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Antimetastatic Effect by Anti-adhesion Therapy with Cell-adhesive Peptide of Fibronectin in Combination with Anticancer Drugs

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We have investigated the therapeutic effect of CH-271 fusion polypeptide containing both cell-binding domain (C-274) and heparin-binding domain (H-271) of fibronectin in combination with anticancer drugs such as doxorubicin (DOX) or mitomycin C (MMC) on tumor metastasis of different types of tumors. CH-271 fusion polypeptide alone significantly inhibited both liver and lung metastasis when it was co-injected with L5178Y-ML25 T-lymphoma, RAW117-H10 B-lymphoma or B16-BL6 melanoma cells, and spontaneous lung metastasis of B16-BL6 melanoma cells when administered i.v. seven times before or after surgical excision of the primary tumors. Combined treatments with CH-271 and either DOX or MMC significantly inhibited liver and lung metastasis of lymphoma or melanoma cells respectively, as compared with either treatment alone or the untreated control. Administrations of CH-271 and DOX in combination substantially prolonged the survival time of mice injected i.v. with L5178Y-ML25 cells. CH-271 or DOX was effective for inhibiting the invasion of L5178Y-ML25 cells into Matrigel in a concentration-dependent manner. Our previous study has shown that CH-271-mediated inhibition of tumor invasion may be due in part to the anti-cell adhesive property without affecting the cell growth, whereas the anti-invasive effect of DOX was established to have resulted from the growth inhibition of tumor cells. Moreover, the combination of CH-271 with DOX provided a more effective inhibition of tumor invasion into Matrigel than did either alone. Thus, we have demonstrated that the combination of anti-cell adhesive CH-271 and anticancer drugs such as DOX or MMC, i.e. anti-adhesion therapy and chemotherapy, is a new approach that offers enhanced (additive) inhibitory effects on tumor metastasis and invasion.

Key words: Metastasis — Recombinant fibronectin fragment — Anticancer drug — Combined therapy

Metastasis in one of the major causes of mortality in cancer. During the metastatic cascade, metastasizing tumor cells interact with various host cells (platelets, lymphocytes or endothelial cells) and/or extracellular matrices and basement membrane components (fibronectin, laminin, etc.).[1–4] Such an encounter or adhesive interaction may lead to the enhancement of survival, arrest, or invasiveness of tumor cells and is a fundamental event in the metastatic cascade.[2,4–7] Therefore, an understanding of the regulatory mechanism of metastasis may help in the development of antimetastatic therapies.

DNA technology has allowed us to identify the primary structures of some cell adhesive proteins such as fibronectin,[8] laminin[9,10] and vitronectin,[11] and the cell surface receptors for some adhesive molecules including integrins. A common and characteristic Arg-Gly-Asp (RGD[4]) sequence in the cell-binding domain of fibronectin and other related adhesion molecules has been shown to contribute to cell functions including adhesion, spreading and migration.[12–14] Several studies have suggested that some synthetic peptides derived from adhesion molecules that are present in cell matrices, basement membranes or plasma can modulate the mechanism involved in the metastasizing function of tumor cells. Among the attempts to regulate the mechanism involved in cell adhesion during the metastatic process (referred to as "anti-adhesion therapy"), administration of fibronectin-derived peptides such as RGDS,[15,16] CS1[17,18] of alternative splicing type III connecting segment (III CS), and purified 33-kDa tryptic/catheptic heparin-binding fragment[19,20] has been used to inhibit experimental tumor metastasis in murine tumor systems. Try-Ile-Gly-Ser-Arg (YIGSR) derived from laminin has also been shown to inhibit lung metastasis when co-injected i.v. with tumor cells.[21] Recently, some attempts to control tumor metastasis have been extensively carried out by using synthetic cell-adhesive peptide analogues including cyclic RGDS or cyclic YIGSR peptides.[22–24]

We have previously reported that poly(RGD), which contains a repetitive structure of RGD sequence, in-

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[4] The abbreviations used are: RGD, Arg-Gly-Asp; YIGSR, Tyr-Ile-Gly-Ser-Arg; MEM, Eagle's minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DOX, doxorubicin; MMC, mitomycin C; IUdR, iododeoxyuridine.

hibited experimental and spontaneous lung metastases of murine melanoma cells, as well as cell-adhesive properties, more effectively than RGD-containing oligopeptides.[25-27] More recently, we 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-271) or their mixture (C-274+H-271) when they were co-injected with tumor cells or separately injected after tumor inoculation.[28] Furthermore, treatment with CH-271 substantially prolonged the survival time of mice injected i.v. with lymphoma cells.[29]

In the present study, we extended our previous study to examine the inhibitory effect of the fusion polypeptide CH-271 on metastasis of different types of tumors. In addition, we focused our attention on the combined effect of CH-271 and anticancer agents on tumor metastasis, i.e., on the ability of the combination of anti-adhesion therapy and chemotherapy to offer augmented therapeutic potential.

MATERIALS AND METHODS

Mice Specific pathogen-free female C57BL/6 and CDF1 (BALB/c×DBA2) mice, 7–10 weeks old, were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. Male BALB/c ByJJcl mice were obtained from CLEA Japan Inc., Tokyo. Mice were maintained in the Laboratory for 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,[30] were kindly provided by Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX. Liver metastatic L5178Y-ML25 T-lymphoma cells[31] (partially metastasizing to the spleen), were kindly provided by Dr. A. Okura, Banyu Pharmaceutical Co. Ltd., Tokyo. RAW117-H10 B-lymphoma cells, which selectively metastasize to the liver,[32] were obtained from Dr. G. L. Nicolson, M.D. Anderson 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, nonessential amino acids, and L-glutamine. L5178Y-ML25 and RAW117-H10 cells were maintained in RPMI-1640 supplemented with 7.5% FBS and L-glutamine.

Recombinant fibronectin fragments We prepared three kinds of recombinant fibronectin fragments (C-274, H-271, CH-271) by expressing human fibronectin cDNA in *E. coli*, using an expression vector pUC118N/119N first described by Maki *et al.*[33] C-274 and H-271 correspond to cell- and heparin-binding domains of fibronectin, respectively, while the other polypeptide, CH-271, is a fusion protein with both cell- and heparin-binding domains (Fig. 1). Two plasmids, pLF5 and pLF2435, were used as a source of cDNA.[34] 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

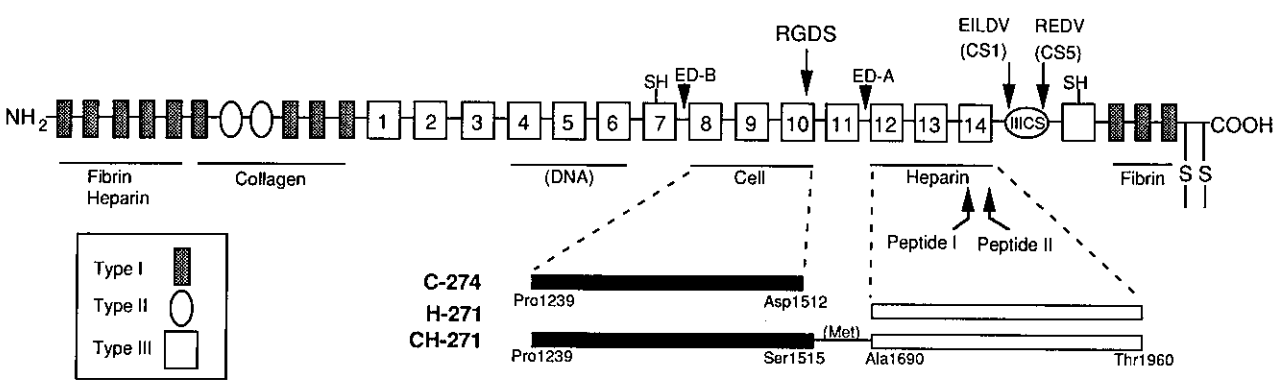

Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open and closed bars. The cell-binding polypeptide C-274 [Pro1239-Asp1512; the sequence is numbered according to the system of Kornblihtt *et al.*[8]] covers three units of type III homology at the 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 RGDS, EILDV (CS1) and REDV (CS5) sites (↓). ED-A and ED-B indicate that extra domains arise from alternative splicing, respectively.

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 pLF 2435 and pUC118N. The fusion protein CH-271 was expressed by use of a recombinant plasmid pCH-101; this had been constructed from pHD 101 and pTF-7121. Detailed accounts of these constructions and expressions have been given elsewhere.[35] 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.[28] 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). 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).

Chemical reagents Reconstituted basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) was purchased from Collaborative Research Inc., MA. Doxorubicin (DOX) and mitomycin C (MMC) were purchased from Kyowa Hakko Co. Ltd., Tokyo.

Assay for liver metastasis of lymphoma cells CDF1 or BALB/c mice were given i.v. injections of L5178Y-ML25 T-lymphoma cells (4×10^4) or RAW117-H10 B-lymphoma cells (5×10^3), respectively, with or without recombinant fragments of fibronectin in PBS. In the therapeutic experiments, mice were treated i.v. with recombinant fibronectin fragment CH-271 in combination with or without anticancer agents at various time points after tumor inoculation. Nine to sixteen days later, the mice were killed and the weights of liver and spleen were recorded to evaluate tumor metastasis as previously described.[28] The survival time of the animals given i.v. injections of tumor cells and treated with the combination of CH-271 fusion polypeptide and anticancer agents 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 percent of survivors was calculated as a function of time.

Assay for experimental and spontaneous lung metastases of melanoma cells C57BL/6 mice were given i.v. injection of B16-BL6 melanoma cells (5×10^4) admixed with recombinant fragment of fibronectin. Fourteen days after the inoculation of tumor cells, the mice were killed and the number of lung tumor colonies was recorded (experimental metastasis). In a spontaneous lung metastasis assay, mice were given s.c. injections of B16-BL6 melanoma cells (5×10^5) into the right hind footpad. The recombinant fibronectin fragments were administered i.v. on various days before or after the surgical excision of primary tumors on day 21. Mice were killed 14 days after the amputation. The lungs were fixed in Bouin's solution and the lung tumor colonies were counted under a dissecting microscope. In the combination experiments, CH-271 and/or anticancer agents in combination were administered i.v. at various time points after i.v. inoculation of melanoma cells.

Invasion assay The invasive activity of tumor cells was assayed in Transwell cell culture chambers (No. 3422; Costar, Cambridge, MA) according to the methods previously described[36] with some modifications. Matrigel (100 μg/filter) in a volume of 10 μl was applied to the lower surface of polyvinylpyrrolidone-free polycarbonate filters with 8 μm pore size (Nucleopore, Pleasanton, CA), and kept for 30 min in a 5% CO_2 atmosphere. L5178Y-ML25 cells in an exponential growth phase were incubated for 24 h in RPMI-1640 containing 7.5% 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 with PBS to remove unbound radiolabel. Labeled tumor cells (2×10^5) with or without agents in a volume of 100 μl were added to the upper compartment of a chamber, and incubated for an appropriate number of hours at 37°C in a 5% CO_2 atmosphere. Matrigel containing labeled cells on the lower surface was wiped and absorbed on cotton swabs, and monitored for radioactivity by γ-counting. The number of cells that had invaded through the filter into the Matrigel was calculated from the radioactivity, and each assay was performed in triplicate.

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 CH-271 fusion polypeptide on metastasis of different types of tumors Our previous study demonstrated that CH-271 fusion polypeptide with the cell- and heparin-binding domains was more effective for the reduction of liver metastasis of L5178Y-ML25 T-lymphoma cells (i.e. liver weight) than C-274+H-271 (similar molar ratio to CH-271, 1:1) when co-injected i.v. with tumor cells.[28,29] Histological analysis revealed that

enlarged liver in the untreated group exhibited diffuse infiltration of tumor cells and no liver cells were seen,[28] indicating the relationship with the increase in liver weight. We first investigated the effect of CH-271 fusion polypeptide on liver and lung metastasis produced by i.v. injection of L5178Y-ML25, RAW117-H10 lymphoma or melanoma cells, and on spontaneous lung metastasis produced by s.c. injection of B16-BL6 melanoma cells (Tables I and II). When L5178Y-ML25 or RAW117-H10 cells were injected i.v. into the corresponding mice, liver weights of the mice were increased approximately 2- to 4-fold compared with those of normal mice (Table I). The co-injection of L5178Y-ML25 or RAW117-H10 cells with recombinant fusion polypeptide CH-271 significantly inhibited liver metastasis. C-274+H-271 at similar molar ratio to CH-271 (1:1) did not exhibit antimetastatic activity. Similarly, CH-271 significantly reduced the number of tumor colonies in the lungs when given by co-injection with B16-BL6 cells (Expt. 1 of Table II). In the spontaneous lung metastasis model using B16-BL6 melanoma cells (Expt. 2 and 3 of Table II), seven intermittent administrations of CH-271 before or after surgical excision of primary tumors on day 21 achieved a statistically significant reduction of lung tumor colonies, but the administrations of C-274+H-271 apparently had no effect. Experiment 2 of Table II shows that i.v. administration of the polypeptides before the amputation did not affect the primary tumor size (growth) at the time of amputation (on day 21). The present results and our previous studies using recombinant fibronectin fragments[28,29] indicated that CH-271 fusion polypeptide was active in inhibiting liver and lung metastases of three different types of tumors when co-injected or separately injected with tumor cells, thus implying the therapeutic potential of CH-271 for tumor metastasis.

Table I. Effect of Recombinant Domain Polypeptides of Fibronectin on Liver Metastasis by i.v. Injection of Murine Lymphoma Cells

Administered i.v. with:	Dose (μg/mouse)	Mean weight (g) ± SD	
		Liver	Spleen
L5178Y-ML25			
Untreated (PBS)		4.56±0.52	0.20±0.03
CH-271	500	1.72±0.68**	0.12±0.03*
C-247+H-271	250+250	4.03±0.70	0.17±0.03
(normal)		1.21±0.09	0.07±0.01
RAW117-H10			
Untreated (PBS)		1.82±0.04	0.28±0.02
CH-271	500	1.15±0.11**	0.14±0.01**
(normal)		1.05±0.08	0.12±0.02

Five CDF1 or BALB/c mice per group were injected i.v. with L5178Y-ML25 (4×10^5) or RAW117-H10 cells (5×10^3), respectively, with or without recombinant polypeptides of fibronectin. Mice were killed 16 (CDF1) or 9 days (BALB/c) after tumor inoculation.
*: $P<0.01$. **: $P<0.001$.

Table II. Effect of Recombinant Polypeptides of Fibronectin on Experimental and Spontaneous Lung Metastasis of B16-BL6 Melanoma Cells

Administered i.v. with:	Dose (μg/mouse ×times)	Primary tumor size on day 21 (mm±SD)	No. of lung metastasis on day 35	
			Mean±SD (Range)	
Expt. 1				
Untreated (PBS)			76±15	(58–98)
CH-271	500		42±12	(32–12)*
	1000		29±7	(23–39)**
	2000		21±6	(16–30)**
Expt. 2				
Untreated (PBS)		10±3	53±15	(37–68)
CH-271	100×7	10±3	29±10	(22–43)*
C-H274+H-271	(50+50)×7	10±3	60±13	(45–75)
Expt. 3				
Untreated (PBS)		(11±2)	43±7	(35–51)
CH-271	100×7		22±5	(16–28)**

Five C57BL/6 mice per group were inoculated i.v. with B16-BL6 cells (5×10^4) admixed with or without CH-271. Tumor colonies in the lungs were counted 2 weeks after tumor inoculation (Expt. 1). Five C57BL/6 mice per group were administered i.v. with fibronectin polypeptides on days 7, 9, 11, 13, 15, 17 and 19 (Expt. 2) or days 22, 24, 26, 28, 30, 32 and 34 (Expt. 3) after s.c. injection of B16-BL6 cells (5×10^5). Primary tumors were removed on day 21 and mice were killed 2 weeks after tumor excision. *: $P<0.02$. **: $P<0.005$.

Combined effects of CH-271 fusion peptide and anticancer agents on tumor metastasis We have previously shown that antimetastatic activity mediated by such cell-adhesive peptides, including CH-271, was partly due to interference with the adhesive interaction of tumor cells with extracellular matrix or basement membrane components, influencing tumor cell adhesion, migration and invasion, but was not due to direct antiproliferative activity.[25, 26, 28] This indicates that the mechanism involved in the inhibition of tumor metastasis by anti-cell adhesive peptide CH-271 is different from that of chemotherapeutic agents such as doxorubicin (DOX) and mitomycin C (MMC). In order to augment the antimetastatic effect of CH-271 more effectively, therefore, we examined the therapeutic effect of multiple administrations of CH-271 fusion peptide in combination with an antiproliferative agent (DOX or MMC) on tumor metastasis of L5178Y-ML25 or B16-BL6 cells. CDF1 mice were treated i.v. with 200 μg of DOX and/or 250 μg of CH-271 at various days after i.v. injection of L5178Y-ML25 cells (Table III). Administrations of either DOX on day 5 or CH-271 on days 1, 2, 3 and 4 after tumor inoculation caused significant inhibition of liver metastasis of L5178Y-ML25 cells, while the administrations of DOX on day 9 or CH-271 on days 5, 6, 7 and 8 or days 10, 11, 12 and 13 did not have any inhibitory effect. This result implies that treatment with DOX or CH-271 during the early period after tumor inoculation is effective in the inhibition of tumor metastasis in this system. On the other hand, various combination administrations of DOX and CH-271 used in this study resulted in significant reductions of liver weight as compared with the untreated control. In particular, the administration of CH-271 on days 1, 2, 3 and 4 followed by DOX on day 5, or the administration of DOX on day 5 followed by CH-271 on days 5, 6, 7 and 8 dramatically inhibited liver metastasis as compared with the administration of either DOX or CH-271 alone. Similar results on the inhibitory effect on liver metastasis were obtained when the combination of CH-271 and MMC in place of DOX was used (Table IV). Combined treatments with MMC on day 5

Table III. Combined Effect of Doxorubicin (DOX) and CH-271 on Liver Metastasis by i.v. Injection of L5178Y-ML25 T-Lymphoma Cells

Treatment	(Days after tumor inoculation)	Liver weight(g) ± SD on day 16
Untreated (PBS)		4.51 ± 0.31
DOX (day 5)		3.49 ± 0.25**
DOX (day 9)		4.02 ± 0.24
CH-271 (days 1, 2, 3, 4)		2.55 ± 1.05**
CH-271 (days 5, 6, 7, 8)		3.94 ± 0.58
CH-271 (days 10, 11, 12, 13)		4.47 ± 0.28
DOX (day 5) +CH-271 (days 1, 2, 3, 4)		1.73 ± 0.74***
DOX (day 5) +CH-271 (days 6, 7, 8, 9)		1.75 ± 0.71***
DOX (day 9) +CH-271 (days 5, 6, 7, 8)		3.65 ± 0.44*
DOX (day 9) +CH-271 (days 10, 11, 12, 13)		3.86 ± 0.32*
(Normal)		1.04 ± 0.03

Five CDF1 mice per group were administered i.v. with DOX (200 μg) and/or CH-271 (250 μg) at the indicated days after the i.v. injection of L5178Y-ML25 cells (4×10^4). Mice were killed 16 days after tumor inoculation. *: $P<0.05$. **: $P<0.05$. ***: $P<0.001$. n.s: not significant.

Table IV. Combined Effect of Mitomycin C (MMC) and CH-271 on Liver Metastasis by i.v. Injection of L5178Y-ML25 T-Lymphoma Cells

Treatment	(Days after tumor inoculation)	Liver weight(g) ± SD on day 16
Untreated (PBS)		4.52 ± 0.81
MMC (day 5)		2.85 ± 0.54**
CH-271 (days 1, 2, 3, 4)		1.92 ± 0.89**
CH-271 (days 6, 7, 8, 9)		3.81 ± 0.33
MMC (day 5) +CH-271 (days 1, 2, 3, 4)		1.76 ± 0.62***
MMC (day 5) +CH-271 (days 6, 7, 8, 9)		1.68 ± 0.70***

Five CDF1 mice per group were administered i.v. with MMC (20 μg) and/or CH-271 (250 μg) at the indicated days after the i.v. injection of L5178Y-ML25 cells (4×10^4).
*: $P<0.05$. **: $P<0.01$. ***: $P<0.001$. n.s.: not significant.

Table V. Combined Effect of Doxorubicin (DOX) and CH-271 on Experimental Lung Metastasis by i.v. Injection of B16-BL6 Melanoma Cells

Treatment	(Days after tumor inoculation)	No. of lung metastasis on day 14	
		Mean ± SD	(Range)
Untreated (PBS)		186 ± 40	(117–227)
DOX (day 5)		110 ± 13	(98–128)*
CH-271 (days 1, 2, 3, 4)		77 ± 9	(68–93)*
DOX (days 5) +CH-271 (days 1, 2, 3, 4)		42 ± 8	(34–50)*

Five CDF1 mice per group were treated i.v. with DOX (200 μg) and/or CH-271 (250 μg) at the indicated days after the i.v. injection of B16-BL6 melanoma cells (5×10^4). Mice were killed 14 days after tumor inoculation. *: $P<0.001$.

and CH-271 on days 1, 2, 3 and 4 or days 6, 7, 8 and 9 after tumor inoculation achieved significant inhibition of liver metastasis as compared with either solitary treatment or the untreated control.

Furthermore, we examined the effect of combination therapy with DOX and CH-271 on lung metastasis produced by i.v. injection of B16-BL6 melanoma cells. Table V shows that the combination of CH-271 on days 1, 2, 3 and 4 and DOX on day 5 reduced the number of lung tumor colonies dramatically as compared with the administrations of DOX or CH-271, or the untreated control. These results clearly indicated that the combination of anti cell-adhesive peptide CH-271 and anticancer agent (DOX or MMC) showed an enhanced therapeutic effect on liver and lung metastases of two different tumors as compared with either separately. The survival rate of mice given i.v. administrations of CH-271 and/or DOX after the inoculation of L5178Y-ML25 cells was also determined (Fig. 2). In this experiment, all the untreated control mice succumbed to the tumor burden within 20 days after the injection. Similar enhanced survival rates were observed in the group of mice which received either CH-271 on days 1, 2, 3 and 4 or DOX on day 5. The group that received CH-271 followed by DOX showed a significantly prolonged survival time ($0.01 < P < 0.05$ by Mann-Witney's U-test), but 9 out of 10 mice had succumbed within 25 days of the tumor inoculation.

Effect of CH-271 and/or DOX 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, 37] To investigate the

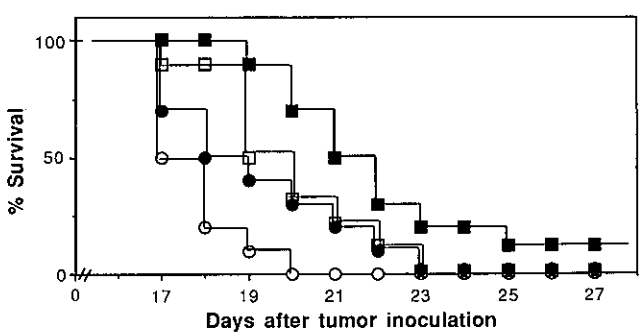

Fig. 2. Effect of combination administrations of CH-271 peptide and DOX on the survival of CDF1 mice injected with L5178Y-ML25 lymphoma cells. Ten mice per group were administered i.v. with PBS (○), 500 μg of CH-271 on days 1, 2, 3 and 4 (●), 200 μg of DOX on day 5 (□) or the combination of 500 μg of CH-271 on days 1, 2, 3 and 4 and 200 μg of DOX on day 5 (■) after i.v. injection of tumor cells. Animal survival was monitored as a function of time. $P < 0.01$; CH-271+DOX vs. untreated control, DOX vs. untreated control. $0.01 < P < 0.05$; CH-271+DOX vs. DOX.

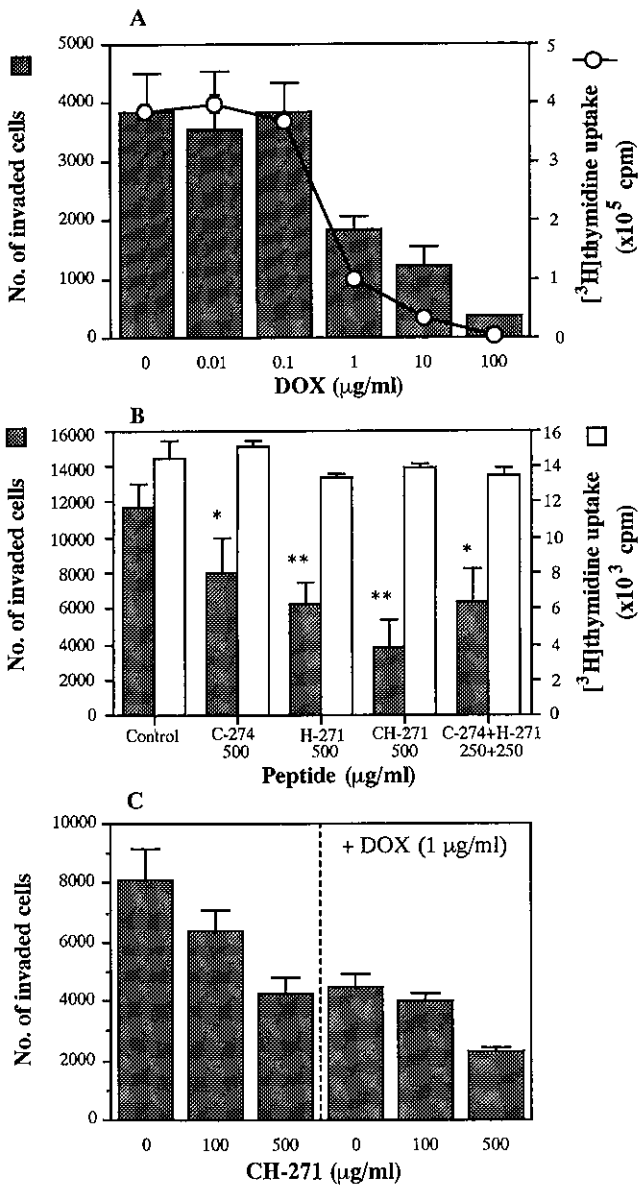

Fig. 3. Effect of recombinant fibronectin peptides and/or DOX or tumor cell invasion into Matrigel-coated filters. The filter of a Transwell cell culture chamber was precoated with 100 μg of Matrigel on the lower surface. ^{125}I-labeled L5178Y-ML25 cells (2×10^5/well) in 0.1% BSA-medium were seeded with various concentrations of DOX (A), with recombinant fibronectin fragments (B) or with CH-271 in combination with 1 μg/ml DOX (C) into the upper compartment. After an 8-h incubation, radioactivity of the cells that had invaded the Matrigel was measured by γ-counting (hatched bars). In the assay for tumor growth inhibition, L5178Y-ML25 cells (1×10^4) were incubated for 8 h with or without various concentrations of DOX or recombinant fibronectin peptides in the presence of 0.5 μCi of [^{3}H]thymidine, and the radioactivity was counted (open circles or bars). *: $P < 0.05$. **: $P < 0.01$.

mechanism of the inhibitory effect of CH-271 and DOX in combination on tumor metastases of different tumors, we examined whether or not antimetastatic peptide CH-271 and/or the anticancer agent DOX were able to inhibit the invasion of L5178Y-ML25 cells into reconstituted basement membrane Matrigel. The invasion of tumor cells through Matrigel-coated filters was significantly inhibited by the addition of DOX in the upper compartment of the chamber in a concentration-dependent manner (Fig. 3A). DOX at the concentration of 1 μg/ml showed approximately 50% inhibition of the invasive activity of tumor cells. At the same time, the incorporation of [^{3}H]thymidine into the tumor cells was also suppressed by the 8-h incubation with DOX in parallel with the anti-invasive effect. On the other hand, as shown in Fig. 3B, the recombinant fibronectin fragments used in this study significantly inhibited tumor cell invasion into Matrigel, although they did not affect the tumor cell growth *in vitro*. In particular, CH-271 fusion peptide was more effective for the inhibition of tumor cell invasion than C-274, H-271 or C-274+H-271 (similar molar ratio to CH-271, 1:1). Addition of CH-271 together with 1 μg/ml DOX to the upper compartment resulted in a more effective inhibition of tumor cell invasion than could be obtained with either DOX or CH-271 alone (Fig. 3C). These results indicated that the anti-invasive effect of DOX was due to its growth inhibition of tumor cells, and that the combination of CH-271 with DOX showed enhanced inhibition of tumor cell invasion into Matrigel. Although the co-presence of CH-271 may promote the growth-inhibitory effect of DOX, the combination of CH-271 at a concentration ranging from 1 to 500 μg/ml with DOX (0.001 to 10 μg/ml) showed a similar pattern of growth inhibition to that of tumor cells treated with DOX alone (data not shown).

DISCUSSION

Tumor adhesion and motility to extracellular matrix and basement membrane components including fibronectin are important in the process of tumor metastasis.[1-6] Previous studies have utilized proteolytic fragments or synthetic peptides including RGDS and YIGSR derived from fibronectin or laminin to inhibit experimental lung metastasis following co-injection with tumor cells.[15, 16, 21] We have attempted to elucidate the regulatory mechanisms involved in cell functions such as adhesiveness, motility and invasiveness during the metastatic process. Our previous study indicated that synthetic poly(RGD), which contains a repetitive RGD sequence, CSl in IIICS region of fibronectin or recombinant fibronectin fragments with functional domains exhibited inhibitory effects on experimental and spontaneous lung metastases of murine melanoma, tumor cell adhesion to extracellular matrix, the penetration of tumor cells through reconstituted basement membranes *in vitro* and tumor-induced angiogenesis in syngeneic mice.[18, 26-29, 38] More recently, a recombinant fusion polypeptide containing the cell- and heparin-binding domains of fibronectin, CH-271, has been shown to inhibit liver metastasis of L5178Y-ML25 lymphoma and to prolong the survival rate of mice when it was co-injected or separately injected after tumor inoculation.[28, 29]

To extend our previous observations on the inhibition of tumor metastasis by synthetic and recombinant polypeptides, we have examined whether or not CH-271 fusion peptide was able to inhibit lung and liver metastases of different types of tumors, and also whether or not the combination of anti-cell adhesive CH-271 peptide with anticancer drugs such as DOX and MMC can lead to enhancement of the inhibitory effect on tumor metastasis and invasion. Co-injection on L5178Y-ML25, RAW117-H10 or B16-BL6 cells with CH-271 resulted in marked suppression of liver and lung metastases as compared with the mixture of C-274 and H-271 (similar molar ratio of CH-271, 1:1) (Tables I and II). Multiple administrations of CH-271 before or after surgical excision of the primary tumors significantly inhibited spontaneous lung metastasis of B16-BL6 melanoma cells without affecting the primary tumor size (growth) at the time of amputation (Table II). The present results indicated that CH-271 fusion peptide of fibronectin was active for the inhibition of liver and lung metastases of three different tumors. The reason for the enhanced antimetastatic effect by CH-271 remains to be determined, but it may include the participation of other receptors than integrins for RGD peptide, or alteration of the binding affinity, because there was no difference in the clearance or stability in the circulation among CH-271, C-274 and H-271 and no cytotoxic effect on normal cells as well as tumor cells.[28] Although some studies of proteolytic fragments of laminin or fibronectin, or synthetic RGD-containing peptides of fibronectin have been performed with an experimental metastasis model involving co-injection with tumor cells,[15, 16, 20-23, 39] it is particular note that, in our experiments with a spontaneous metastasis model, CH-271 showed therapeutic potential against tumor metastasis following systemic administration.

To enhance further the CH-271-mediated anti-metastatic effect, we tested combined therapy with CH-271 and anticancer drugs. The administrations of CH-271 on days 1, 2, 3 and 4 followed by DOX on day 5 or the administrations of DOX on day 5 followed by CH-271 on days 5, 6, 7 and 8 dramatically inhibited liver metastasis of lymphoma cells as compared with the administrations of either DOX or CH-271 alone (Table III). Similar results for the enhancement of inhibitory effect on tumor metastasis were also obtained with the combi-

nation of CH-271 and DOX in a pulmonary metastasis model involving i.v. injection of B16-BL6 melanoma cells (Tables IV and V). In addition, the mice that received CH-271 on days 1, 2, 3 and 4 followed by DOX on day 5 showed a significant prolongation of survival time as compared with either CH-271 or DOX alone, or the untreated control, but the effect was not great (Fig. 2). These results indicate that the combination of CH-271 and either DOX or MMC, i.e. anti-adhesion therapy and chemotherapy, clearly produced an enhancement of inhibitory effect on tumor metastasis. However, the design of combined treatments, including timings, doses and schedules, will need to be improved and optimized to enhance further the antimetastatic effect.

Tumor cell invasion through several connective tissue barriers which contain adhesion molecules is an important step in the process of tumor metastasis.[1, 2, 4, 5, 37] Fig. 3B and C showed that recombinant fibronectin fragments significantly inhibited the invasion of L5178Y-ML25 cells into reconstituted basement membrane Matrigel without showing direct cytotoxicity or affecting the growth of tumor cells. CH-271 fusion peptide was particularly effective for the inhibition of tumor cell invasion as compared with C-274, H-271 and C-274+H-271. We have also shown that CH-271 was able to inhibit the adhesion of lymphoma cells to substrates precoated with fibronectin, laminin and Matrigel through RGDS-dependent or -independent mechanisms.[29] Thus, CH-271-mediated inhibition of tumor metastasis may be partly due to interference with the adhesive interaction of tumor cells with extracellular matrix or basement membrane components, including tumor cell adhesion and invasion, but not due to direct cytotoxicity and the inhibition of tumor cell growth (Fig. 3B). In contrast, DOX showed an inhibitory effect on tumor cell invasion in parallel with the inhibition of tumor cell growth *in vitro* (Fig. 3A). The combination of CH-271 and DOX resulted in more effective inhibition of tumor invasion than either CH-271 or DOX alone (Fig. 3C). Thus, the antimetastatic effect of CH-271 and DOX in combination may be associated with the operation of distinct inhibitory mechanisms.

In conclusion, we have demonstrated that the recombinant fusion polypeptide of fibronectin, CH-271, has therapeutic potential against liver and lung metastases of three different types of tumors. Combined treatment with CH-271 and anticancer drugs (DOX or MMC) resulted in an enhanced inhibitory effect on tumor metastasis and invasion, and a significantly prolonged survival of mice injected with lymphoma cells. This indicates that anti-adhesion therapy with cell-adhesive peptide CH-271 in combination with chemotherapy may be potentially useful in the prevention of cancer metastasis and invasion. However, further study will be needed to examine in detail the optimum timings and schedules for the combination treatment, and to examine the mechanisms of the inhibitory effect.

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REFERENCES

1) Fidler, I. J. The Ernst W. Bertner Memorial Award Lecture: the evolution of biological heterogeneity in metastatic nepolasmas. *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**, 331–316 (1986).
6) McCarthy, J. B. and Furcht, L. T. Laminin and fibronectin promote the haptotactic migration of B16-BL6 mouse melanoma cell *in vitro*. *J. Cell Biol.*, **98**, 1414–1480 (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 least 10 polypeptides from a single gene. *EMBO J.*, **4**, 1755–1759 (1985).
9) 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).
10) 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).
11) Suzuki, S., Oldberg, A., Hayman, E. G., Pierschbacher, M. D. and Ruoslahti, 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).
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) 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).
16) 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 (1986).
17) Humphries, M. J., Komoriya, A., Akiyama, S. K., Olden, K. and Yamada, K. M. Indentification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.*, **262**, 6886–6892 (1987).
18) 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 domain. *Jpn. J. Cancer Res.*, **81**, 1003–1011 (1990).
19) 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).
20) McCarthy, J. B., Skubitz, A. P. N., Palm, S. L. and Furcht, L. T. Metastasis inhibition of different tumor types by purified laminin fragment and a heparin-fragment of fibronectin. *J. Natl. Cancer Inst.*, **80**, 108–116 (1988).
21) 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 metastasis formation. *Science*, **238**, 1132–1134 (1987).
22) Kumagai, H., Tajima, M., Ueno, Y., Giga-Hama, Y. and Ohba, M. Effect of cyclic RGD peptide on cell adhesion and tumor metastasis. *Biochem. Biophys. Res. Commun.*, **177**, 74–82 (1991).
23) Kleinman, H. K., Graf, J., Iwamoto, Y., Sasaki, M., Schasteen, C. S., Yamada, Y., Martin, G. R. and Robey, F. A. Identification of a second active site in laminin for promotion of cell adhesion and migration and inhibition of *in vivo* melanoma lung colonization. *Arch. Biochem. Biophys.*, **272**, 39–45 (1989).
24) Tressler, R. J., Belloni, P. N. and Nicolson, G. L. Correlation of inhibition of adhesion of large lymphoma and hepatic sinusoidal endothelial cells by RGD-containing peptide polymers with metastatic potential: role of integrin-dependent and -independent adhesion mechanisms. *Cancer Commun.*, **1**, 55–63 (1989).
25) Saiki, I., Iida, J., Murata, J., Ogwa, 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).
26) Saiki, I., Murata, 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).
27) 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).
28) 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 L-5178Y-ML25 lymphoma cells. *Jpn. J. Cancer Res.*, **82**, 1120–1129 (1991).
29) Matsumoto, Y., Saiki, I., Makabe, T., Yoneda, J., Murata, J., Kimizuka, F., Ishizaki, Y., Kato, I. and Azuma, I. Inhibitory effect of antimetastatic fusion polypeptide of human fibronectin or tumor cell adhesion to extracellular matrices. *Jpn. J. Cancer Res.*, **82**, 1130–1138 (1991).
30) Hart, I. R. The selection and characterization of an invasive variant of the B16 melanoma. *Am. J. Pathol.*, **97**, 587–592 (1979).
31) 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).
32) Brunson, K. W. and Nicolson, G. L. Selection and biologic properties of malignant variants of a murine lymphosarcoma. *J. Natl. Cancer Inst.*, **61**, 1499–1503 (1978).

33) 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).
34) 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 a and b subunits of plasma fibronectin. *Biochemistry,* **25,** 4936–4951 (1986).
35) 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 adhesion function within the cell-binding domain of fibronectin. *J. Cell Biol.*, **5,** 3045–3051 (1991).
36) 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).
37) Liotta, L. A., Rao, C. N. and Wewer, U. M. Biochemical interaction of tumor cells with the basement membrane. *Ann. Rev. Biochem.*, **55,** 1037–1057 (1986).
38) Saiki, I., Murata, J., Makabe, T., Nishi, N., Tokura, S. and Azuma, I. Inhibition of tumor angiogenesis by a synthetic cell-adhesive polypeptide containing the Arg-Gly-Asp (RGD) sequence of fibronectin, poly(RGD). *Jpn. J. Cancer Res.*, **81,** 668–675 (1990).
39) 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).