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Recombinant Fusion Polypeptide with Cell- and Heparin-binding Domains of Fibronectin Inhibits Liver Metastasis of L5178Y-ML25 Lymphoma Cells

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We have investigated the effect of recombinant polypeptides with cell-binding domain (C-274) or with heparin-binding domain (H-271) and their fusion polypeptide (CH-271) on liver metastasis of murine lymphoid tumor. The polypeptides containing heparin-binding domain, H-271 and CH-271, were able to inhibit liver metastasis when co-injected i.v. with L5178Y-ML25 T-lymphoma cells, while C-274 with cell-binding domain showed much weaker antimetastatic activity. Treatment with H-271 or CH-271 substantially prolonged the survival time of mice injected i.v. with L5178Y-ML25 cells. CH-271, containing cell- and heparin-binding domains, was more antimetastatic than H-271. The reason why CH-271 was more effective in inhibiting liver metastasis than H-271 can not be explained in terms of a difference in the stability in the circulation or in the molecular size of the polypeptide. The polypeptides used in this study did not affect the tumor cell growth or viability *in vitro*. CH-271 was found to be still active in inhibiting liver metastasis even when natural killer cells or macrophages were removed from this system. Furthermore, multiple administrations of CH-271 after tumor implantation effectively inhibited liver metastasis and enhanced the survival rate as compared with H-271, C-274 and untreated control. Thus, the fusion of H-271 with C-274 (i.e. CH-271) augments the antimetastatic property of H-271, possibly through the interaction between tumor cells and the heparin-binding domain of fibronectin.

Key words: Recombinant fibronectin fragment — Metastasis — Lymphoma cell

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). Such an encounter may lead to enhancement of survival, arrest, or invasiveness of tumor cells,[1-3] and is therefore a fundamental event in the metastatic process.

DNA technology has allowed us to identify the primary structures of some cell adhesion proteins such as fibronectin,[4] vitronectin,[5] and laminin,[6,7] and the receptors for some adhesive molecules on the cell surface.[8] 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 cell functions including adhesion, spreading and migration.[9,10] Several studies have suggested that some synthetic peptides corresponding to fragments of 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 or synthetic fragment of laminin has been used to inhibit experimental metastasis.[11-14] Humphries *et al.*[15,16] 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 lung metastasis of murine melanoma. We have recently reported that poly(RGD), which consists of the repeated RGD sequence, inhibited experimental and spontaneous lung metastases of murine melanoma cells more effectively than RGD-containing oligopeptides, as well as showing anti cell-adhesive properties.[17-21]

On the other hand, McCarthy *et al.*[14] 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. More recently, we demonstrated that CS1 peptide, which is present within type III homology connecting segment (IIICS) of 33-kDa heparin-binding fragment of fibronectin and promotes melanoma cell adhesion,[23] is active in inhibiting lung metastasis of murine melanoma in spontaneous and experimental metastasis models.[24]

Recent studies showed that 33-kDa heparin-binding fragment of fibronectin promoted the adhesion of lymphocytes or lymphoid tumors as well as melanoma.[25-28] Tumor lines reported to produce hepatic metastases preferentially are mostly lymphoid tumors such as RAW117-

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H10, MDAY-D2 and L5178Y-ML.[29,30] However, the inhibitory effect on liver metastasis of murine lymphoid tumors by peptides or fragments of fibronectin remains undefined. In the present study, we examined the effect of recombinant fibronectin fragments containing heparin- or cell-binding domains and their chimeric polypeptide on liver metastasis of murine L5178Y-ML25 lymphoid tumor.

MATERIALS AND METHODS

Animals Specific-pathogen-free CDF_1 mice (BALB/c× DBA/2) 8–13 weeks old, were purchased from the 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 Liver metastatic L5178Y-ML25 T lymphoma cells, obtained from L5178Y parent cells by *in vivo* selection for invasion,[29] were kindly provided by Dr. A. Okura, Banyu Pharmaceutical Co. Ltd., Tokyo. L5178Y-ML25 cells were maintained in RPMI-1640 supplemented with 7.5% fetal bovine serum (FBS) and L-glutamine.

Recombinant fibronectin fragments and other reagents We prepared three kinds of recombinant fibronectin fragments (C-274, H-271 and CH-271) by expressing human fibronectin cDNA in *E. coli,* using an expression vector pUC118N/119N first described by Maki *et al.*[31] C-274 and H-271 correspond to cell- and heparin-binding domains of fibronectin, respectively, while another polypeptide, CH-271, is a fusion protein with both a cell- and a heparin-binding domain (Fig. 1). Two plasmids, pLF5 and pLF2435, were used as a source of cDNA.[32] 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 use of a recombinant plasmid pHD101; this had been constructed from pLF2435 and pUC118N. The fusion protein CH-271 was expressed by use of a recombinant plasmid pCH-101; this had been constructed from pHD101 and pTF-7121. Detailed accounts of these constructions and expressions will be given elsewhere.[33] The recombinant fragment C-274 expressed in *E. coli* was purified from the cell extract by DEAE ion exchange chromatography, followed by SP ion exchange chromatography. Fragments H-271 and CH-271 were purified by CM ion exchange chromatography, followed by affinity chromatography with heparin as a ligand. The purity of these polypeptides was verified by SDS-polyacrylamide gel electrophoresis. The amino-terminal sequence was checked with an automated peptide sequencer, model 477A (Applied Biosystems Inc., Foster City, CA). The carboxyl-terminal amino acid was also determined by use of carboxypeptidase P (Takara Shuzo Co. Ltd., Kyoto). Poly (RGD), which consists of a repeated Arg-Gly-Asp (RGD) sequence, was prepared by the synthesis of the monomer RGD peptide by the conventional method followed by polymerization with diphenylphosphoryl azide, as described previously.[17–19] Poly (RGD) was estimated to have an approximate average molecular weight of 10,000, as assessed by viscometric measurements, SDS-polyacrylamide gel electrophoresis and gel permeation chromatography. These polypeptides were

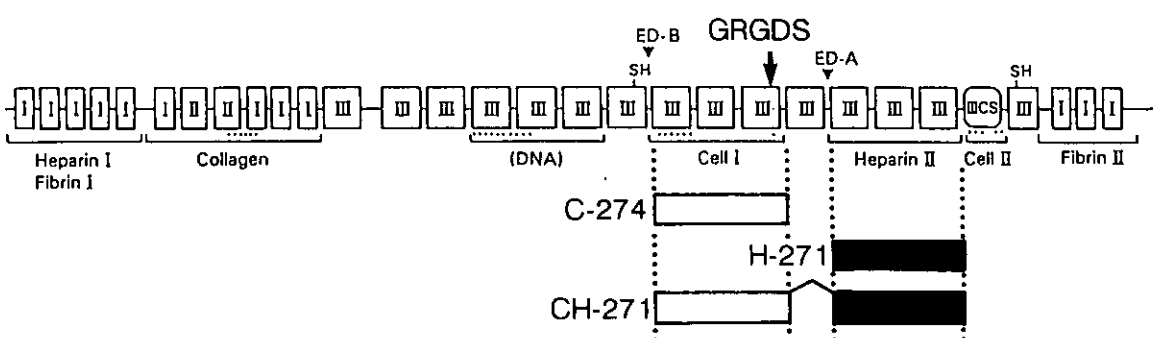

Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open and closed bars. The cell-binding polypeptide C-274 (Pro1239-Asp1512; the sequence is numbered according to the system of Kornblihtt[4]) covers three units of type III homology at the cell-binding domain. The heparin-binding polypeptide H-271 (Ala1690-Thr1960) covers the complete region of the heparin-binding domain. The fusion polypeptide CH-271 (Pro1239-Ser1515-Met-Ala1690-Thr1960) contains both the cell- and heparin-binding domains. The boxes at the top represent the locations of the type I, II and III homology repeats. The vertical arrow indicates the GRGDS site. ED-A and ED-B indicate that extra domains arise from alternative splicing.

dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) before use. All the reagents and media in this study were endotoxin-free (< 1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co. Ltd., Tokyo).

Assay for liver metastasis of lymphoid tumors CDF_1 mice were given i.v. injection of L5178Y-ML25 lymphoma (4×10^4) with or without recombinant fragments of fibronectin in PBS. Fourteen to seventeen days later, the mice were killed and the weights of liver and spleen were recorded to evaluate tumor metastasis as previously described in detail.[29] The survival time of the animals given i.v. injection of tumor cells with or without recombinant fibronectin fragments was also determined by allowing the animals to live until they succumbed naturally from the tumor burden. Animals were autopsied at the time of death to verify the presence of the tumor in the liver. The % survivors was calculated as a function of time.

Labeling of recombinant fibronectin polypeptides CH-271 and H-271 polypeptides were iodinated by using Bolton-Hunter reagent according to the conventional procedure. Briefly, the polypeptide (1 mg) was dissolved in 100 μl of PBS, and added to 1 mCi of Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxy-3,5-[^{125}I]diiodophenyl)propionate; specific activity 2000 Ci/mmol; New England Nuclear) freshly dried from a solution in benzene. After agitation of the mixture at 4°C overnight, the reaction was quenched by addition of 5 μl of 1 *M* glycine in borate buffer. Iodinated polypeptide was separated from by-products by gel filtration on Sephadex G-25, equilibrated and eluted with 0.05 *M* phosphate buffer (pH 7.5). The absorbance at 280 nm of the ^{125}I-labeled polypeptide thus obtained was measured with a spectrophotometer.

Procedure for study of clearance of ^{125}I-labeled polypeptide *in vivo* CDF_1 mice were given i.v. injections of ^{125}I-labeled CH-271 or H-271 polypeptide (3.46×10^6 cpm/20 μg or 5.65×10^6 cpm/20 μg, respectively) in a volume of 0.2 ml of PBS. After various times, mice were exsanguinated, and the lungs, liver, kidneys, spleen, and blood were collected and rinsed in 70% ethanol. The radioactivity in each organ was measured in a gamma counter.

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

RESULTS

Effect of recombinant polypeptides on liver metastasis of lymphoid tumor We first examined the effect of the polypeptides with functional domains on liver metastasis of L5178Y-ML25 lymphoma cells. Polypeptides were co-injected i.v. with L5178Y-ML25 lymphoma cells into CDF_1 mice (Table I). The polypeptides containing the heparin-binding domain, H-271 and CH-271, significantly inhibited liver and spleen metastases of L5178Y-ML25 lymphoma cells at concentrations ranging from 50 to 500 μg per mouse. CH-271, a fusion polypeptide

Table I. Effect of Recombinant Fibronectin Fragments on Liver Metastases by i.v. Injection of L5178Y-ML25 Lymphoma Cells

Co-injected i.v. with	Dose (μg/mouse)	Mean weight (g) ± SD	
		Liver	Spleen
Untreated (PBS)		4.35±0.50	0.24±0.04
C-274	100	3.78±0.62	0.23±0.03
	250	4.14±0.84	0.22±0.03
	500	2.42±0.58**	0.21±0.05
H-271	100	2.72±0.80*	0.17±0.05
	250	2.24±0.75**	0.14±0.05*
	500	1.42±0.35**	0.11±0.02**
CH-271	50	1.83±0.92**	0.18±0.05**
	100	0.90±0.05**	0.08±0.01**
	250	0.90±0.07**	0.08±0.01**
	500	0.90±0.08**	0.08±0**
Poly(RGD)	500	2.72±1.04*	0.16±0.06
(Normal mice)		0.81±0.09	0.08±0

Five CDF_1 mice per group were inoculated i.v. with L5178Y-ML25 (4×10^4) with or without recombinant fibronectin fragments or poly(RGD). Mice were killed 16 days after tumor inoculation. *; $P<0.02$. **; $P<0.001$.

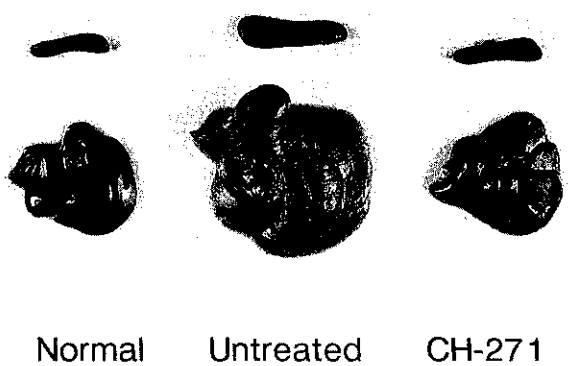

Fig. 2. Effect of CH-271 polypeptide on liver and spleen metastases of L5178Y-ML25 lymphoma cells. CDF_1 mice were given i.v. tumor cells (4×10^4) admixed with or without 100 μg of polypeptide. Mice were killed 14 days after tumor inoculation.

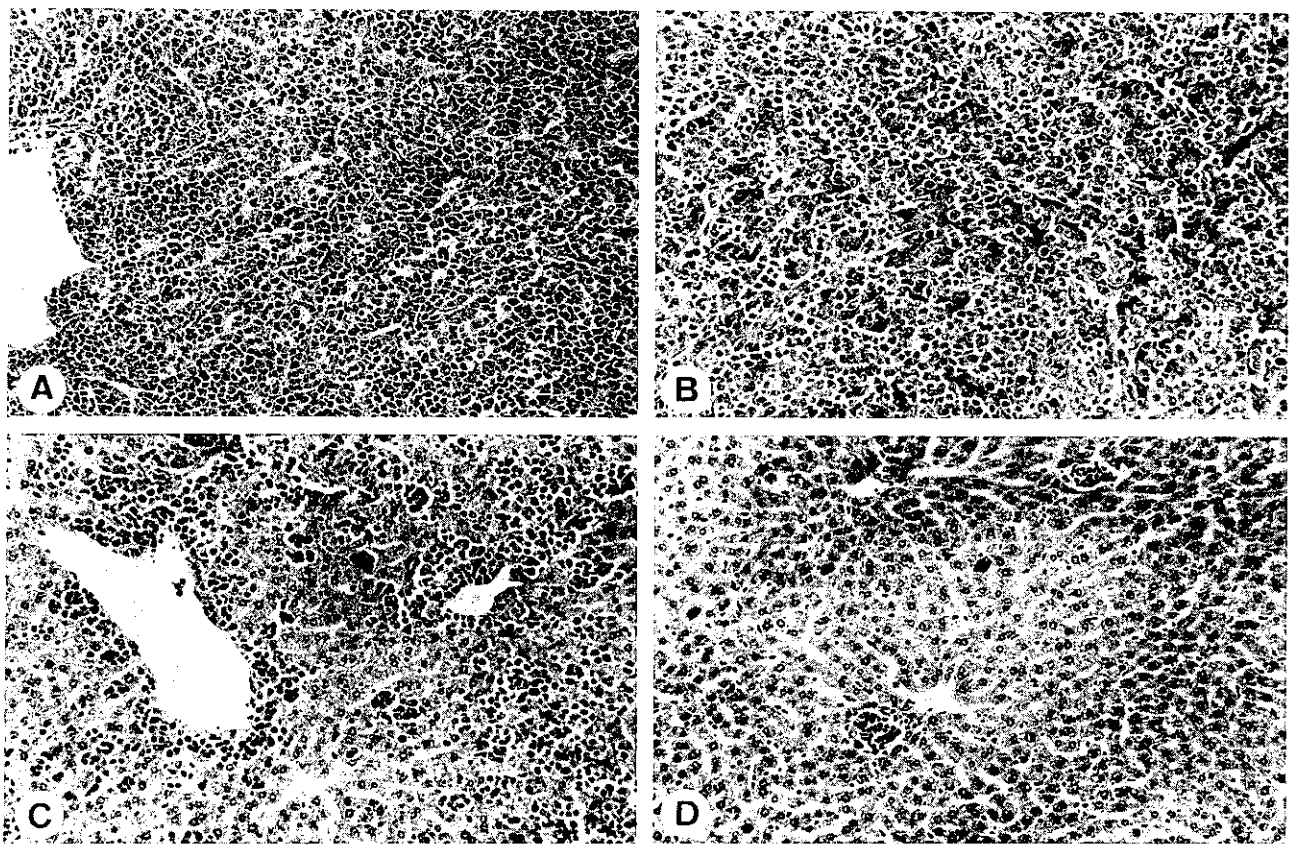

Fig. 3. Liver metastasis of L5178Y-ML25 lymphoma cells. A, Control (untreated). All the liver cells are replaced by tumor metastases. B, C-274 (500 μg)-treated. A small number of liver cells remains among proliferating tumor cells. C, H-271 (500 μg)-treated. Diffuse and focal tumor metastases are present around the vessels and in the sinusoids. D, CH-271 (100 μg)-treated. Only a few mononuclear foci but no tumor cells scattered in the liver tissue. Hematoxylin-eosin staining $\times$160.

with both cell- and heparin-binding domains, was more active than H-271 in inhibiting tumor metastasis and decreased the liver and spleen weights to the normal level (Table I and Fig. 2). In contrast, C-274 with the cell-binding domain and poly(RGD), which consists of repeated RGD sequences (in the cell-binding domain of fibronectin), achieved less reduction of liver and spleen weights at any dose than did H-271 and CH-271. Histological analysis revealed that the enlarged liver in the control (untreated) group exhibited diffuse infiltration of tumor cells, and no liver cells were seen (Fig. 3A). The C-274-treated group showed many size variations of foci of tumor metastasis from large to small. A few liver cells remained among the metastatic foci (Fig. 3B). In the H-271-treated group (Fig. 3C), tumor cells proliferated around the vessels and in the sinusoids, but they were far smaller and fewer than those of the C-274-treated group. No metastasis was found in the CH-271-treated group but there were quite a few small foci of mononuclear cells (Fig. 3D). The survival rate of mice given i.v. injection of L5178-ML25 lymphoma cells admixed with the polypeptides was also determined in the experimental liver metastasis model (Fig. 4). In this experiment, 50% of untreated mice succumbed to the tumor burden within 16 days of tumor cell inoculation. Similar survival rates were observed in the group of mice which received L5178Y-ML25 cells admixed with C-274 polypeptide. The group that received H-271 showed an enhanced survival rate, but 6 out of 10 mice had succumbed within 50 days of the tumor cell inoculation. All the mice given CH-271 were still alive at 50 days following tumor cell injection. Table II shows that the incubation of tumor cells with various concentrations of polypeptides did not affect the incorporation of [^{3}H]thymidine into tumor cells or the cell viability determined by the trypan blue dye exclusion test. The peptidic anticancer drug neocarcinostatin (10 μg/ml), used as a positive control, potently inhibited the cell growth *in vitro*. This result

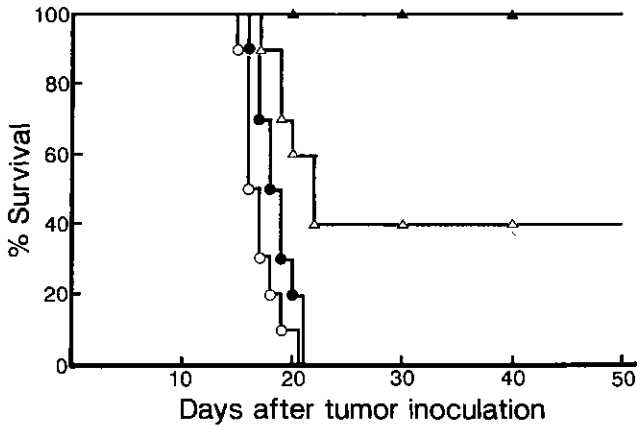

Fig. 4. Effect of recombinant fragments of fibronectin on the survival of CDF_1 mice co-injected i.v. with L5178Y-ML25 lymphoma cells. Mice were injected i.v. with L5178Y-ML25 (4×10^4) together with PBS (○), or 250 μg of C-274 (●), H-271 (△) or CH-271 (▲) and animal survival was monitored as a function of time.

indicates that the polypeptides were not cytotoxic and did not inhibit cell growth.

Effect of anti-asialo GM1 serum or 2-chloroadenosine on CH-271-mediated inhibition of tumor metastasis Since NK cells or macrophages in the circulation play an important role in the inhibition of tumor metastasis,[34, 35] we investigated whether or not the action of CH-271 polypeptide requires NK or macrophages to induce the inhibition of tumor metastasis. Anti-asialo GM1 serum can selectively eliminate NK cells[36] and 2-chloroadenosine is a macrophage-toxic substance.[37] Table III shows that pretreatment of mice with anti-asialo GM1 serum is likely to enhance slightly the frequency of liver metastasis as compared with the frequency found among untreated mice but pretreatment with 2-chloroadenosine does not enhance it significantly. The co-injection with CH-271 led to a significant reduction of liver metastasis in both untreated and treated mice.

Retention of ^{125}I-labeled polypeptide in the circulation We next examined the behavior of H-271 or CH-271 polypeptide in the circulation and various organs. Figure 5 indicates that there is no discernible difference between H-271- and CH-271-injected mice in the clearance of labeled polypeptide in the blood and organs after the injection. The clearance of labeled polypeptide from the circulation was biphasic and rapid during the early phase after the injection. A very rapid initial decrease in polypeptide was obtained within 5 min after the injection, implying that the initial decrease would probably be due to rapid equilibration or a dilution effect in the body fluid. After the initial equilibration, the clearance of labeled polypeptide was rapid up to 1 h and thereafter the labeled polypeptide was gradually cleared. The half-lives

Table II. Effect of Recombinant Fibronectin Fragments on the Growth and Viability of L5178Y-ML25 Lymphoma Cells

Treatment	Concentration (μg/ml)	Incorporation of [^{3}H]thymidine into the cells (cpm)	Viability (%)
Untreated (medium)		227111±10635	98
C-274	0.1	183221±24430	
	1	185022±1171	
	10	183331±36593	
	100	188147±6650	98
H-271	0.1	211488±868	
	1	200610±15067	
	10	215323±9250	
	100	240020±5063	100
CH-271	0.1	193450±4204	
	1	218670±9688	
	10	189178±10883	
	100	193912±26188	97
Neocarcinostatin	10	1409±457	2

L5178Y-ML25 cells (5×10^3) were incubated with medium, recombinant fibronectin fragments or neocarcinostatin for 3 days at 37°C. The culture was pulsed with 0.5 μCi of [^{3}H]-thymidine for the last 4 h before termination. Cell viability was assessed by the trypan blue dye exclusion method 3 days after the co-incubation.

Table III. Effect of Anti-asialoGM1 IgG or 2-Chloroadenosine on CH-271-mediated Inhibition of Liver Metastases of L5178Y-ML25 Lymphoma Cells

Treatment of mice	CH-271	Mean weight (g)±SD	
		Liver	Spleen
None	−	2.99±0.53	0.19±0.02
	+	0.97±0.09*	0.08±0.02*
Rabbit anti-asialoGM1 IgG, 20 μl, i.v.	−	3.72±0.36	0.26±0.05
	+	0.96±0.06*	0.10±0.02*
2-chloroadenosine, 50 μg, i.v.	−	3.14±0.62	0.19±0.04
	+	1.40±0.25*	0.10±0.01
(Normal mice)		0.98±0.07	0.08±0

L5178Y-ML25 cells (4×10^4) were injected i.v. with or without 100 μg of CH-271 into groups of control CDF_1 mice or mice pretreated 24 h earlier with antibody or 2-chloroadenosine. Mice were killed 14 days after tumor inoculation.
*; $P<0.001$.

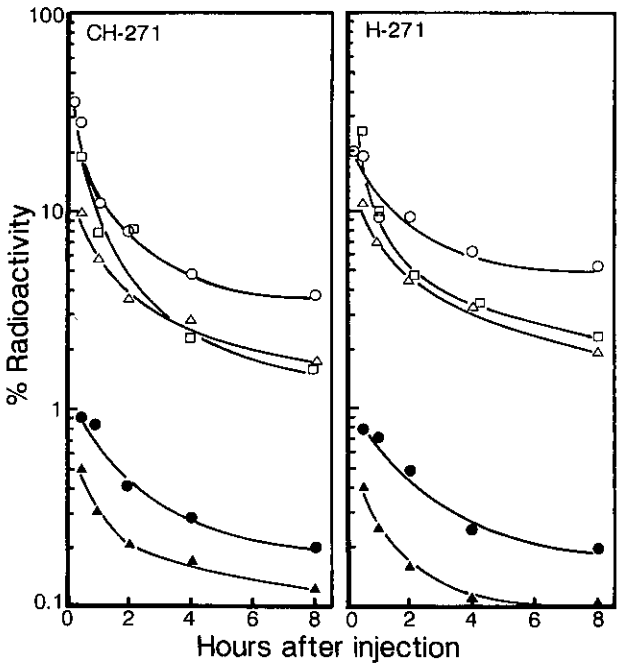

Fig. 5. Clearance of ^{125}I-labeled polypeptides from the circulation in CDF_1 mice. The levels of radioactivity in blood; (○), lungs; (●), liver; (△), spleen; (▲) and kidneys; (□) were determined as described in "Materials and Methods." The first samples were collected after 5 min. Each point represents the mean of a group of three mice.

of the polypeptide during the early and late phases were approximately 15 min and 3 h.

Effect of administration timing of CH-271 fusion polypeptide on liver metastasis of L5178Y-ML25 lymphoma cells We next examined the effect of administration protocol of the CH-271 polypeptide on liver metastasis of L5178Y-ML25 cells. CH-271 at a dose of 100 μg significantly suppressed the increase of liver and spleen weights upon co-injection (admixing) with L5178Y-ML25 cells (Table IV). In addition, i.v. administration of CH-271 polypeptide 1 h before or after tumor injection significantly inhibited the liver metastasis. Pretreatment of L5178Y-ML25 cells with 100 μg of CH-271 for 30 min also achieved the inhibition of metastasis.

Therapeutic effect of multiple administrations of polypeptides on liver metastasis of L5178Y-ML25 lymphoma cells Multiple treatments with polypeptides at the dose of 250 μg were performed daily for 7 days after tumor inoculation. Table V shows that the i.v. administrations of CH-271 significantly suppressed the increase of liver and spleen weights. Multiple treatments with H-271 or C-274 also showed significant suppression of the increase of liver and spleen weights, but were less effective than CH-271 fusion polypeptide. The survival rate of mice given multiple i.v. administrations of polypeptides for 7 days after tumor inoculation was also determined (Fig. 6). In this experiment, 50% of untreated control mice succumbed to the tumor burden within 20 days of the injec-

Table IV. Effect of Injection Timing of CH-271 Polypeptide on Liver Metastases by i.v.-Injected L5178Y-ML25 Lymphoma Cells

Administered i.v. with	Timing	Dose (μg/mouse)	Mean weight (g) ± SD	
			Liver	Spleen
Expt. I				
Untreated (PBS)			3.22±0.86	0.19±0.03
CH-271	Admix	100	1.01±0.08**	0.10±0.01**
	Separate[a]			
	1 h before	100	1.67±0.21**	0.11±0.01**
	1 h after	100	1.71±0.51*	0.14±0.02
(Normal mice)			0.95±0.04	0.09±0.01
Expt. II				
Untreated (PBS)			3.36±0.42	0.18±0
CH-271	Admix	100	0.91±0.10**	0.10±0.01**
	Pretreatment[b]	100	0.88±0.08**	0.08±0.01**

Five CDF_1 mice per group were inoculated i.v. with L5178Y-ML25 (4×10^4) cells with or without CH-271. Mice were killed 14 days after tumor inoculation.

a) CH-271 was administered i.v. at the indicated time points before or after the i.v. injection of L5178Y-ML25 cells.

b) The cells were incubated with CH-271 for 30 min and then washed three times before injection.

*; $P<0.01$. **; $P<0.001$.

Table V. Therapeutic Effect of Recombinant Fibronectin Fragments on Liver Metastasis by L5178Y-ML25 Lymphoma Cells

Administered with	Dose (μg/mouse)	Mean weight (g) ± SD	
		Liver	Spleen
—	—	5.16±0.42	0.26±0.02
CH-271	250×7	1.45±0.23*	0.14±0.01*
H-271	250×7	3.56±0.37*	0.20±0.02
C-274	250×7	3.25±0.64*	0.18±0.06
(Normal)		1.12±0.11	0.08±0

Five CDF_1 mice were implanted i.v. with 4×10^4 L5178Y-ML25 lymphoma cells, and administered i.v. with or without 250 μg of the polypeptides for 7 days after tumor inoculation. The treatments with polypeptides were started 1 day after tumor inoculation. Mice were killed 13 days after tumor inoculation. *, $P<0.001$.

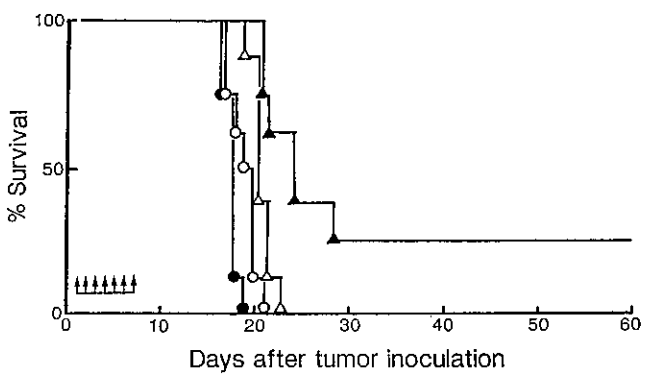

Fig. 6. Effect of multiple administrations of polypeptides on the survival of CDF_1 mice injected with L5178Y-ML25 lymphoma cells. Mice were administered i.v. with PBS (○), C-274 (●), H-271 (△) or CH-271 (▲) 250 μg per day for 7 days (↑) after i.v. injection of tumor cells. The treatments with polypeptides were started 1 day after tumor inoculation. Animal survival was monitored as a function of time. $P<0.01$; CH-271 vs. untreated control.

tion. Similar survival rates were observed in the group of mice which received C-274 or H-271 polypeptide. The group that received CH-271 showed a significantly enhanced survival rate ($P<0.01$ by Mann-Whitney's U test), but 6 out of 8 mice had succumbed within 30 days of the tumor inoculation.

DISCUSSION

We have attempted to elucidate the mechanisms involved in cell functions such as adhesion and motility during the metastatic process. Tumor cell adhesion to components of the extracellular matrix, in particular fibronectin, is an important aspect of several steps of metastasis.[1-3] Previous studies have utilized proteolytic fragments or synthetic peptides of laminin or fibronectin to inhibit experimental metastasis of tumor cells in mice.[11-16] We also demonstrated that synthetic peptides derived from fibronectin such as poly(RGD), CS1 or recombinant fragments with functional domains can be used to inhibit experimental and spontaneous lung metastases of murine melanoma, tumor cell adhesion to the extracellular matrix, the penetration of tumor cells through reconstituted basement membrane *in vitro* and tumor-induced angiogenesis in syngeneic mice.[17-21, 24]

To extend our previous observation on the inhibition of tumor metastasis by synthetic and recombinant polypeptides, we have examined the behavior of recombinant polypeptides with the cell- or heparin-binding domain or the fusion polypeptide on liver metastasis of lymphoid tumor *in vivo*. Co-injection of L5178Y-ML25 lymphoma cells with the polypeptides containing heparin-binding domain (H-271 and CH-271) resulted in marked suppression of the increase of liver and spleen weights, but C-274 with the cell-binding domain and poly(RGD) inhibited liver metastasis only at the high dose (500 μg/mouse) (Table I and Figs. 2 and 3). H-271 or CH-271 significantly enhanced the survival rate as compared with untreated control or C-274 (Fig. 4). There results clearly indicate that the heparin-binding domain of fibronectin (H-271 and CH-271) dramatically inhibited liver metastasis of L5178Y-ML25 lymphoma cells. Furthermore, significant inhibition of liver metastasis was observed when CH-271 was injected i.v. 1 h before or after the injection of L5178Y-ML25 cells as well as when a mixture of CH-271 and tumor cells was co-injected. We therefore concluded that the cells do not require a prolonged incubation with polypeptide. The polypeptides used, however, did not exhibit direct cytotoxicity against tumor cells, nor did they affect cell growth (Table II). This suggests that the inhibitory effects of the polypeptides on tumor metastasis cannot be simply explained by direct cytotoxicity toward tumor cells.

Tumor cells in the circulation interact with host cells such as lymphocytes, natural killer (NK) cells and monocytes, which are believed to be particularly important in killing tumor cells.[34, 35] CH-271 significantly inhibited liver metastasis in mice pretreated with anti-asialo GM1 serum or 2-chloroadenosine as well as untreated mice (Table III). Since CH-271 was still active when NK cells and macrophages were removed from our system, its inhibitory mechanism is unlikely to be directly related to the stimulation and activation of these cells. We recently observed that the co-injection of tumor cells with polypeptides containing the heparin-binding domain led to a significantly reduced arrest of tumor cells in lung and liver over 8 h after injection, and we showed that these

polypeptides were able to inhibit tumor cell adhesion to substrates coated with reconstituted basement membrane component, Matrigel.[38] The adhesive interaction of tumor cells with CH -271 was inhibited by the addition of heparin or monoclonal antibodies against the heparin-binding domain of fibronectin.[38] Thus, the inhibitory effect on liver metastasis by CH-271 may be attributable to the interaction between the tumor cell surface and the heparin-binding domain rather than the cell-binding domain in the polypeptide molecule. In support of this notion, the interaction between heparin-like molecules on the cell surface and the heparin-binding domain in fibronectin could modulate haptotactic migration of metastatic melanoma to fibronectin-substrate.[39]

Among these polypeptides, CH-271 fusion polypeptide inhibited liver metastasis of L5178Y-ML25 lymphoma cells more effectively than H-271 on a weight basis (Table I). The clearance rate of ^{125}I-labeled CH-271 from the circulation after the i.v. injection is similar to that of labeled H-271, and the half-life of both polypeptides during the late phase (after 1 h) is approximately 3 h (Fig. 5). These results indicate that the inhibition of metastasis of L5178Y-ML25 cells by CH-271 or H-271 may not depend on a difference in the stability of the polypeptides in the circulation after injection, or in the molecular size. Since we observed that a mixture of H-271 and C-274, or C-274 alone was much less active in inhibiting tumor metastasis than CH-271 fusion polypeptide at a similar molar ratio (Table I and reference 38), the fusion of H-271 with C-274 may facilitate the inhibitory effect on tumor metastasis, or possibly the interaction between tumor cells and the heparin-binding domain (perhaps by altering the binding affininty). Further study is needed.

Multiple administrations of CH-271 after tumor implantation significantly inhibited liver metastasis of L5178Y-ML25 cells and enhanced the suvival rate as compared with the untreated control, whereas multiple treatments with H-271 or C-274 did not (Table V and Fig. 6). The exact mechanism responsible for the inhibition of liver metastasis by CH-271 is not known yet, but may be more complex than a simple interference with tumor cell adhesion to the extracellular matrix. Further study will be needed to determine the inhibitory effect on the invasion and enzymatic degradation of the extracellular matrix by L5178Y-ML25 cells and so on.

Finally, the present study has demonstrated that recombinant fusion polypeptide of fibronectin (CH-271) inhibited liver metastasis of L5178Y-ML25 T-lymphoma cells, possibly through a mechanism mediated by the heparin-binding domain, and enhanced the survival rate. Our previous study showed that C-274 (containing RGD sequence) or CS1 peptide could inhibit pulmonary metastasis of murine melanoma by an RGD-dependent or -independent mechanism, respectively.[24] More recently, we observed that CH-271 also inhibited liver metastasis of RAW117-H10 B-lymphoma cells (data not shown). Thus, since the fusion polypeptide of fibronectin showed no short-term toxicity to the host, it may be potentially useful in the prevention of cancer metastasis.

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