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Inhibitory Effect of Fibronectin and Its Recombinant Polypeptides on the Adhesion of Metastatic Melanoma Cells to Laminin

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We have utilized recombinant fibronectin fragments with cell-binding domain (C-274), heparin-binding domain (H-271) or CS1 peptide in type III connecting segment (IIICS) and their fusion polypeptides such as CH-296 (containing C-274, H-271 and CS1), CH-271 (containing C-274 and H-271) and C-CS1 (containing C-274 and CS1) to investigate the mechanism of the fibronectin-mediated inhibitory effect on tumor cell adhesion to laminin as well as fibronectin. These fragments retained cell adhesion-promoting and/or heparin-binding properties when they were immobilized on a surface. Pretreatment of tumor cells with CH-296 or CH-271 suppressed cell adhesion to both laminin and fibronectin. H-271 at the high concentration of 500 μg/ml slightly inhibited cell adhesion to laminin (but not to fibronectin), whereas C-274, C-CS1 or a mixture of C-274, H-271 and CS1 (similar molar ratio to CH-296) inhibited cell adhesion to fibronectin but not to laminin. On the other hand, tumor cell adhesion to laminin-substrate was also inhibited by heparin or heparan sulfate, which were able to bind to laminin, suggesting that heparin-like molecules on the cell surface may be included among the laminin receptors. These results indicated that the co-presence of cell- and heparin-binding domains of fibronectin may be required for the fibronectin-mediated inhibitory effect on tumor cell adhesion to laminin, and that the interaction of the heparin-binding domain of fibronectin with the cell surface leads to the inhibition of the cell adhesion to laminin.

Key words: Recombinant fibronectin fragment — Cell adhesion — Laminin — Metastasis — Tumor cells

Extracellular matrix proteins such as fibronectin and laminin play a key role in biological processes associated with normal and pathological development, including cell growth, healing and cancer metastasis. Recent studies on cell adhesion molecules and their surface receptors have to some extent elucidated the molecular events involved in cell-substrate adhesion. Fibronectin is a glycoprotein with multiple functional domains which interact with heparin, cells, or other extracellular matrix components. The Arg-Gly-Asp-Ser (RGDS) sequence in the cell-binding domain of fibronectin has been shown to interact with most cells via cell surface integrins.[1-4] Despite the importance of RGD/integrin complex in fibronectin-mediated cell adhesion, further study using proteolytic fibronectin fragments has provided clear evidence indicating the involvement of other biologically active domains on fibronectin. McCarthy *et al.*[5] have reported that 33-kDa heparin-binding fragment of fibronectin, in which active adhesive peptides I and II were recently found to be present,[6] could promote cell attachment by an RGD-independent mechanism. CS1 peptide in the alternatively spliced type III connecting segment (IIICS) has been shown to promote cell adhesion through an RGD-independent and integrin-dependent mechanism.[7,8]

The interaction of metastatic tumor cells with extracellular matrix and basement membranes is a fundamental event in the sequential multisteps of metastasis.[9] Previous studies have shown that metastatic tumor cells have a high affinity for extracellular matrix proteins including collagens, fibronectin, and laminin, and have the ability to degrade them. The incubation of melanoma cells with laminin caused an increase of invasive activity against basement membrane, whereas incubation with fibronectin resulted in the suppression of invasiveness.[10] These changes have been shown to be correlated with the ability of cells to bind to and invade basement membranes.[10] Recently, proteolytic or synthetic peptides of laminin or fibronectin or antibodies against laminin have also been shown to inhibit lung metastasis when they were coinjected with tumor cells.[11-17] Thus, tumor cell interaction with extracellular matrix components may facilitate or control adhesiveness, invasiveness and metastasis.

In the present study, we have found that soluble fibronectin can inhibit tumor cell adhesion not only to fibronectin but also to laminin. Further study using recombinant fibronectin fragments with functional

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domains demonstrated that fibronectin inhibition of cell adhesion to laminin is mediated by the ability of the heparin-binding domain of fibronectin to compete for the cell surface, and that the heparin-binding domain effectively shows such an inhibitory effect when it is co-present with cell-binding domain or CS1.

MATERIALS AND METHODS

Cells Highly metastatic B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion were kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer, Center Houston, Texas. 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.

Recombinant fibronectin fragments and other reagents We prepared various kinds of recombinant fibronectin fragments (C-274, H-271, CH-271, CH-296 and C-CS1) by expressing human fibronectin cDNA in *E. coli,* using an expression vector pUC118/119N first described by Maki *et al.*[18] C-274 and H-271 correspond to cell- and heparin-binding domains of fibronectin, respectively, while other peptides, CH-271, CH-296 and C-CS1, are fusion proteins containing a cell- or a heparin-binding domain or CS1 sequence (DELPQLVTLPHPNLHGPEILDVPST) in type III connecting segment (IIICS) of fibronectin (Fig. 1). All protein sequences here are numbered according to the system of Kornblihtt *et al.*[19] and all nucleotide sequences are numbered as in the EMBL data-bank file HUMFNMC of Kornblihtt.

Two cDNA clones, pLF5 and pLF2435[20] were used for the construction of expression plasmids. Plasmid pLF5 is a derivative of pUC13, and it contains a 1.4-kb insert corresponding to Thr1218-Thr1778, but lacks the extra domain A of fibronectin at Asn1600-Thr1689. The plasmid pLF2435 was reconstructed from pLF2, 3, 4, and 5, which were described elsewhere.[20] It contained the cDNA fragment coding for a portion of the cell-binding domain and the entire sequences of the second heparin-binding domain and the C-terminal fibrin-binding domain of human fibronectin.

The cell-binding polypeptide C-274 with 274 amino acids (Pro1239-Asp1512) was expressed through a recombinant plasmid pTF7221 which had been constructed mainly from pLF5 and pU119N. The heparin-binding polypeptide H-271 with 271 amino acids (Ala 1690-Thr1960) was expressed by use of a recombinant plasmid pHD101. The fusion polypeptide with cell- and heparin-binding domain CH-271, which was expressed by plasmid pCH101, had the Pro1239-Ser1515 sequence bound via methionine to H-271. CH-296 fusion polypeptide, which was expressed by plasmid pCH102, contained Pro1239-Ser1515 sequence bound via methionine to H-296 that had the additional 25 amino acids of CS1 (Asp1961-Thr1985) at the C-terminus of H-271. C-CS1 polypeptide containing cell-binding domain and CS1, expressed by plasmid pCS25, had Pro1239-Ser1515 bound to CS1. Detailed accounts of the construction and

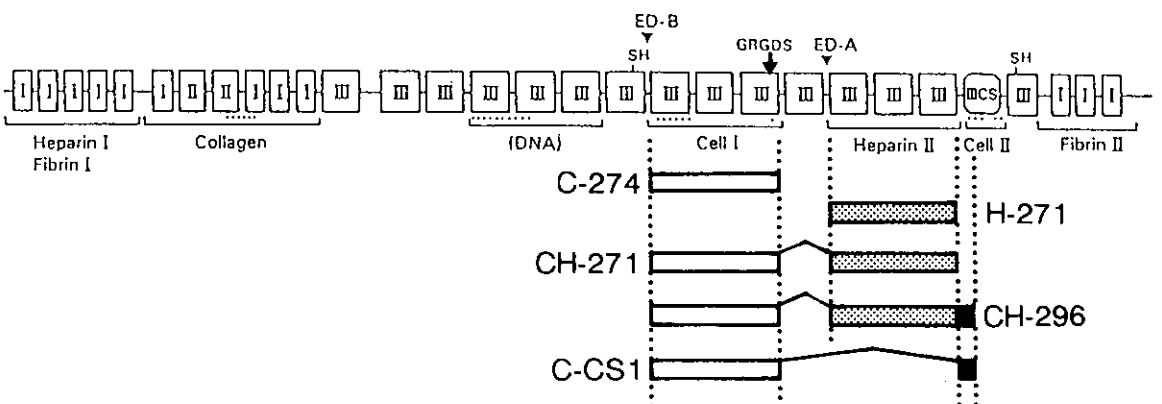

Fig. 1. Schematic diagram of recombinant fibronectin fragments and their fusion polypeptides. Locations of the fragments are shown by open, dotted and closed bars. The cell-binding polypeptide C-274 (Pro1239-Asp1512) covers three units of type III homology at the cell-binding domain. The heparin-binding polypeptide H-271 (Ala1690-Thr1960) covers the complete region of the heparin-binding domain. CS1 peptide (Asp1961-Thr1985, DELPQLVTLPHPNLHGPEILDVPST) is present within IIICS region. The fusion polypeptides CH-271 (Pro1239-Ser1515-(Met)-H-271), CH-296 (Pro1239-Ser1515-(Met)-H-271-CS1) and C-CS1 (Pro-1239-Ser1515-CS1) contain cell- or heparin-binding domains or CS1. The boxes at the top reperesent 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.

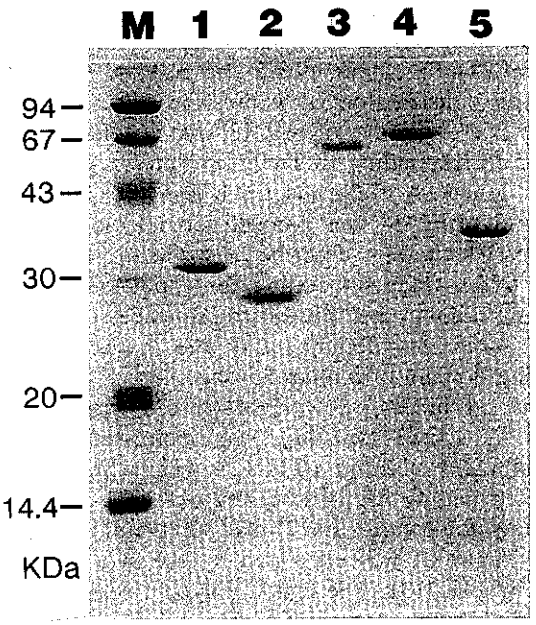

Fig. 2. SDS-polyacrylamide gel electrophoresis of recombinant fibronectin fragments. Purified proteins (2 μg) were analyzed on a 15% polyacrylamide gel under reducing conditions and visualized by CBB staining. Lane M represents molecular weight markers. Lanes 1, 2, 3, 4 and 5 represent C-274, H-271, CH-271, CH-296 and C-CS1, respectively.

expression of these plasmids will be presented elsewhere.[21]

The purity of the recombinant polypeptides was verified by SDS-polyacrylamide gel electrophoresis (Fig. 2). 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 identified by use of carboxypeptidase P (Takara Shuzo Co. Ltd., Kyoto). These polypeptides were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) before use. Purified human fibronectin was purchased from Biomedical Technologies Inc., MA. Purified mouse laminin and purified IgG fraction of rabbit anti mouse laminin were obtained from Collaborative Research Inc., MA. Heparan sulfate (bovine kidney Lot. No. 589901) was purchased from Seikagaku Kogyo Co. Ltd., Tokyo. Heparin sodium salt (Lot TLP3856; specific activity, 197.1 units/mg) was purchased from Wako Pure Chemical Industries, Ltd., Osaka. All the reagents and media in this study were endotoxin-free (approximately <1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co. Ltd.).

Microassay for cell adhesion The cell attachment assay was carried out by the method described already.[16] B16-BL6 melanoma cells in the exponential growth phase were incubated for 24 h in MEM containing 5% FBS supplemented with 0.3 μCi/ml [^{125}I]-iododeoxyuridine ([^{125}I]IUdR) (specific activity, 200 mCi/mmol, New England Nuclear Research Products, Boston, MA). The cells were washed twice in warm PBS to remove unbound radiolabels, harvested by adding 0.02% EDTA for 1 min at 37°C, and resuspended in cold serum-free MEM to form a single cell suspension. [^{125}I]IUdR-labeled tumor cells (2×10^4), at a volume of 0.05 ml/well, were added to microculture wells precoated with laminin, fibronectin or its fragments. The cultures were incubated at 37°C for each time period and then washed four times with PBS to remove unattached cells. The remaining substrate-bound tumor cells were lysed with 75 μl of 0.1 *N* NaOH. The lysate was absorbed on cotton swabs and monitored for radioactivity by gamma counting. The binding capacity (No. of cells bound/substrate) was expressed as follows:

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

Heparin binding assay The binding of [^{3}H]heparin (specific activity, 0.49 mCi/mg, Du Pont-New England Nuclear Research Products) to fibronectin, its related fragments and BSA was quantitated by a solid-phase radioimmunoassay in 96-well tissue culture plates.[22] Recombinant fibronectin fragments (5 μg) in a volume of 50 μl, were added to each well and dried overnight at 4°C. A BSA-containing buffer (5 mg/ml in 6 m*M* phosphate, 0.1 *M* $MgCl_2$, 68 μ*M* $CaCl_2$, pH 6.8) was added to each well followed by a 2 h incubation at 37°C. After removal of the buffer, [^{3}H]heparin (1×10^5 dpm/μg heparin in the same buffer), in a volume of 50 μl, was incubated for various set times at 37°C. Unbound [^{3}H]-heparin was removed by washing 3 times with PBS containing 0.1% Triton X-100. Tritiated heparin was solubilized by incubation with 100 μl of 0.5 *N* NaOH containing 1% SDS for 30 min at 37°C and quantitated in a liquid scintillation counter.

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

RESULTS

Effect of fibronectin on tumor cell adhesion to laminin Recent studies have shown that coinjection of fibronectin, its proteolytic fragments or synthetic peptides with tumor cells inhibits tumor invasion of basement membranes and the formation of pulmonary metastases.[11-17] We therefore examined whether or not soluble fibronectin was able to inhibit tumor cell adhesion to laminin, which is found exclusively in basement mem-

Table I. Modulation of the Adhesion of B16-BL6 Melanoma to Laminin-coated Substrate by Fibronectin

Addition/Treatment		No. of cells bound/substrate
—		7118±179
Fibronectin	500 μg/ml	4716±656 (34%)*
	250	5037±244 (29%)**
	125	5509±60 (23%)**
Anti-laminin	1:40	3979±414 (44%)**
Pretreatment of laminin-substrate with fibronectin,[a]	500 μg/ml	6528±1271
Pretreatment of cells with fibronectin,[b]	500 μg/ml	4980±435 (30%)*

^{125}I-labeled B16-BL6 cells (2×10^4/well) were added to wells precoated with 10 μg/ml laminin and incubated in the presence or absence of fibronectin at 37°C for 30 min.
a) Laminin-coated wells (10 μg/ml) were pretreated with 500 μg/ml fibronectin for 1 h and then washed twice with PBS before the addition of the cells.
b) ^{125}I-labeled B16-BL6 cells (2×10^4/wells) were pretreated with 500 μg/ml fibronectin and then washed twice with PBS before their addition into laminin-coated wells.
*; $P<0.01$. **; $P<0.001$.

brane, to elucidate the inhibitory mechanism of tumor metastasis by fibronectin. Table I shows that the adhesion of B16-BL6 cells to laminin was significantly inhibited by the addition of fibronectin. Rabbit anti mouse laminin serum also inhibited the tumor cells adhesion at the dilution of 1:40. The pretreatment of tumor cells with fibronectin also resulted in a reduction of tumor cell adhesion to laminin, whereas the pretreatment of laminin-substrates did not.

Tumor cell adhesion and [^{3}H]heparin binding to fibronectin and its recombinant fragments To study further the inhibitory effect of tumor cell adhesion to laminin by using recombinant fibronectin fragments with different functional domains and their fusion polypeptides, we first examined whether or not these fragments possessed the functional properties of fibronectin. The polypeptides containing cell-binding domain (C-274, CH-271, CH-296 and C-CS1), like fibronectin, promoted the adhesion of B16-BL6 cells in a concentration-dependent manner when they were immobilized on the wells (Fig. 3). Their promoting activities plateaued at the coating concentration of 5 μg/ml or more. In contrast, H-271, a polypeptide containing only a heparin-binding domain, showed less adhesion-promoting activity than other polypeptides used. The adhesion-promoting activity almost plateaued at a coating concentration of 500 μg/ml. We next examined the binding capacity of [^{3}H]heparin to fibronectin and its recombinant fragments. Table II shows that [^{3}H]heparin bound to the polypeptides containing heparin-binding domain (H-271, CH-271 and CH-296), but not to C-274 and C-CS1 polypeptides (without a heparin-binding domain), or to BSA as a negative control. The binding of [^{3}H]heparin to H-271, CH-271 and CH-296 was completely inhibited by the presence of 100-fold excess of unlabeled heparin. The above results clearly indicate that the recombinant frag-

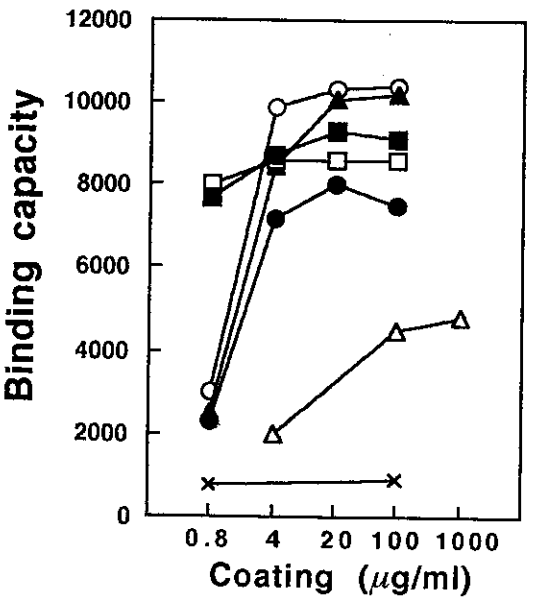

Fig. 3. Tumor cell adhesion to fibronectin and its recombinant fragments. ^{125}I-labeled B16-BL6 melanoma cells (2×10^4) were added to wells precoated with various concentrations of fibronectin (○) and its recombinant fragments; C-274 (●), H-271 (△), CH-271 (▲), CH-296 (□), C-CS1 (■) or BSA (×). After 30-min incubation, nonadherent cells were washed away and the adherent cells were counted.

Table II. Binding of [^{3}H]Heparin to Recombinant Fragments of Fibronectin

Coated with:	[^{3}H]heparin bound (dpm±SD)	
	without	with heparin
C-274	588±69	572±42
H-271	2660±290	480±76
CH-271	4620±860	584±148
CH-296	3220±280	371±59
C-CS1	603±126	446±92
BSA	350±120	451±125

Wells precoated with 5 μg of recombinant fibronectin fragments were incubated for 2 h with [^{3}H]heparin (76175±8411 dpm/0.75 μg) in the presence or absence of 100-fold excess of unlabeled heparin.

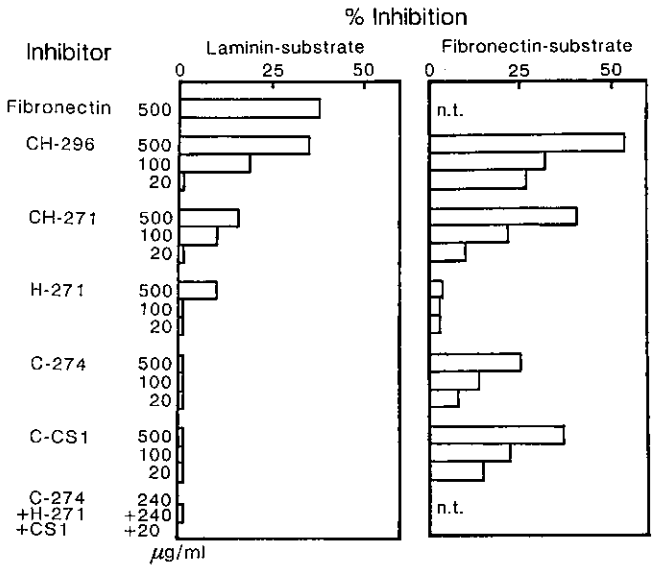

Fig. 4. Effect of fibronectin and its domain polypeptides on tumor cell adhesion to laminin or fibronectin. Labeled B16-BL6 cells (2×10^4), which had been pretreated with or without fibronectin or its domain polypeptides for 30 min and then washed twice, were added to wells precoated with 10 μg/ml laminin or fibronectin. After 30-min incubation, nonadherent tumor cells were washed away and the attached cells were counted.

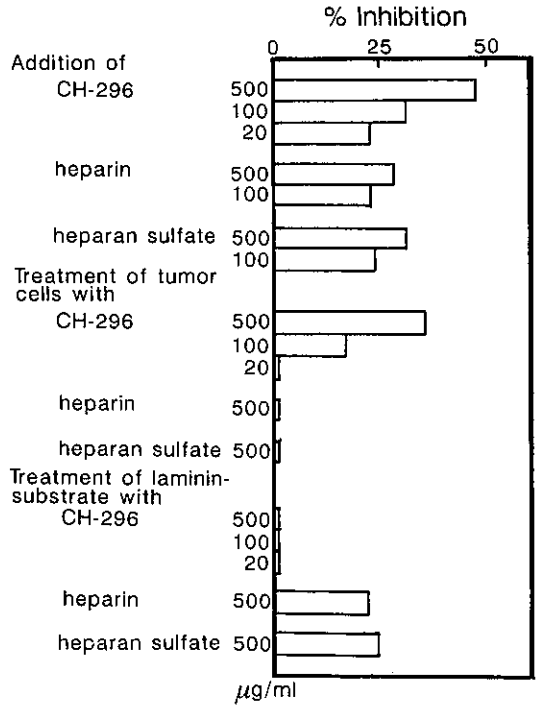

Fig. 5. Inhibition of tumor cell adhesion to laminin-substrate by CH-296, heparin or heparan sulfate. Labeled B16-BL6 cells (2×10^4) were added to wells precoated with 10 μg/ml laminin in the presence of CH-296, heparin or heparan sulfate. Laminin-substrates or tumor cells were pretreated for 30 min with them and then washed twice before addition to the wells. After 30-min incubation, nonadherent tumor cells were washed away and the attached cells were counted.

ments of fibronectin and their fusion polypeptides retain the functional properties of intact fibronectin.

Inhibition of tumor cell adhesion to laminin by fibronectin and its domain polypeptides Table I demonstrates that tumor cell adhesion to laminin was inhibited by the addition of or pretreatment of tumor cells with soluble fibronectin. We next used various recombinant fragments of fibronectin (as described above) to examine the fibronectin-mediated inhibitory effect on tumor cell adhesion to laminin. Tumor cells were pretreated with fibronectin or its recombinant fragments for 30 min and then washed twice before being added to the substrates. Fig. 4 shows that CH-296 or CH-271, in which the heparin-binding domain is located near the cell-binding domain, inhibited the adhesion of tumor cells to laminin-substrates in a concentration-dependent manner. H-271, containing only a heparin-binding domain, showed a slightly inhibitory effect on tumor cell adhesion at the high concentration of 500 μg/ml. In contrast, C-274, C-CS1 without the heparin-binding domain or a mixture of C-274, H-271 and CS1 (similar molar ratio to CH-296; 1:1:0.1) did not affect tumor cell adhesion to laminin. The pretreatment of tumor cells with CH-296 resulted in the inhibition of tumor cell adhesion to laminin, whereas the treatment of laminin-substrates with CH-296 did not affect tumor cell adhesion to laminin-substrate (Fig. 5). On the other hand, tumor cell adhesion to fibronectin-substrate was inhibited by polypeptides containing the cell-binding domain (CH-296, CH-271, C-274 and C-CS1) in a concentration-dependent manner, but was not inhibited by H-271 polypeptide (Fig. 4).

Effect of heparin on tumor cell adhesion to laminin Accumulating evidence indicates that cell surface proteoglycans and glycosaminoglycans such as chondroitin sulfate and heparan sulfate mediate cell attachment and possibly complex adhesive responses on extracellular matrix components, and such interactions between cell surface glycosaminoglycans and the matrix components modulate aspects of cell behavior such as motility, adhesiveness and cell growth.[23,24] We examined whether heparin or heparan sulfate could inhibit tumor cell adhesion to laminin-substrate or not. Tumor cell adhesion to laminin-substrate was inhibited by the addition of hepa-

rin or heparan sulfate (Fig. 5). Pretreatment of laminin-substrate with heparin or heparan sulfate resulted in the inhibition of tumor cell adhesion to laminin, whereas the pretreatment of tumor cells with them did not.

DISCUSSION

Tumor cell adhesion to the extracellular matrix components is an important aspect in several steps of the metastatic process. Previous studies have utilized cell adhesion-promoting fragments of laminin or fibronectin,[11, 12] or their synthetic peptides such as RGD oligopeptides[13, 17] or RGD polypeptides[14–16] to inhibit the experimental metastasis of tumor cells. Heparin or glycosaminoglycans including heparan sulfate have been shown to possess the ability to inhibit cell attachment or migration to extracellular matrices including fibronectin[23, 24] and to inhibit tumor metastasis upon co-injection with tumor cells.[25–27] Terranova *et al.*[10] have reported that fibronectin suppressed the adhesive and invasive activities against basement membrane when tumor cells were exposed in culture to fibronectin, whereas laminin increased the invasiveness. Thus, the regulatory mechanism involved in the adhesive interaction between tumor cells and extracellular matrices may be associated with the prevention of tumor metastasis *in vivo*. In the present study, we found that fibronectin regulates the adhesive interaction between tumor cells and laminin, which is restricted to the basement membrane. The treatment of tumor cells with fibronectin resulted in a reduction of cell adhesion to laminin, suggesting that this inhibitory effect can be attributed to the interaction of fibronectin with tumor cells.

We prepared various recombinant fibronectin fragments to investigate the fibronectin-mediated regulatory mechanism of tumor cell adhesion to laminin in detail. CH-296 and CH-271 inhibited the cell adhesion to laminin, whereas C-274 and C-CS1 did not (Fig. 4). However, not only CH-296 and CH-271, but also C-274 and C-CS1 acted as inhibitors of cell adhesion to fibronectin possibly via VLA-5/RGD interaction and/or VLA- 4/CS 1 interaction.[4, 28] Studies from other laboratories[29] have shown that no inhibitory effect on cell adhesion to laminin was observed upon addition of anti-fibronectin receptor antibodies. Our previous study showed that CS1 peptide at a concentration of more than 100 μg/ml could remarkably inhibit tumor cell adhesion to laminin.[15] Considering the amount of CS1 in C-CS1 fragment, it is expected that C-CS1 may inhibit tumor cell adhesion to laminin at concentrations higher than that in Fig. 4. These results indicated that fibronectin-mediated inhibition of tumor cell adhesion to laminin is not mediated by the ability of fibronectin to compete for cell surface integrin receptors, and that the heparin-binding domain of fibronectin may be associated with the inhibitory effect.

H-271, which showed a poor cell adhesion-promoting property when it was immobilized on the surface (Fig. 3), slightly inhibited tumor cell adhesion to laminin only at the high dose (Fig. 4). Heparin-binding domains of fibronectin or laminin have been shown to possess not only heparin-binding but also cell-binding properties.[6, 22] Cell surface proteoglycans have been shown to be capable of association with extracellular matrix components.[30, 31] Further study using mutant cell lines which are deficient in glycosaminoglycan synthesis has provided evidence that cell adhesion to heparin-binding fragments of fibronectin could be mediated by cell surface proteoglycans.[32] Figure 5 shows that tumor cell adhesion to laminin was inhibited by the addition of, or the treatment of laminin-substrate with, heparin or heparan sulfate. We also observed that treatment of the cell surface proteoglycan with specific antibodies or enzymes resulted in partial inhibition of tumor cell adhesion to laminin (data not shown). This indicates that cell surface proteoglycans can mediate the cell adhesion to extracellular matrices such as fibronectin or laminin. These observations suggested that the association of the heparin-binding domain of fibronectin with tumor cells via cell surface proteoglycans contributed to the fibronectin-mediated inhibitory effect.

CH-296 and CH-271, in which the heparin-binding domain is co-present closely with a cell adhesion-promoting domain, C-274 or CS1, were more effective in inhibiting tumor cell adhesion to laminin than H-271 (with the heparin-binding domain), or a mixture of C-274, H-271 and CS1 (Fig. 4). These results indicated that although the adhesive interaction between the heparin-binding domain (H-271) and tumor cells may not be sufficient for cell attachment (Fig. 3), the localization of H-271 near cell adhesion-promoting domains such as C-274 and CS1 within the molecule may augment the interaction of H-271 with tumor cell surface proteoglycan.

Recently the exact role of proteoglycan in tumorigenesis and metastasis has been examined. For example, progression of Kirsten ras$^+$ tumor resulted in extensive catabolism of proteoglycan and alteration of fibronectin function in cell adhesion.[33] Mutants defective in the synthesis of heparan sulfate proteoglycan did not form tumors.[34] There was a reciprocal correlation between the cell surface level of heparan sulfate proteoglycan and the metastatic potential of murine melanoma cells.[35] Our preliminary results using tumor cell variants with different metastatic potential showed that the degree of inhibitory effect exerted by fibronectin on tumor cell adhesion to laminin is inversely proportional to the metastatic ability of tumor cell variants. These results suggest that lack of cell surface proteoglycan in part

allows tumor cells to evade the fibronectin-mediated inhibitory mechanism for cell adhesion to laminin, and consequently increases the metastatic properties.

In conclusion, we have demonstrated that soluble fibronectin inhibits the tumor cell-laminin interaction as well as the cell-fibronectin interaction. Such a regulatory effect may depend on interference with the interaction between cell surface proteoglycans and laminin by the heparin-binding domain of fibronectin.

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