Jpn. J. Cancer Res. 81, 1003–1011, October 1990

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

Ikuo Saiki,[1,3] Jun Murata,[1] Takashi Makabe,[1] Yoshihiro Matsumoto,[1] Yoichi Ohdate,[2] Yasutoshi Kawase,[2] Yuki Taguchi,[2] Tomoko Shimojo,[2] Fusao Kimizuka,[2] Ikunoshin Kato[2] and Ichiro Azuma[1]

[1]*Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060 and* [2]*Biotechnology Research Laboratories, Takara Shuzo Co. Ltd., Seta 3-4-1, Otsu 520-01*

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 (IIICS) 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] vitronectin[9] 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 fragment[22] and lies between the carboxyl-terminal heparin- and fibrin-binding domains, has been shown to promote cell adhesion and spreading,[23-25] 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

[3] To whom correspondence should be addressed.

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.*[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.[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 carboxyl-terminus of C-274. The heparin-binding polypeptide H-271 was expressed by used of a recombinant plasmid pHD101; 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 (DELPQLVTLPNLHGPEILDVPST) which is present within type III connecting segment (IIICS) of human fibronectin,[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,[28] were kindly provided by Dr. I. J. Fidler, M.D. Anderson

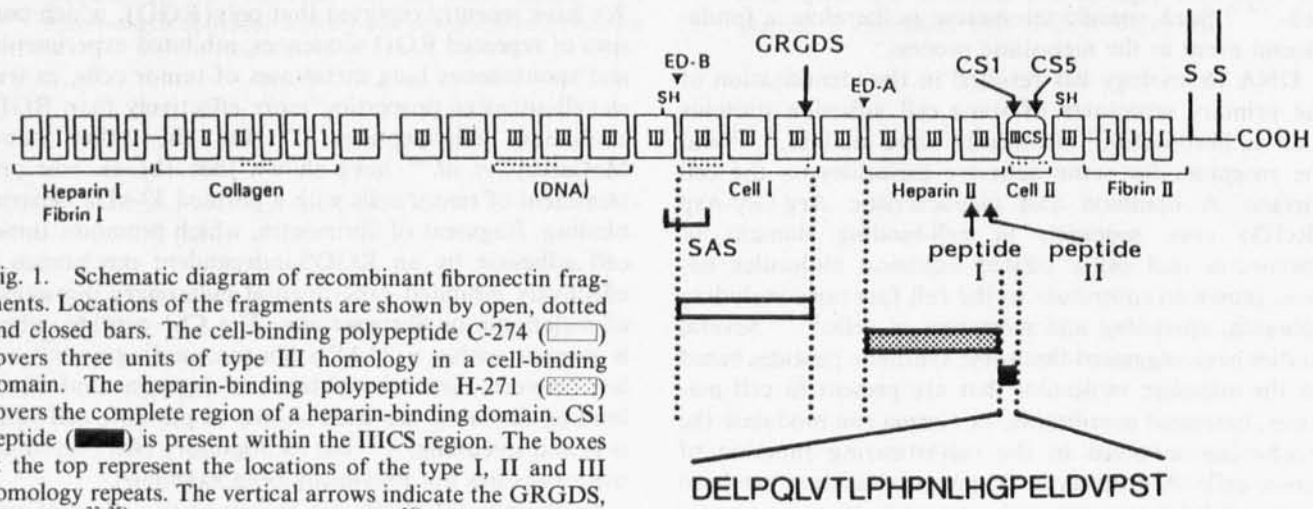

Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open, dotted and closed bars. The cell-binding polypeptide C-274 (▭) covers three units of type III homology in a cell-binding domain. The heparin-binding polypeptide H-271 (▩) covers the complete 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,[23-25] and peptide I and II sites.[36] 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.[19] 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 [^{125}I]iododeoxyuridine ([^{125}I]IUdR) (specific activity, 200 mCi/mmol, New England Nuclear, Boston, MA). The cells were washed twice in warm PBS to remove unbound radiolabels, 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. [^{125}I]IUdR-labeled tumor cells (2×10^4) 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;

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

Haptotactic migration assay Tumor cell migration along a gradient of substratum-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.*,[29] with some modifications.[30] 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 m*M* 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% CO_2 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.*,[31] with some modifications.[30] 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 (3×10^4) 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		90±20 (69–113)
	200	42±10 (25–51)	
	500		59±19 (41–85)*
	1000	13±16 (3–32)*	28±14 (14–48)**
H-271	40	51±6 (44–55)	
	100		76±13 (60–92)
	200	32±14 (19–48)	
	500		66±20 (44–90)
	1000	34±4 (29–40)	105±16 (81–124)
CS1	40	30±6 (25–40)	
	200	12±6 (6–21)**	
	1000	8±6 (4–18)**	23±2 (22–25)**

Five C57BL/6 mice per group were inoculated iv with B16-BL6 cells (3×10^4) 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)	$P^{a)}$
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
		7, 10, 13, 16, 19	100×5	10±2	36±10 (23–44)	<0.02
		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
		22, 25, 28, 31, 34	100×5	10±2	29±2 (26–31)	<0.01
		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
			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.

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 [^{3}H]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 (2×10^4 U/ml)		22110±5063

B16-BL6 cells (5×10^3) 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 [^{3}H]thymidine for the last 4 h before the termination.

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**	
	500	10±2**	58±4
H-271	500	49±13	61±14
CS1	100	32±6*	43±6*
	500	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 (2×10^5/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.
*; $P<0.01$, **; $P<0.001$

iment, 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 100 μg of CS1 as well as 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 [^{3}H]-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.[1-5, 32, 33] 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 CS1 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,[2, 4-7] 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

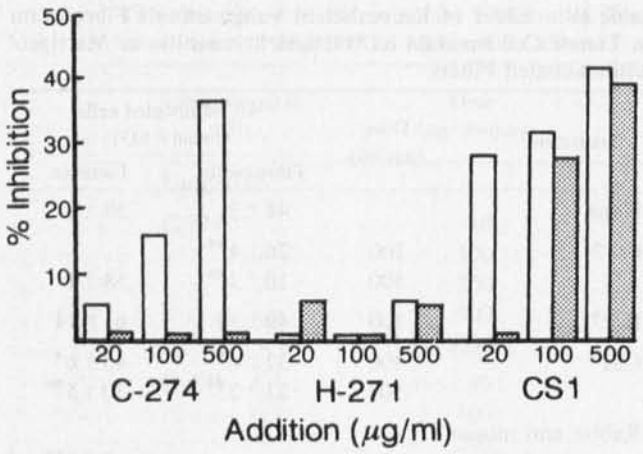

Fig. 2. Inhibition of tumor cell adhesion to fibronectin or laminin by the domain polypeptides. ^{125}I-labeled B16-BL6 cells (2×10^4) were added to wells precoated with 1 μg of fibronectin (▭) or 1 μg of laminin (▨) in the presence or absence of C-274, H-271 or CS1 peptides. After a 30-min incubation, nonadherent cells were washed away and the adherent cells were counted. Percent inhibition of cell adhesion was quantitated as: 1 − (the number of cells bound in the presence of inhibitor/the number of cells bound in the absence of the inhibitor) × 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**	
	100	29 ± 3**	
	500	22 ± 4**	93 ± 6
H-271	20	54 ± 10	
	100	48 ± 5	
	500	45 ± 10	96 ± 10
CS-1	20	42 ± 4*	96 ± 6
	100	30 ± 4**	75 ± 6**
	500	24 ± 4**	60 ± 6**
Rabbit anti mouse laminin IgG	10		30 ± 3**

Filters in a Transwell chamber were precoated with 5 μg of fibronectin or laminin on their lower surfaces. B16-BL6 cells (2×10^5) were incubated with the domain polypeptides in the upper compartment or with anti mouse laminin IgG in the lower compartment for 4 h. The migrant cells on the lower surfaces were counted visually.
*; $P<0.01$, **; $P<0.001$.

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.[15-18] 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[15] or fibronectin,[16] or synthetic RGD-containing peptides of fibronectin[17,18] 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 therapeutial 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.*[16,22] 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 antimetastatic 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.*[23,24] 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.[34,35,37] 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 $\alpha_3\beta_1$ 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.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture, for the Comprehensive 10-Year Strategy for Cancer Control from the Japanese Ministry of Health and Walfare and for Developmental Scientific Research (No. 62870023) from the Japanese Ministry of Education, Science and Culture, as well as by grants from the Osaka Foundation for Promotion of Clinical Immunology, the Naito Foundation for Promotion of Science, the Yamanouchi Foundation for Research on Metabolic Disorders, and the CIBA-GEIGY Foundation for the Promotion of Science, and by Grants-in-Aid for Special Project Research from Hokkaido University, Japan. The authors thank Ms. M. Sato for typing the manuscript.

(Received May 12, 1990/Accepted July 25, 1990)

REFERENCES

1) Fidler, I. J. The Ernst, W. Bertner Memorial Award Lecture: the evolution of biological heterogeneity in metastatic neoplasms. *In* "Cancer Invasion and Metastases: Biologic and Therapeutic Aspects," ed. G. L. Nicolson and L. Milas, pp. 5–26 (1984). Raven Press, New York.
2) Nicolson, G. L. Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncofetal expression. *Cancer Res.*, **47**, 1473–1487 (1987).
3) Hart, I. R. "Seed and soil" revisited: mechanisms of site specific metastasis. *Cancer Metastasis Rev.*, **1**, 5–16 (1982).
4) Liotta, L. A., Rao, C. V. and Barsky, S. H. Tumor invasion and the extracellular matrix. *Lab. Invest.*, **49**, 636–649 (1983).
5) Terranova, V. P., Hujanen, E. S. and Martin, G. R. Basement membrane and the invasive activity of metastatic tumor cells. *J. Natl. Cancer Inst.*, **77**, 311–316 (1986).
6) McCarthy, J. B. and Furcht, L. T. Laminin and

fibronectin promote the haptotactic migration of B16 mouse melanoma cells *in vitro*. *J. Cell Biol.*, **98**, 1474–1480 (1984).
7) Karpathlan, S. and Pearlstein, E. Heterogenous mechanism of tumor cell-induced platelet aggregation with possible strategy toward prevention of metastases. *In* "Hemostasis Mechanisms and Metastasis," ed. K. V. Honn and B. F. Sloane, pp. 139–169 (1984). Martinus Nijhoff, Boston.
8) Kornblihtt, A. R., Umezaka, K., Vibe-Pedrsan, K. and Baralle, F. E. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J.*, **4**, 1755–1759 (1985).
9) Suzuki, S., Oldberg, A., Hayman, E. G., Pierschbacher, M. D. and Rouslahti, E. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO J.*, **4**, 2519–2524 (1985).
10) Sasaki, M., Kato, S., Kohno, K., Martin, G. R. and Yamada, Y. Sequence of the cDNA encoding the laminin B1 chain reveals a multidomain protein containing cysteine rich repeats. *Proc. Natl. Acad. Sci. USA*, **84**, 935–938 (1987).
11) Sasaki, M. and Yamada, Y. The laminin B2 chain has a multidomain structure homologous to the B1 chain. *J. Biol. Chem.*, **262**, 17111–17117 (1987).
12) Pierschbacher, M. D. and Ruoslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*, **309**, 30–33 (1982).
13) Ruoslahti, E. and Pierschbacher, M. D. Arg-Gly-Asp: a versatile cell recognition signal. *Cell*, **44**, 517–518 (1986).
14) Graf, J., Iwamoto, Y., Sasaki, M., Martin, G. R., Kleinman, H. K., Robey, F. A. and Yamada, Y. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell*, **48**, 989–996 (1987).
15) Barsky, S. H., Rao, C. N., Williams, J. E. and Liotta, L. A. Laminin molecular domains which alter metastasis in a murine model. *J. Clin. Invest.*, **74**, 843–848 (1984).
16) McCarthy, J. B., Skubitz, A. P. N., Palm, S. L. and Furcht, L. T. Metastasis inhibition of different tumor types by purified laminin fragments and a heparin-binding fragment of fibronectin. *J. Natl. Cancer Inst.*, **80**, 108–116 (1988).
17) Humphries, M. J., Olden, K. and Yamada, K. M. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science*, **233**, 467–470 (1986).
18) Humphries, M. J. Yamada, K. M. and Olden, K. Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells. *J. Clin. Invest.* **81**, 782–790 (1988).
19) Saiki, I., Iida, J., Murata, J., Ogawa, R., Nishi, N., Sugimura, K., Tokura, S. and Azuma, I. Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequence of cell-adhesive molecules. *Cancer Res.*, **49**, 3815–3822 (1989).
20) Saiki, I., Murata, J., Iida, J. Nishi, N., Sugimura, K. and Azuma, I. The inhibition of murine lung metastasis by synthetic polypeptides [poly(arg-gly-asp) and poly(tyr-ile-gly-ser-arg)] with a core sequence of cell adhesion molecules. *Br. J. Cancer*, **59**, 194–197 (1989).
21) Saiki, I., Murata, J., Iida, J., Sakurai, T., Nishi, N., Matsuno, K. and Azuma, I. Antimetastatic effects of synthetic polypeptides containing repeated structures of the cell adhesive Arg-Gly-Asp (RGD) and Tyr-Ile-Gly-Ser-Arg (YIGSR) sequences. *Br. J. Cancer*, **60**, 722–728 (1989).
22) McCarthy, J. B., Hagen, S. T. and Furcht, L. T. Human plasma fibronectin contains multiple adhesion- and motility-promoting domains for metastatic melanoma cells. *J. Cell Biol.*, **102**, 179–188 (1986).
23) Humphries, M. J., Komoriya, A., Akiyama, S. K., Olden, K. and Yamada, K. M. Identification of two distinct regions of the type IIICS connectin that promote cell type-specific adhesion. *J. Biol. Chem.*, **262**, 6886–6892 (1987).
24) Humphries, M. J., Akiyama, S. K., Komoriya, A., Olden, K. and Yamada, K. M. Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. *J. Cell Biol.*, **106**, 1289–1297 (1988).
25) Dufour, S., Duband, J-L., Humphries, M. J., Obara, M., Yamada, K. M. and Thiery, J. P. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple sites on fibronectin molecules. *EMBO J.*, **7**, 2661–2671 (1988).
26) Maki, M., Takano, E., Mori, H., Sato, A., Murachi, T. and Hatanaka, M. All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpeins I and II. *FEBS Lett.*, **223**, 174–180 (1987).
27) Sekiguchi, K., Klos, M. A., Kurachi, K., Yoshitake, S. and Hakomori, S. Human liver fibronectin complementary DNAs: identification of two different messenger RNAs possibly encoding the α and β subunits of plasma fibronectin. *Biochemistry*, **25**, 4936–4941 (1986).
28) Hart, I. R. The selection and characterization of an invasive variant of the B16 melanoma. *Am. J. Pathol.*, **97**, 587–592 (1979).
29) McCarthy, J. B., Palm, S. L. and Furcht, L. T. Migration of haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. *J. Cell Biol.*, **97**, 772–777 (1983).
30) Saiki, I., Murata, J., Watanabe, K., Fujii, H., Abe, F. and Azuma, I. Inhibition of tumor cell invasion by ubenimex (bestatin) *in vitro*. *Jpn. J. Cancer Res.*, **80**, 873–878 (1989).
31) Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M. and McEwan, R. N. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.*, **47**, 3239–3245 (1987).

32) Liotta, L. A., Garbisa, S. and Tryggvason, K. Biochemical mechanisms involved in tumor cell penetration of the basement membrane. *In* "Tumor Invasion and Metastasis," ed. L. A. Liotta and T. R. Hart, pp. 319–334 (1982). The Hague: Martinus Nijhoff, Boston.

33) Nicolson, G. L., Nakajima, M. and Irimura, T. Invasion of vascular endothelium and organ tissue *in vitro* by B16 melanoma variants. *In* "Mechanisms of Metastasis: Potential Therapeutic Implications," ed. K. V. Honn, W. E. Powers and B. F. Sloane, pp. 275–297 (1985). The Hague: Martinus Nijhoff, Boston.

34) Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A. and Carter, W. G. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS1) in plasma fibronectin. *J. Cell. Biol.*, **109**, 1321–1330 (1989).

35) Guan, J-L. and Hynes, R. O. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. *Cell*, **60**, 53–61 (1990).

36) McCarthy, J. B., Chelberg, M. K., Mickelson, D. J. and Furcht, L. T. Localization and chemical synthesis of fibronectin peptides with melanoma adhesion and heparin binding activities. *Biochemistry*, **27**, 1380–1388 (1988).

37) Mould, A. P., Wheldon, L. A., Komoriya, A., Wayner, E. A., Yamada, K. M. and Humphries, M. J. Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha_4\beta_1$. *J. Biol. Chem.*, **265**, 4020–4024 (1990).