS1 is not well understood. Polypeptide-mediated inhibition of tumor metastasis may be related to interference with cellular adhesive interactions in the multistep metastatic process, including tumor invasion into the surrounding tissues during the intravasation and extravasation steps or at a target organ. Some possibilities include the acceleration of the release of arrested tumor cells from the lung and the inhibition of their lodgment by the polypeptides. Further study will be needed to examine the optimum administration timings of CS1 and C-271 and the detailed mechanism of the inhibitory effect.

On the other hand, H-271 polypeptide containing a heparin-binding domain II was unable to inhibit lung metastasis, although McCarthy et al. have reported that a purified 33 kDa heparin-binding fragment of fibronectin, which includes H-271 and CS1 polypeptides, inhibited experimental metastases. The difference in anti-metastatic effects between H-271 and the proteolytic 33 kDa fragment of fibronectin may arise because the 33 kDa fragment contained a partial sequence of an IIICS insert of fibronectin, i.e. a CS1 portion that inhibited tumor metastasis as shown in Tables I and II, whereas H-271 did not contain the sequence.

Despite the importance of RGD sequences in the cell-binding domain of fibronectin, CS1 in the IIICS insert of fibronectin deserves particular attention regarding its possible contributions to fibronectin functions. Humphries et al. have reported that CS1 peptide contains a cell-adhesion-promoting activity for different cell types. Adhesion of T or B lymphocytes as well as melanoma cells to this region of fibronectin has been demonstrated to involve an $\alpha_4\beta_1$ integrin receptor which is RGD-independent. We recently observed that CS1 or H-271 inhibited liver metastasis of L5178Y-ML25 lymphoma cells in CDF1 mice (manuscript in preparation). This may imply that multiple receptors including $\alpha_4\beta_1$, $\alpha_5\beta_1$ or $\alpha_5\beta_1$ integrins interact with fibronectin, apparently at different sites in the molecule. Interestingly, our results showed that CS1 inhibited tumor cell invasion, adhesion and migration not only to fibronectin but also to laminin in an RGD-independent manner. This suggests that CS1 peptide derived from fibronectin is able to modulate the interaction between tumor cells (presumably surface receptor) and laminin as well as fibronectin. We are now examining in detail the regulatory mechanisms that allow the polypeptides to inhibit tumor metastasis and invasion.

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