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Inhibitory Effect of Antimetastatic Fusion Polypeptide of Human Fibronectin on Tumor Cell Adhesion to Extracellular Matrices

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We investigated the inhibitory mechanism of liver metastasis by using recombinant fragments with cell- and/or heparin-binding domains (C-274, H-271 or the fusion fragment CH-271). Intravenous co-injection of L5178Y-ML25 cells with CH-271 was more effective for the inhibition of liver metastasis than C-274, H-271 or C-274+H-271. Reduction of the arrest and retention of the radiolabeled tumor cells in the liver of mice was found when CH-271 was co-injected with tumor cells. L5178Y-ML25 cells adhered both concentration- and time-dependently to the substrates precoated with fibronetin, laminin and reconstituted basement membrane, Matrigel. The tumor cell adhesions to the substrates were inhibited in the presence of CH-271. The tumor cell interaction with CH-271-substrate was inhibited by heparin, and monoclonal antibodies (IST-1 or IST-2) against the heparin-binding domain of fibronectin. However, monoclonal antibodies against the cell-binding domain failed to block the interaction. Similarly, CH-271-mediated antimetastatic activity was also inhibited by the treatment of CH-271 with IST-1 before the co-injection with tumor cells, whereas monoclonal antibody against the cell-binding domain had no effect. Thus, the antimetastatic effect of CH-271 fusion fragment on liver metastasis of L5178Y-ML25 cells may be partly due to interference with the adhesive interaction of tumor cells with extracellular matrix or basement membrane components by a heparin-binding domain-dependent mechanism.

Key words: Recombinant fibronectin fragment — Liver metastasis — Cell adhesion — Lymphoma

Metastasis is one of the characteristics by which malignant tumors differ from benign neoplasms, and is a complex, multistep process. The ability of circulating tumor cells to form metastases depends, among other factors, on their tendency to undergo homotypic aggregation or heterotypic aggregation with host cells to form emboli. Specific interactions of solitary tumor cells or tumor emboli with capillary endothelial cells and with the underlying extracellular matrix (ECM) are thought to be important for organ-specific metastases and invasion.[1-5]

Recent studies on cell adhesion molecules and their cell surface receptors have to some extent elucidated the molecular events involved in tumor adhesive interaction.[6-9] One class of molecules involved in such interaction is the integrin superfamily of adhesion receptors which consist of heterodimeric structures of distinct α- and common β-chains.[6] Some members of the integrins use the Arg-Gly-Asp (RGD) sequence as the primary recognition site for binding to ECM components such as fibronectin and vitronectin.[8,9] Cell-cell adhesion is also mediated by the binding of certain integrins to cell surface molecules such as leukocyte function-associated antigen-1 (LFA-1) and intercellular-adhesion molecule-1 (ICAM-1). Roossien *et al.*[10] have shown that LFA-1 is involved in adhesion to hepatocytes by a lymphoma cell line and is likely to be important for the formation of liver metastases. Several studies have demonstrated that *ex vivo* pretreatment of tumor cells with proteolytic fragments or synthetic peptides of fibronectin including RGDS-oligopeptide inhibited experimental lung metastasis of murine melanoma.[11-13] We previously showed that poly(RGD), which contains repetitive structure of RGD, inhibited experimental and spontaneous lung metastasis of murine melanoma cells upon systemic administration,[14] and that CS1 peptide of alternative splicing type III connecting segment (IIICS) in fibronectin inhibited lung metastasis through an RGD-independent mechanism.[15]

Recent studies showed that 33-kDa tryptic/catheptic fibronectin fragment, which contains the carboxy-terminal heparin-binding domain and CS1 peptide of IIICS, promoted the adhesion of lymphocytes or lymphoid tumors as well as melanoma when it was immobilized on the surface,[16] and resulted in a reduction of lung tumor colonies when co-injected with tumor cells.[11] We also demonstrated that recombinant fusion polypeptide of fibronectin containing the cell- and heparin-binding domain (referred to as CH-271) was more active in inhibiting liver metastasis of L5178Y-ML25 lymphoma cells than polypeptide with the cell-binding domain (C-274), polypeptide with the heparin-binding domain (H-

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271) or their mixture (C-274+H-271) when they were co-injected with tumor cells or separately injected after tumor inoculation. Furthermore, treatment with CH-271 substantially prolonged the survival time of mice injected i.v. with L5178Y-ML25 cells.[34]

In the present study, we extend our previous study to examine the inhibitory effect of the fusion polypeptide on liver metastasis of L5178Y-ML25 cells. Therefore, we focused our attention of the inhibitory effect of recombinant fibronectin fragments on tumor adhesive properties to ECM components.

MATERIALS AND METHODS

Animals Specific pathogen-free CDF_1 mice, (BALB/c×DBA/2) 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 5 to 9 weeks old and sex-matched.

Cells Liver metastatic L5178Y-ML25 T-lymphoma cells (partially metastasizing to the spleen), obtained from L5178Y parent cells by *in vivo* selection for invasion,[17] were kindly provided by Dr. A. Okura, Banyu Pharmaceutical Co. Ltd., Tokyo. L5178Y-ML25 cells were maintained in PRMI-1640 supplemented with 7.5% fetal bovine serum and L-glutamine.

Recombinant fragments of human fibronectin We prepared three kinds of recombinant fragments of human fibronectin (C-274, H-271 and CH-271) by expressing cDNA of human fibronectin in *E. coli*, using the expression vector pUC118N/119N described by Maki *et al.*[18] C-274 and H-271 correspond to the cell- and heparin-binding domains of fibronectin, respectively, while another fragment, CH-271, is a fusion protein with both cell- and heparin-binding domains (Fig. 1). The molecular weights of C-274, H-271 and CH-271 are 29,882, 29,592 and 59,899 respectively. Two cDNA clones, pLF5 and pLF2435, were used as the source of cDNA.[19] The cell-binding fragment C-274 was expressed by a recombinant plasmid pTF7221, derived from pTF7121, which expresses a cell-binding fragment C-279 with five additional amino acids at the carboxyl-terminus of C-274. The heparin-binding fragment 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 a recombinant plasmid pCH101; this had been constructed from pHD101 and pTF7121. Detailed accounts of these constructions and expressions have appeared elsewhere.[20] 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-acid sequence was checked with an automated peptide sequencer, model 477A (Applied Biosystems Inc., Foster City, CA). The carboxyl-terminal amino acid sequence was determined by use of carboxypeptidase P (Takara Shuzo Co. Ltd., Kyoto). These fragments were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) before use. All the polypeptides in this study were endotoxin-free (< 1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co. Ltd., Tokyo).

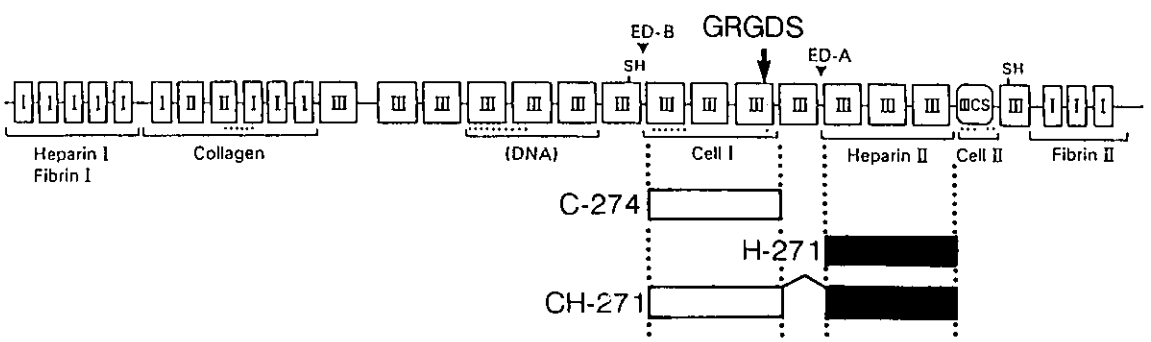

Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open and closed bars. The cell-binding polypeptide C-274 (Pro1239-ASP1512, the sequence numbered by the system of Kornblihtt *et al*,[33]) covers three units of type III homology at the cell-binding 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 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, respectively.

Antibodies and other reagents Mouse control IgG was purchased from ZYMED, CA. Mouse monoclonal antibodies (ascites) IST-1 and IST-2,[21] against heparin-binding domain of human fibronectin (both are IgG_1), were purchased from Sera-Lab, Clawry Down, UK. Monoclonal antibodies, F12-8 and FN30-8,[22] against the cell-binding domain of human fibronectin were obtained from Takara Shuzo Co. Sodium heparin was purchased from Green Cross Co., Osaka. Bovine serum albumin (BSA) was purchased from Seikagaku Kogyo, Tokyo. Purified mouse fibronectin was purchased from Biomedical Technologies Inc., MA. Purified mouse laminin and reconstituted basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) were purchased from Collaborative Research Inc., MA.

Assay for liver metastasis of lymphoma cells CDF_1 mice were given i.v. injection of 4×10^4 L5178Y-ML25 T-lymphoma cells admixed with or without various doses of the recombinant fragments of fibronectin in a volume of 200 μl of PBS. Fourteen to seventeen days after tumor inoculation, mice were killed and the weights of liver and spleen were recorded to evaluate tumor metastasis as previously described in detail.[17]

Radioiodination of tumor cells L5178Y-ML25 cells were radiolabeled by incubation for 12 h in culture medium containing 5% FBS and 1.5 μCi/ml [^{125}I]iododeoxyuridine (specific activity, 200 mCi/mmol, Du Pont-New England Nuclear, Boston, MA). The cells were washed twice with PBS to remove unincorporated radiolabel and resuspended in PBS or serum-free culture medium containing 0.1% BSA.

Organ distribution and retention of radiolabeled tumor cells Radiolabeled tumor cells (1.5×10^5 cpm/3×10^5 cells) admixed with or without domain polypeptides in a volume of 0.2 ml of PBS were injected into the lateral tail veins of CDF_1 mice. Mice were killed at times ranging from 1 to 8 h after the injection. The lungs, liver, spleen, and 100 μl of blood were collected from each mouse. The radioactivity in each organ was measured in a gamma counter.

Cell adhesion assay Micro cell adhesion assay was carried out by the method described previously.[14] L5178Y-ML25 cells were radiolabeled as described above. Radiolabeled tumor cells suspended in serum-free culture medium containing 0.1% BSA were adjusted to a concentration of 2×10^6/ml. The cells in a volume of 50 μl were seeded onto Immulon-2 microculture wells precoated with the ECM components and polypeptides. Non-specific sites and control wells were blocked for 2 h by treating the wells with 1% BSA in PBS. The cultures were incubated at 37°C for various periods of time and then the wells were washed twice with PBS to remove non-adherent cells. The remaining substrate-bound tumor cells were lysed with 50 μl of 0.1 *N* NaOH. The cell lysates were adsorbed on cotton swabs, and monitored for radioactivity by gamma counting. The % adhesion was calculated as follows; Adhesion % = (cpm of bound cells/cpm of added cells) × 100. In the inhibition assay for tumor cell adhesion by polypeptides or antibodies, the % inhibition was calculated as follows; % Inhibition = (1 − cpm of bound cells with inhibitor/cpm of bound cells without inhibitor) × 100.

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

RESULTS

Effects of recombinant fragments of human fibronectin on liver metastasis of murine lymphoma cells We first examined the effects of recombinant fragments corresponding to cell- or/and heparin-binding domains of human fibronectin on experimental liver metastasis of L5178Y-ML25 T-lymphoma cells in mice (Table I). When the tumor cells were injected i.v. into CDF_1 mice, liver and spleen weights of the mice were increased more than 3-fold compared to those of normal mice. The co-injection of the tumor cells with recombinant fragments containing heparin-binding domain, H-271 and CH-271, significantly inhibited liver metastases in a dose-dependent manner. On the other hand, the fragment containing cell-binding domain alone, C-274, had no effect. CH-271, a fusion fragment containing both cell- and heparin-binding domains, was more effective than H-271 alone or the mixture of C-274 and H-271 (same molar ratio as CH-271, 1:1). This result indicates that the heparin-binding domain of fibronectin probably contributes to the antimetastatic activity, and that the fusion of H-271 with C-274 may lead to the effective expression of the activity. This is in good agreement with our previous findings using these recombinant fragments of fibronectin.[34]

Organ distribution and retention of tumor cells Our previous study indicated that the antimetastatic activity of CH-271 was due to neither a direct effect against tumor cell growth nor activation of natural killer cells and macrophages to the tumoricidal state.[34] To investigate the mechanism responsible for the inhibitory effect of CH-271 on liver metastasis of L5178Y-ML25 cells, we examined the organ distribution and retention of ^{125}I-labeled tumor cells to see whether the co-injection of tumor cells with recombinant fragments can lead to a decrease in the arrest of tumor cells in the capillary bed of the chosen organ. Significantly lower values were found in the liver and lungs of mice over 8 h after co-injection with CH-271 fusion polypeptide (Fig. 2). There are no discernible differences between control and

Table I. Effects of Recombinant Fragments of Fibronectin on Experimental Liver Metastasis Caused by i.v. Injection of L5178Y-ML25 Lymphoma Cells

Co-injected with[a]	μg/mouse (nmol)	Mean weight (g) ± SD[b]	
		Liver	Spleen
—	—	4.09±0.32	0.24±0.03
CH-271	50 (0.8)	2.24±0.30***	0.18±0.02**
	100 (1.7)	1.80±0.41***	0.15±0.03**
	200 (3.3)	1.31±0.21***	0.13±0.02***
C-274+H271	25+25 (0.8+0.8)	3.74±0.54	0.27±0.02
	50+50 (1.7+1.7)	3.82±0.45	0.28±0.03
	100+100 (3.3+3.3)	3.34±0.64*	0.24±0.03
C-274	25 (0.8)	4.43±0.16	0.28±0.02
	50 (0.7)	3.95±0.54	0.26±0.02
	100 (3.3)	4.07±0.64	0.27±0.05
H-271	25 (0.8)	3.60±0.73	0.25±0.06
	50 (1.7)	3.18±0.89	0.23±0.04
	100 (3.3)	3.10±0.50**	0.23±0.04
(Normal)		0.85±0.03	0.07±0.01

a) L5178Y-ML25 cells were co-injected i.v. with or without the indicated doses of fragments into CDF_1 mice.
b) Mice were killed 14 days after the tumor inoculation. ***, $P<0.001$. **, $P<0.01$. *, $P<0.05$.

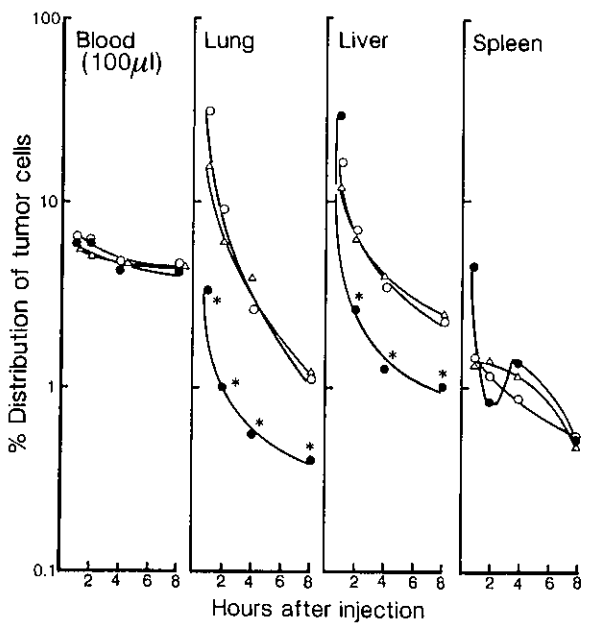

Fig. 2. ^{125}I-labeled L5178Y-ML25 cells (3×10^5 per mouse) were injected with 250 μg of CH-271 (●), 125 μg of C-274+125 μg of H-271 (△) or PBS (○) into the lateral tail vein of CDF_1 mice. At the indicated times, mice were killed and radioactivity retained in the blood, lung, liver or spleen was measured. The results represent % of cells remaining calculated from the input radioactivity (1.5×10^5 cpm per 3×10^5). *; $P<0.01$.

CH-271-injected mice in the arrest of labeled tumor cells in spleen and blood. In contrast, co-injection with a mixture of C-274 and H-271 (similar molar ratio to CH-271) did not affect the arrest and retention of labeled tumor cells in any organ as compared with untreated control.

Adhesive property of tumor cells to ECM components and basement membrane Since tumor cell adhesion plays an important role in the multistep process of tumor metastasis,[1-5] we tested the ability of L5178Y-ML25 cells to adhere to components of ECM and basement membrane, such as mouse fibronectin, laminin, collagen types I and IV, and reconstituted basement membrane (Matrigel). Figure 3A and B shows that the tumor cells adhered time- and concentration-dependently to the substrates precoated with Matrigel, laminin and fibronectin. We also observed that the tumor cells adhered to the substrates coated with collagen types I and IV only at the basal level, as much as in the case of BSA (data not shown). Figure 4 shows that the recombinant fragment containing the heparin-binding domain, CH-271, inhibited the tumor cell adhesion to Matrigel, fibronectin or laminin. The cell adhesion to laminin, in particular, was inhibited strongly by the addition of CH-271. In contrast, the fragment containing the cell-binding domain alone, C-274, could not inhibit tumor cell adhesion to any substrate. These results indicate that CH-271 fusion fragment was more effective in inhibiting the adhesion

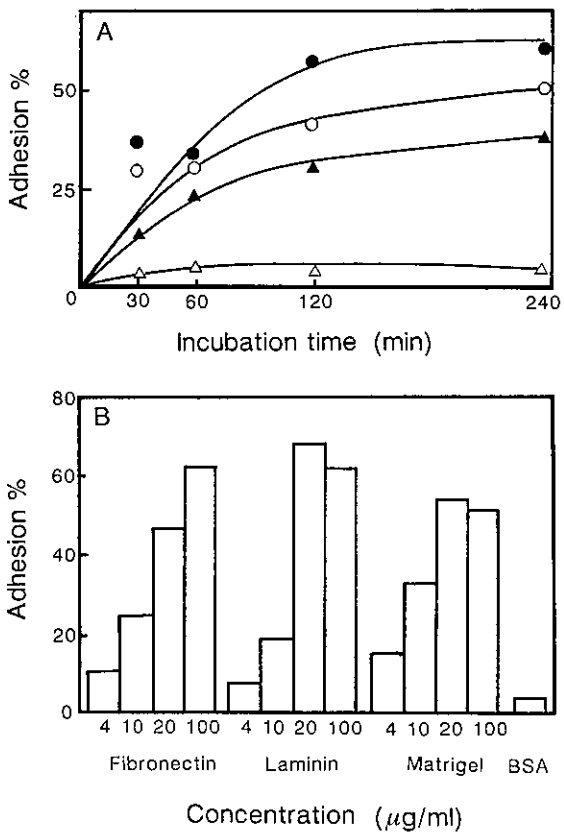

Fig. 3. Time course and concentration-response relationship of the adhesion of L5178Y-ML25 cells to the components of ECM or basement membrane. A: ^{125}I-labeled L5178Y-ML25 cells (1×10^5) were added to wells precoated with 20 μg/ml of Matrigel (○), laminin (●), fibronectin (▲) or BSA (△). After various times of incubation, nonadherent cells were washed away and adherent cells were counted. B: Labeled tumor cells (1×10^5) were added to wells precoated with various concentrations of Matrigel, laminin, fibronectin or BSA. After 2-h incubation, nonadherent cells were washed away and the adherent cells were counted.

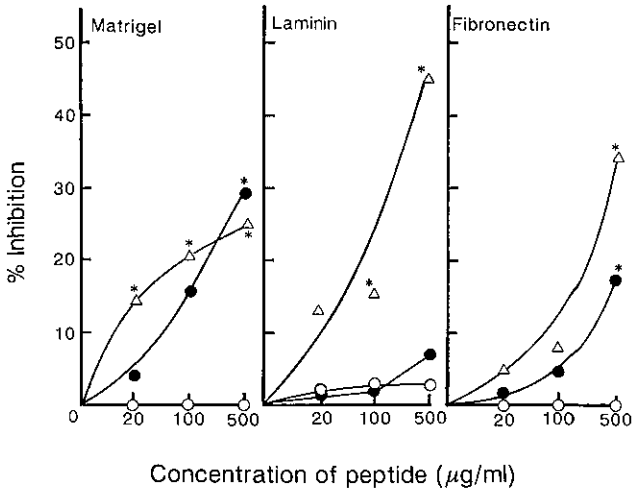

Fig. 4. Inhibition of tumor cell adhesion to Matrigel-, laminin- or fibronectin-substrates by the fibronectin fragments. Labeled L5178Y-ML25 cells (1×10^5) were added to wells precoated with 20 μg/ml Matrigel, laminin or fibronectin in the presence of various concentrations of C-274 (○), H-271 (●) or CH-271 (△). After 2-h incubation, nonadherent tumor cells were washed away and attached cells were counted. *; $P<0.01$.

of L5178Y-ML25 cells to these substrates than other fragments.

Adhesive interaction between CH-271 and tumor cells

Since the addition of recombinant fragment, especially CH-271, inhibited tumor cell adhesion to ECM and basement membrane components (Fig. 4), we next examined the interaction between tumor cells and the fragments of fibronectin. Figure 5A shows that the tumor cells adhered to wells precoated with 20 μg/ml CH-271, H-271 and C-274 in a time-dependent manner. Suboptimal tumor cell adhesions were observed approximately 2 h after the incubation. Figure 5B shows that the tumor cell adhesions to the immobilized fragments are concentration-dependent for coating. CH-271 showed much greater adhesion-promoting activity than other fragments. These results suggest that the tumor cells show an effective adhesive interaction with CH-271 fusion fragment. To analyze further the adhesive interaction between the tumor cells and CH-271, we tested the effect of recombinant domain fragments or heparin on tumor cell adhesion to CH-271-substrate. The tumor cell adhesion to CH-271 was inhibited in a concentration-dependent manner by H-271 or heparin, which binds to the heparin-binding region (Fig. 6A). C-274, however, did not inhibit the tumor cell interaction with CH-271. We also carried out adhesion inhibition assay using monoclonal antibodies against fibronectin in order to confirm the mediation of the heparin-binding domain (Fig. 6B). Monoclonal antibodies against different epitopes in the heparin-binding domain, IST-1 and IST-2, blocked the adhesive interaction of the tumor cells with CH-271 in a concentration-dependent manner, while two monoclonal antibodies, FN12-8 and FN30-8, against cell-binding domain, as well as control IgG did not block such interaction. These results suggest that the interaction of the tumor cell with heparin-binding domain of CH-271 caused the inhibition of tumor cell adhesion to ECM components, which may consequently lead to the inhibition of liver metastasis of L5178Y-ML25 cells. We next examined whether the blocking of the heparin-bind-

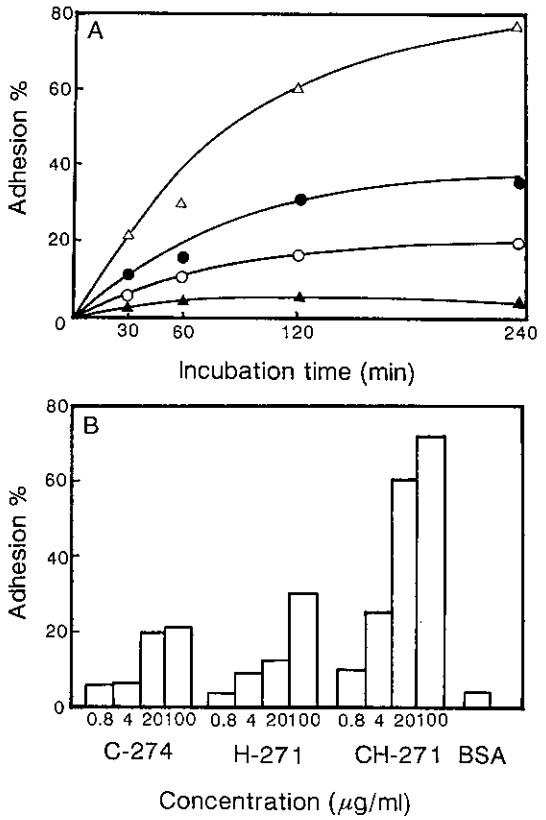

Fig. 5. Time course and concentration-response relationship of the adhesion of L5178Y-ML25 cells to the immobilized fibronectin fragments. A: ^{125}I-labeled L5178Y-ML25 cells (1×10^5) were added to wells precoated with 20 μg/ml of C-274 (○), H-271 (●), CH-271 (△) or BSA (▲). After various times of incubation, nonadherent cells were washed away and adherent cells were counted. B: Labeled tumor cells (1×10^5) were added to wells precoated with various concentrations of C-274, H-271, CH-271 or BSA. After 2-h incubation, nonadherent cells were washed away and the adherent cells were counted.

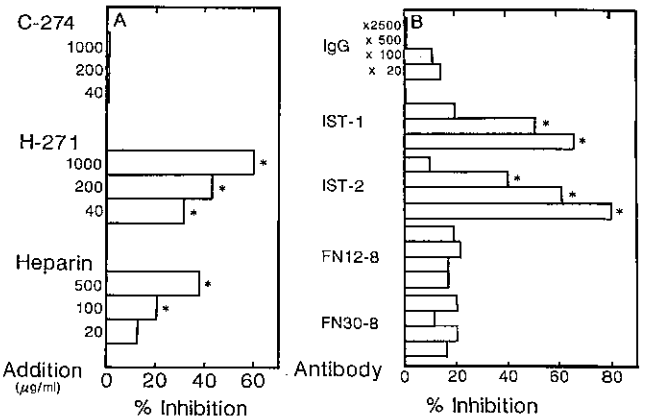

Fig. 6. Inhibition of tumor cell adhesion to the immobilized CH-271 fragment by various inhibitors. A, Labeled L5178Y-ML25 cells (1×10^5) were added to wells precoated with 20 μg of CH-271 fragment in the presence of various concentrations of C-274, H-271 or heparin. B, CH-271-coated substrates were incubated with various dilutions of monoclonal antibodies for 30 min before the addition of ^{125}I-labeled tumor cells. After 2-h culture, nonadherent tumor cells were washed away and the adherent cells were counted. *; $P<0.01$.

Table II. Effects of Anti-fibronectin Monoclonal Antibodies on CH-271-mediated Inhibition of Liver Metastasis of L5178Y-ML25 Lymphoma Cells

Co-injected with[a]		Mean weight (g) ± SD[b]		
CH-271 (μg)	mAb	Liver	Spleen	
Expt. I				
—	—	3.30±0.94	0.17±0.01	
—	IST-1	3.36±0.77	0.17±0.03	
—	IST-2	3.85±0.93	0.18±0.03	
100	—	1.13±0.23	0.11±0.02	
100	IST-1	3.11±0.52	0.20±0.03	
100	IST-2	1.62±0.45	0.12±0.02	
Expt. II				
—	—	2.90±0.47	0.18±0.03	
—	FN12-8	3.35±0.53	0.20±0.03	
—	FN30-8	2.78±0.55	0.17±0.03	
100	—	1.36±0.34	0.12±0.02	
100	FN12-8	1.59±0.27	0.14±0.03	
100	FN30-8	1.37±0.10	0.11±0.01	

a) L5178Y-ML25 cells were co-injected i.v. with or without CH271 pretreated with or without diluted (1:20) monoclonal antibodies against fibronectin.
b) Mice were killed 15 days after tumor inoculation.
*, $P<0.01$. **, $P<0.001$.

ing domain can suppress the CH-271-mediated antimetastatic effect *in vivo* (Table II). Although co-injection of 100 μg of CH-271 with tumor cells caused a reduction of liver weight as shown in Table I, preincubation of CH-271 with IST-1 resulted in the suppression of the CH-271-mediated antimetastatic activity. In contrast, IST-2 as well as FN12-8 and FN30-8 did not affect the CH-271-mediated antimetastatic activity. Also the co-injection of tumor cells with four monoclonal antibodies showed no inhibitory effect on liver metastases of L5178Y-ML25 cells. These results suggest that the IST-1-binding region of CH-271 plays an important role in the development of the antimetastatic effect of CH-271, and that the linkage of the heparin-binding domain with the cell-binding domain contributes to the augmentation of CH-271-mediated inhibitory effect.

DISCUSSION

Tumor cell adhesion to ECM and basement membrane components including fibronectin is an important step in the process of tumor metastasis.[1-5] Previous studies have shown that proteolytic fragments or synthetic peptides of fibronectin or laminin inhibited experimental lung metastases upon co-injection with tumor cells.[11-15] We have attempted to elucidate the regulatory mechanism involved in cell functions, such as adhesiveness, that are important during the metastatic process. Our recent study indicated that a recombinant fusion polypeptide containing cell- and heparin-binding domains, CH-271, exhibited an inhibitory effect on liver metastasis of L5178Y-ML25 T-lymphoma cells and prolonged the survival rate of mice when it was co-injected or separately injected after tumor inoculation.[34] In the present study, we report the inhibitory mechanism of liver metastasis of the lymphoid cells by CH-271.

CH-271 fusion fragment was much more effective in inhibiting liver metastasis of L5178Y-ML25 cells than H-271 (Heparin-binding domain), C-274 (cell-binding domain), or a mixture of C-274 and H-271 (Table I). These results indicate that CH-271-mediated inhibition of liver metastasis is not due to synergism between C-274 and H-271. The difference of antimetastatic effect between CH-271 and either H-271 or a mixture of C-274 and H-271 can not simply be explained in terms of the stability (clearance) of the polypeptides in the circulation or the molecular size, because there was no discernible difference between CH-271- and H-271-treated mice in the clearance of the radiolabeled polypeptide from the circulation after i.v. injection.[34] On the other hand, C-274 showed no inhibitory effect on liver metastasis of L5178Y-ML25 cells at concentrations ranging from 25 to 100 μg (Table I). Co-injection of 500 μg of C-274, however, led to approximately 45% reduction in the liver weight of untreated tumor-bearing mice (2.42 ± 0.58 vs. 4.35 ± 0.50).[34] Also, the pretreatment of the cell-binding domain (C-274) of CH-271 with two monoclonal antibodies against this domain (FN12-8 and FN30-8) before the co-injection with tumor cells did not affect the CH-271-mediated inhibition of liver metastasis (Table II). In contrast, the pretreatment of CH-271 with IST-1 antibody against heparin-binding domain resulted in the suppression of the CH-271-mediated antimetastatic property. Thus, the heparin-binding domain contributes to the expression of antimetastatic activity, and the fusion of H-271 with C-274, although C-274 by itself showed only a weak inhibitory effect on liver metastasis, may lead to augmentation of the antimetastatic effect. Further study will be needed to examine the role of the cell-binding domain (C-274) in the fusion polypeptide in CH-271-mediated antimetastatic action.

The co-injection of L5178Y-ML25 cells with CH-271 remarkably reduced the arrest and retention of tumor cells in liver and lung, as compared with the control or a mixture of C-274 and H-271 (Fig. 2). Significantly lower values were still found in the liver and lungs of CH-271-treated mice at 24 h after the co-injection (data not shown). CH-271 fragment also inhibited the tumor cell adhesion to substrates precoated with Matrigel, fibronectin or laminin (Fig. 4). These results indicate that anti-adhesive properties of CH-271 to ECM components may be associated with the inhibition of tumor cell arrest in the liver and consequently the prevention of liver metastasis. Tumor cells attached themselves to the immobilized CH-271 on the surface in a coating concentration-dependent manner (Fig. 5). Such tumor cell adhesion to CH-271-coated substrate was inhibited by the addition of H-271 fragments, heparin or monoclonal antibodies against heparin-binding domain, IST-1 and IST-2 (Fig. 6). Thus, the inhibitory effect can be attributed to the adhesive interaction between tumor cells and the heparin-binding domain in CH-271 fragment. IST-1 antibody, which recognizes an epitope within the first 158 amino acids of the domain,[21, 23] suppressed the antimetastatic and anti-adhesive properties mediated by CH-271. Although IST-2 antibody suppressed the inhibitory effect of CH-271 on the adhesion of L5178Y-ML25 cells, it did not inhibit the CH-271-mediated antimetastatic activity (Table II). The reason for this discrepancy is not known, but the difference of the inhibitory effects by IST-2 may be attributable to alteration of the conformation of the CH-271 molecule between the immobilized (coated) and soluble forms, or the reactivity of IST-2 to CH-271. McCarthy *et al.*[24, 25] have reported that two synthetic hydrophilic peptides (termed peptides I and II) within the carboxyl-terminal heparin-binding domain of fibronectin possessed heparin-binding activity and promoted CS1- and RGD-independent adhesion of melanoma cells. Since both peptides I and II are localized within the IST-2 binding region of the heparin-binding domain, neither peptide I nor II may contribute to the antimetastatic activity of CH-271 against L5178Y-ML25 lymphoma cells. These results suggest that the antimetastatic effect depends on the interaction of tumor cells with a particular region in the heparin-binding domain (i.e. IST-1 binding region).

The observation that the heparin-binding fragment can inhibit liver metastasis suggests that heparin-like molecules or other proteoglycans on the cell surface play an important role in mediating the adhesion of metastasizing tumor cells. Previous studies have reported that these cell surface proteoglycans promoted the adhesion of a variety of normal and transformed cell types.[26-29] However, we observe that pretreatment of L5178Y-ML25 cells with a monoclonal antibody HepSS-1, which binds

heparan sulfate glycosaminoglycans of a variety of cells,[30] failed to inhibit either tumor cell adhesion to H-271 *in vitro* or liver metastasis *in vivo*. Moreover, enzymatic degradations of polysaccharides on the cell surface by heparitinase or chondroitinase ABC did not affect the tumor cell adhesion and liver metastasis of L5178Y-ML25 cells (data not shown). These results suggest that the interaction of L5178Y-ML25 cells with H-271 or CH-271, although it remains to be elucidated in detail, is not mediated by heparan sulfate and chondroitin sulfate glycosaminoglycans, but is mediated by other molecules such as other glycans or integrin receptors. Recently, Liao *et al.* have shown that some lymphoid cell lines adhere to the heparin-binding domain in an RGD-independent manner, and that the interaction is not mediated by cell surface glycosaminoglycans but is mediated by a certain protein.[31]

We have demonstrated that the inhibitory effect of CH-271 fusion polypeptide on liver metastasis of L5178Y-ML25 cell is partly mediated through interference with the adhesive interaction of tumor cells with ECM or basement membrane components via a heparin-binding domain-dependent mechanism. Our previous study showed that RGD-containing polypeptides such as poly(RGD) and C-274 could inhibit pulmonary metastasis of various types of tumors (melanoma, Lewis lung carcinoma and colon carcinoma) by an RGD-dependent mechanism.[14, 15, 32] More recently, CH-271 was also observed to inhibit liver metastasis of RAW117-H10 B-lymphoma cells through a C-274-dependent mechanism (data not shown). The present study clearly indicated that the inhibitory effects of domain polypeptides on liver metastasis of L5178Y-ML25 cells are reversed as compared with those described in our previous reports.[14, 15, 32] These results imply that multiple cell surface receptors or molecules including integrins interact with fibronectin, apparently at different sites in the molecules. The difference of such interactions may depend on the cell types of tumors or target organs and tissues for metastasis. Further study will be needed to identify the regulatory mechanisms and the molecules involved in detail. We recently found that multiple administrations of CH-271 after inoculation of L5178Y-ML25 cells can inhibit liver metastasis and significantly improve the survival rate of mice. Thus, CH-271 fusion polypeptide may provide a therapeutic benefit for the prevention of metastasis of various types of tumors.

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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. Cancer metastasis: organ colonization and the cell surface properties of malignant cells. *Biochim. Biophys. Acta,* **695,** 113–176 (1982).
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. N. and Barsky, S. H. Tumor invasion and the extracellular matrix. *Lab. Invest.,* **49,** 636–649 (1983).
5) McCarthy, J. B., Basara, M. L., Palm, S. L., Sas, D. F. and Furcht, L. T. The role of cell adhesion proteins, laminin and fibronectin, in the movement of malignant and metastatic cells. *Cancer Metastasis Rev.,* **4,** 125–152 (1985).
6) Hynes, R. O. Integrins: a family of cell surface receptors. *Cell,* **48,** 549–554 (1987).
7) Pierschbacher, M. D. and Rouslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of molecule. *Nature,* **309,** 30–33 (1984).
8) Ruoslahti, E. and Pierschbacher, M. D. Arg-Gly-Asp: a versatile cell recognition signal. *Cell,* **44,** 517–518 (1986).
9) Ruoslahti, E. and Pierschbacher, M. D. New perspective in cell adhesion: RGD and integrins. *Science,* **238,** 491–497 (1987).
10) Roossien, F. F., de Kuiper, P. E., de Rijk, D. and Roos, E. Invasive and metastatic capacity of revertants of LFA-1-deficient mutant T-cell hybridomas. *Cancer Res.,* **50,** 3509–3513 (1990).
11) 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).
12) Iwamoto, Y., Robey, F. A., Graf, J., Sasaki, M., Kleinman, H. K., Yamada, Y. and Martin, G. R. YIGSR, a synthetic laminin pentapeptide, inhibits experimental me-

tastasis formation. *Science,* **238,** 1132–1134 (1987).
13) 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).
14) 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 sequences of cell-adhesive molecules. *Cancer Res.,* **49,** 3815–3822 (1989).
15) Saiki, I., Murata, J., Makabe, T., Matsumoto, Y., Ohdate, Y., Kawase, Y., Taguchi, Y., Shimojo, T., Kimizuka, F., Kato, I. and Azuma, I. Inhibition of lung metastasis by synthetic and recombinant fragments of human fibronectin with functional domains. *Jpn. J. Cancer Res.,* **81,** 1003–1011 (1990).
16) McCarthy, J. B., Hagen, S. T. and Furcht, L. T. Human fibronectin contains distinct adhesion- and motility-promoting domains for metastatic melanoma cells. *J. Cell Biol.,* **102,** 179–188 (1986).
17) Watanabe, Y., Okura, A., Naito, K. and Kobayashi, M. Murine liver metastasis model using L5178Y-ML lymphoma and the effect of antitumor agents on the metastasis. *Jpn. J. Cancer Res.,* **79,** 1208–1216 (1988).
18) 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).
19) 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).
20) Kimizuka, F., Ohdate, Y., Kawase, Y., Shimojo, T., Taguchi, Y., Hashino, K., Goto, S., Hashi, H., Kato, I., Sekiguchi, K. and Titani, K. Role of type III homology repeats in cell adhesive function within the cell-binding domain of fibronectin. *J. Biol. Chem.,* **266,** 3045–3051 (1991).
21) Carnemolla, B., Borsi, L., Zardi, L., Owens, R. J. and Baralle, F. Localization of the cellular-fibronectin-specific epitope recognized by the monoclonal antibody IST-9 using fusion proteins expressed in *E. coli. FEBS Lett.,* **215,** 269–273 (1987).
22) Katayama, M., Hino, F., Odate, Y., Goto, S., Kimizuka, F., Kato, I., Titani, K. and Sekiguchi, K. Isolation and characterization of two monoclonal antibodies that recognize remote epitopes on the cell-binding domain of human fibronectin. *Exp. Cell Res.,* **185,** 229–236 (1989).
23) Castellani, P., Siri, A., Rosellini, C., Infusini, E., Borsi, L. and Zardi, L. Transformed human cell release different fibronectin variants than do normal cells. *J. Cell Biol.,* **103,** 1671–1677 (1986).
24) 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).
25) McCarthy, J. B., Skubitz, A. P. N., Zhao, Q., Ya, X-Y., Mickelson, D. J., Klein, D. J. and Furcht, L. T. RGD-independent cell adhesion to the carboxy-terminal heparin-binding fragment of fibronectin involves heparin-dependent and -independent activities. *J. Cell Biol.,* **110,** 777–787 (1990).
26) Cole, G. J., Schubert, D. and Glaster, L. Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. *J. Cell Biol.,* **100,** 1192–1199 (1985).
27) Cole, G. J., Loewy, A. and Glaser, L. Neuronal cell-cell adhesion depends on interaction of N-CAM with heparin-like molecules. *Nature,* **320,** 445–447 (1986).
28) Edgar, D., Timpl, R. and Thoenen, H. The heparin binding domain of laminin is responsible for its effects on neurite outgrowth and survival. *Eur. Mol. Biol. Organ J.,* **3,** 1463–1468 (1984).
29) Stamatoglou, S. C. and Keller, J. M. Correlation between cell substrate attachment *in vitro* and cell surface heparan sulfate affinity for fibronectin and collagen. *J. Cell Biol.,* **96,** 1820–1823 (1983).
30) Kure, S. and Yoshie, O. A syngeneic monoclonal antibody to murine Meth-A sarcoma (HepSS-1) recognizes heparan sulfate glycosaminoglycan (HS-GAG): cell density and transformation dependent alternation in cell surface HS-GAG defined by HepSS-1. *J. Immunol.,* **137,** 3900–3906 (1986).
31) Liao, N., John, J. S., McCarthy, J. B., Furcht, L. T. and Cheung, H. T. Adhesion of lymphoid cells to the carboxyl-terminal heparin-binding domains of fibronectin. *Exp. Cell Res.,* **181,** 348–361 (1989).
32) 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).
33) Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, 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).
34) Saiki, I., Matsumoto, Y., Murata, J., Makabe, T., Yoneda, J., Okuyama, H., Kimizuka, F., Ishizaki, Y., Kato, I. and Azuma, I. Recombinant fusion polypeptide with cell- and heparin-binding domains of fibronectin inhibits liver metastasis of L5178Y-ML25 lymphoma cells. *Jpn. J. Cancer Res.,* **82,** 1120–1129 (1991).