Interaction with Heparin and Matrix Metalloproteinase 2 Cleavage Expose a Cryptic Anti-adhesive Site of Fibronectin†

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ABSTRACT: We recently found that fibronectin (FN) had a functional site [YTIYVIAL sequence in the heparin-binding domain 2 (Hep 2)] that was capable of suppressing the integrin-mediated cell adhesion to extracellular matrix. However, our results also indicated that this anti-adhesive site seemed to be usually buried within the Hep 2 domain structure because of its hydrophobic nature, raising a question as to the physiological significance of the cryptic anti-adhesive activity of FN. The present study demonstrates that the cryptic anti-adhesive activity can be exposed through the physiological processes. A 30-kDa chymotryptic FN fragment derived from Hep 2 domain (Hep 2 fragment), which had no effect on adhesion of MSV-transformed nonproducer 3T3 cell line ($KN_7$8) to FN, expressed the anti-adhesive activity after treatment with 6 M urea. Light scattering and circular dichroism measurements showed that the urea treatment induced the conformational change of the Hep 2 fragment from a more compact form to an unfolded one. Incubation of the Hep 2 fragment with heparin also induced similar conformational changes and expression of anti-adhesive activity. Additionally, both the urea and heparin treatments made the Hep 2 fragment and intact FN much more accessible to the polyclonal antibody (αIII14A), with a recognition site near the anti-adhesive site of FN. Specific cleavage of either the Hep 2 fragment or intact FN by matrix metalloproteinase 2 (MMP-2) released a 10-kDa fragment with the anti-adhesive activity, which was shown to have the exposed anti-adhesive site on the amino-terminal region. Thus, the cryptic anti-adhesive activity of FN can be expressed upon conformational change and proteolytic cleavage of Hep 2 domain.

Fibronectin (FN)[1] mediates adhesion of various types of cells to extracellular matrix (ECM) via several cell adhesive sites distributed in the type III homologous repeating modules. The central cell-binding (CCB) domain is the major cell adhesive domain of FN, the activity of which is attributed to the Arg-Gly-Asp (RGD) motif in the 10th type III module (III10) (*1*) and the synergistic motif in the III9 module (*2*). These cell adhesive sites are recognized by cell adhesion receptor integrin α5β1 (*3*). The carboxy-terminal heparin-binding domain (Hep 2), consisting of III12−III14 modules, contains at least six major cell adhesive sites, represented by the synthetic peptides termed H1 (*4*) and FN-C/H-I, -II, -III, -IV, and -V (*5*−*9*), that support heparin-dependent, RGD-independent cell adhesion (*7*). Moreover, there exist additional cell adhesive sites in the domain adjacent to the Hep 2 domain, termed the type III connecting segment (IIICS), including the CS1 and CS5 regions, which are recognized by integrin α4β1 (*4*). On the basis of such a diversity of cell adhesive sites, FN participates in regulation of cellular processes such as growth, differentiation, migration, and apoptosis.

In contrast to these general aspects, we recently found that FN also had a functional site opposing cell adhesion to ECM (*8*): a 30-kDa chymotryptic fragment derived from Hep 2 domain of FN (Hep 2 fragment), which in itself showed no significant effect on Mardin-Darby canine kidney (MDCK) cell adhesion to FN, expressed an ability to suppress the integrin-mediated cell adhesion to ECM after exposure to 6 M urea. Unlike the functional peptides derived from cell adhesive sites of FN such as RGD, CS1, and FN-C/H-I-V, this urea-treated Hep 2 fragment suppresses cell adhesion effectively even when coated on a culture dish as an insoluble

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[1] Abbreviations: FN, fibronectin; ECM, extracellular matrix; RGD, Arg-Gly-Asp; MDCK, Madin-Darby canine kidney; MMP, matrix metalloproteinase; CCB, central cell-binding domain; Hep 2, carboxy-terminal heparin-binding domain; DLS, dynamic light scattering; CD, circular dichroism; EIA, enzyme immunoassay; LM, laminin; TSP, thrombospondin.

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substrate as well as when added as a soluble supplement, indicating that the urea-treated Hep 2 fragment expresses the anti-adhesive activity in a noncompetitive fashion. More recently, this functional site (so-called anti-adhesive site) in Hep 2 domain was located within a peptide (termed III14-2) comprising 21 amino acid residues (1835−1855) that is present in the III14 module (*9*). An analogous peptide in which the YTIYVIAL hydrophobic sequence was completely scrambled (III14-2scr), failed to express the anti-adhesive activity, indicating that the YTIYVIAL sequence is indispensable for the anti-adhesive activity (*9*). Furthermore, our recent results indicate that peptide III14-2, but not III14-2scr, has the potential to modulate a number of cellular processes including cell growth, differentiation, and apoptosis (*10*, *11*).

On the other hand, peptide III14-2 and the Hep 2 fragment were shown to suppress integrin-mediated cell adhesion to FN without interaction with either FN or integrin $\alpha5\beta1$ (*8*), supporting the previous assumption that the anti-adhesive site of Hep 2 domain exerts its activity in a noncompetitive fashion. More recently, we found the presence of a 55-kDa membrane protein specifically binds with peptide III14-2, but not with the inactive peptide III14-2scr (*12*). Since binding ability of analogous peptides to the 55K protein closely correlated with their strength of anti-adhesive activity (*12*), we proposed that this 55K membrane protein serves as a receptor protein mediating the anti-adhesive activity of peptide III14-2.

Despite such reliability of the anti-adhesive site in FN, our previous data showing that the anti-adhesive activity was only detected after exposure of the Hep 2 fragment to protein denaturant (6 M urea) (*8*) also raise a question as to whether the anti-adhesive site of FN can exerts its function in vivo. To answer this question, here we show that the cryptic anti-adhesive activity can be expressed through physiological processes, including interaction of FN with heparin and proteolytic cleavage by matrix metalloproteinase (MMP) 2.

MATERIALS AND METHODS

Materials. Interstitial collagenase (MMP-1) (*13*), gelatinase A (MMP-2) (*14*), stromelysin 1 (MMP-3) (*15*), and matrilysin (MMP-7) (*16*) were purified as described previously. The peptides III14-2, TEATITGLEPGTEYTIYVIALC, and III14A, EATITGLEPGTEYT, were purchased from Sawady Technology. Polyclonal antibody recognizing near the anti-adhesive site in the Hep 2 domain was prepare with peptide III14A as the antigen. Rabbits were immunized with peptide III14A−keyhole limpet hemocyanin conjugate in complete Freund's adjuvant for the first immunization and in incomplete Freund's adjuvant for subsequent boosts. Titer of the antiserum was determined by conventional enzyme-linked immunosorbent assay (ELISA). Monoclonal antibody FNH3-8, which recognizes in Hep 2 domain of FN, was obtained from Takara Biomedicals.

Preparation of the Hep 2 Fragment and Its Treatment with Urea. Plasma FN was isolated from human plasma by gelatin affinity chromatography under mild conditions without use of any protein denaturants (*17*). A 30-kDa fragment derived from only the Hep 2 domain (Hep 2 fragment) was prepared and isolated, through two-step proteolytic digestions of the purified FN with thermolysin and chymotrypsin, as described previously (*8*, *18*). To induce the conformational change, the Hep 2 fragment was incubated with 6 M urea at room temperature for 2 h. After thorough dialysis against PBS(−) to remove urea, the Hep 2 fragment was used as the urea-treated Hep 2 fragment.

Digestion of the Native Hep 2 Fragment and Intact FN by MMP-2. The purified latent form of MMP-2 was activated by incubating with 1 mM *p*-aminophenylmercuric acetate at 37 °C for 1 h (*19*). The Hep 2 fragment or intact FN in 20 mM HEPES buffer (pH 7.5) was incubated at 37 °C for the indicated periods of time with the activated MMP-2 (1/50 w/w) in the presence of 2 mM $CaCl_2$. The reaction was stopped by the addition of *o*-phenanthroline (10 mM).

Cell Culture. Kirsten MSV-transformed nonproducer 3T3 cell line ($KN_7$8) was kindly provided by Dr. Masayoshi Hiragun, The Tokyo Metropolitan Institute of Medical Science. The cells were cultured in HD medium (1:1 mixture of Ham F-12 and Dulbecco's modified Eagle media) supplemented with 10% fetal bovine serum and 2 mM glutamine.

Assay of Anti-adhesive Activity. Ninety-six-well tissue culture plates were coated with FN (2 μg/mL) at 37 °C for 1 h. After being washed with PBS(−), the plates were overcoated with the samples to be assayed under the indicated conditions. In some experiments, samples were added into the culture medium as a soluble supplement instead of overcoating as above. The plates were then blocked with 0.2% heat-denatured BSA at 37 °C for 1 h. An aliquot (100 μL) of cell suspension [(1−2) × 10^4 cells] in the serum-free medium was added to each well, and the plates were incubated at 37 °C for 1 h. An aliquot (100 μL) of 5% formaldehyde in PBS(−) containing 5% sucrose was poured gently into each well to fix the cells that were adhered to substrates. After the plates were washed, the adherent cells were stained with crystal violet. The number of spreading cells in five different randomly chosen areas was counted under the microscope at 100× magnification.

Assay of Cell Adhesive Activity. Ninety-six-well tissue culture plates were coated with FN (2 μg/mL) or the samples to be assayed at 37 °C for 1 h. The plates were blocked with the heat-denatured BSA. An aliquot (100 μL) of cell suspension [(1−2) × 10^4 cells] in serum-free medium was added to each well, and the plates were incubated at 37 °C for 1 h. After the cells were fixed with formalin, the number of spreading cells was counted as described above.

Dynamic Light Scattering Studies. DLS measurements were carried out on a Photal DLS-700 dynamic light scattering spectrophotometer with an Ar+ laser at 488 nm. Samples in PBS(−) solution to be analyzed were filtered with a 0.45-μm pore size cellulose acetate membrane filter. The measurements were performed at 25 °C with a 5-mm path length cuvette, a sampling time of 0.8 μs, and scattering angles of 45° or 90°. The diffusion coefficient (*D*) of the Hep 2 samples was estimated by analyzing the measured intensity autocorrelation function in homodyne mode by the cumulant method (*20*). We assumed the shape of the FN fragments to be a sphere, and the Stokes radius (*r*) of the molecule was calculated from the Stokes−Einstein equation (*21*):

$$r = (K_B T)/(6D\eta\pi) \quad (1)$$

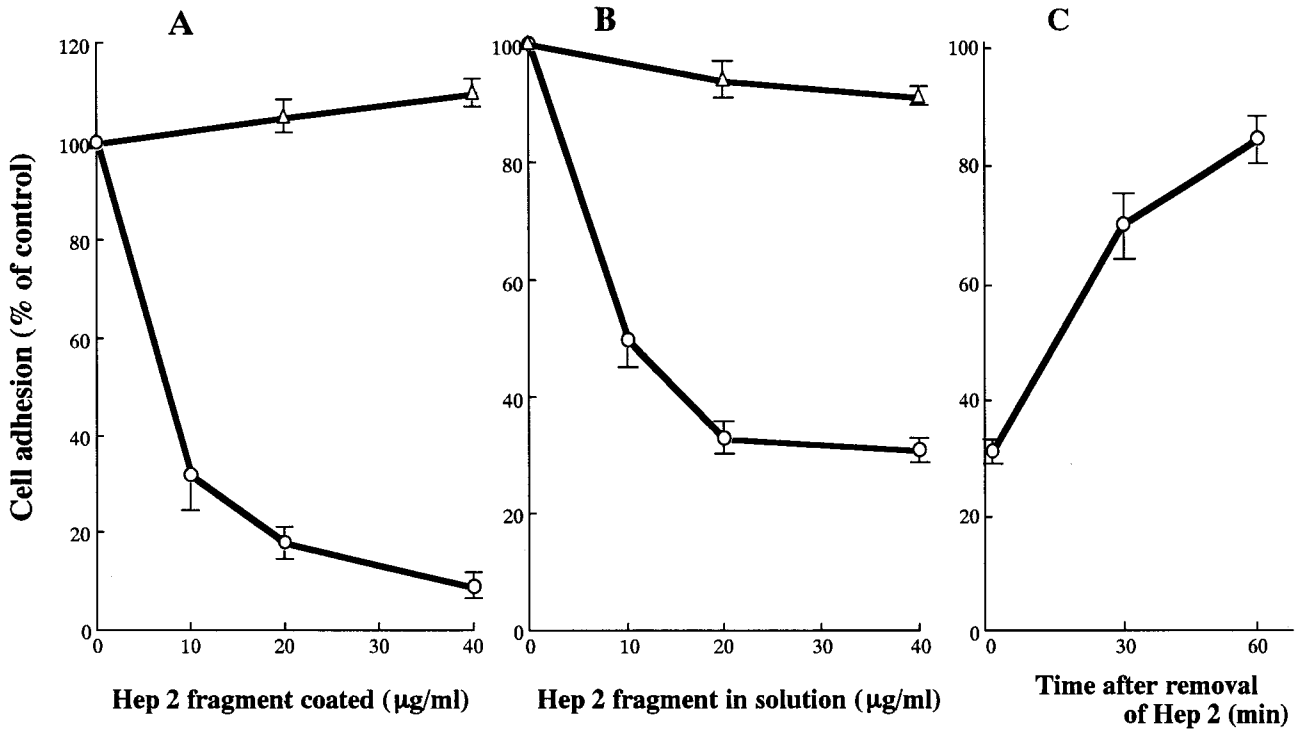

FIGURE 1: Urea treatment induces the anti-adhesive activity of the Hep 2 fragment. Anti-adhesive activity of the Hep 2 fragment treated with (○) or without (△) 6 M urea was assayed as described under Materials and Methods. (A), Hep 2 fragment in solution. (B), Hep 2 fragment coated. (C) Reversibility of anti-adhesive effect of the urea-treated Hep 2 fragment. After the incubation of cells in the presence of the urea-treated Hep 2 fragment (40 μg/mL) (see panel B), half volumes of the supernatants were changed for the fresh medium free of the Hep 2 fragment. This medium change was repeated 3 times and the cells were cultured further for the indicated periods of time. Data show the means ± SE of four determinants, represented as percent of cell adhesion on the FN substrate without Hep 2 fragment.

where K_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, and π is the ratio of the circumference of a circle to its diameter.

Circular Dichroism Measurements and Analysis. CD spectra were obtained on a Jasco J-600 spectropolarimeter. All CD spectra were measured with a 10-mm path length cuvette, a time constant of 2 s, and a scan rate of 50 nm min^{-1}. CD data that were obtained by scanning five times and averaging were used for calculations. The mean residual ellipticity ([θ]) was calculated from

$$[\theta] = 100\theta_{obs}/lc \quad (2)$$

where θ_{obs} is the observed ellipticity (in degrees), l is the path length of the cell (in centimeters), and c is the residual molar concentration of the sample. The mean residue molecular weight of FN was assumed to be 116 (*22*). The secondary structure of the proteins was estimated from [θ] by the analyzing method described by Sreerama and Woody (*23*).

Enzyme Immunoassay. A sandwich-type enzyme immunoassay was carried out, in which the mAb directed to Hep 2 domain (FNH3-8) (0.2 μg/100 μL of PBS) was coated onto 96-well microtiter plates as a primary antibody, and the Hep 2 fragment treated under the indicated conditions was sandwiched by the biotinylated αIII14A antibody. The wells were washed with PBS(−) containing 0.2% Tween-20, and 100 μL of a 1/1000 dilution of HRP-conjugated streptavidin (Dako) was incubated for 1 h, followed by further washing and addition of peroxidase substrate.

Separation of the Anti-adhesive Peptide from the MMP-2 Digest of Intact FN. The MMP-2 digest of intact FN prepared as above was subjected to molecular-sieve chromatography on a Superdex 200 pg column (Pharmacia Biotech) equilibrated with 0.1 M ammonium acetate buffer (pH 7). Aliquots (4 mL) of the eluates were lyophilized, dissolved in the serum-free HD medium, and examined for cell adhesive and anti-adhesive activities as described above.

Amino Acid Sequencing. The amino-terminal end of FN fragment was sequences on an Applied Biosystems gas-phase sequenator 476A.

RESULTS

Conformational Change of Hep 2 Domain Causes Exposure of the Anti-adhesive Site. The Hep 2 fragment, after treatment with 6 M urea, suppressed the $KN_7$8 cell adhesion to FN substrate either when coated on the culture plates (Figure 1A) or when added as a soluble supplement into the culture medium (Figure 1B). Anti-adhesive effect of the urea-treated Hep 2 fragment was reversed by removing the urea-treated Hep 2 fragment from the culture medium (Figure 1C). Appearance of the anti-adhesive activity following the urea treatment suggested that the cryptic anti-adhesive site might be exposed upon conformational change of the Hep 2 domain induced by the urea treatment. To evaluate the structural changes in the Hep 2 fragment, we first assessed the molecular dimensions of the Hep 2 fragment before and after the urea treatment by DLS. The diffusion coefficient of the Hep 2 fragment clearly decreased after the urea treatment (Table 1). Apparent Stokes radii calculated from the diffusion coefficient values suggested that the urea treatment caused the shape of the Hep 2 domain to change from a more compact globular form to an unfolded expanded one. We

Table 1: Diffusion Coefficient (D) and Stokes Radius (r) of the Native Hep 2 Fragment Treated with or without 6 M Urea

treatment	D ($\times 10^{-11}$,^{2} s^{-1})	r (nm)
none	9.45	2.75
6 M urea	6.02	4.25

Table 2: Secondary Structure Elements of the Native Hep 2 Fragment Treated with 6 M Urea or Heparin

	proporation of secondary structure (%)			
treatment	α-helix	β-sheet	β-turn	unordered
none	25.9	38.8	12.1	23.0
6 M urea	5.9	65.0	14.1	15.4
heparin[a]	4.8	62.5	12.5	16.5

[a] The native Hep 2 fragment was incubated with 0.1 mg/mL heparin.

next evaluated the secondary structure by CD. As summarized in Table 2, upon urea treatment, most of the α-helix structure of the Hep 2 fragment decreased, but the β-sheet structure increased instead.

Heparin Induces Exposure of the Anti-adhesive Site of Hep 2 Domain. We investigated whether the cryptic anti-adhesive site can be exposed under physiological conditions. Two possible processes, conformational change and proteolytic cleavage of Hep 2 domain, are presumed. We first examined the former process. Since the anti-adhesive site (YTIYVIAL) is situated between previously identified functional sites, FN-C/H-I and -II, in Hep 2 domain, both of which bind with heparin and promote cell adhesion, the cryptic anti-adhesive site may be exposed by the interaction of these heparin-binding sites with heparin. Then, the Hep 2 fragment was incubated with increasing concentrations of heparin, and its effect on cell adhesion was examined. As shown in Figure 2, addition of heparin caused the appearance and increase in the anti-adhesive activity depending on the added heparin concentrations. Heparin did not affect the functional properties of peptide III14-2 and its inactive control peptide III14-2scr (data not shown). Furthermore, heparin itself had no anti-adhesive activity (Figure 2). These results suggested that the effect of heparin may be due to induction of a structural change in the Hep 2 domain. We next assessed the secondary structure of the Hep 2 fragment by CD. Table 2 shows that most of the α-helix structure in the Hep 2 fragment converted to β-sheet structure upon incubation with heparin. Interestingly, the proportions of the secondary structure elements of the Hep 2 fragment incubated with heparin were similar to those of the urea-treated Hep 2 fragment, suggesting that heparin and urea induced similar conformational changes in the Hep 2 fragment.

Next, we measured the immunoreactivity of the Hep 2 fragment with the polyclonal antibody αIII14A (see Materials and Methods) that was raised against peptide III14A containing a part of the anti-adhesive site. Figure 3 shows that αIII14A antibody hardly bound to the Hep 2 fragment, whereas accessibility of the Hep 2 fragment to this antibody increased remarkably following both urea treatment and incubation with heparin. Furthermore, heparin also induced increased accessibility of αIII14A antibody to intact FN.

Proteolytic Cleavage with MMP-2 Releases the Anti-adhesive Fragment. Another possible way to expose the anti-adhesive site is proteolytic degradation of the Hep 2 domain structure. We previously reported that MMP-2 effectively

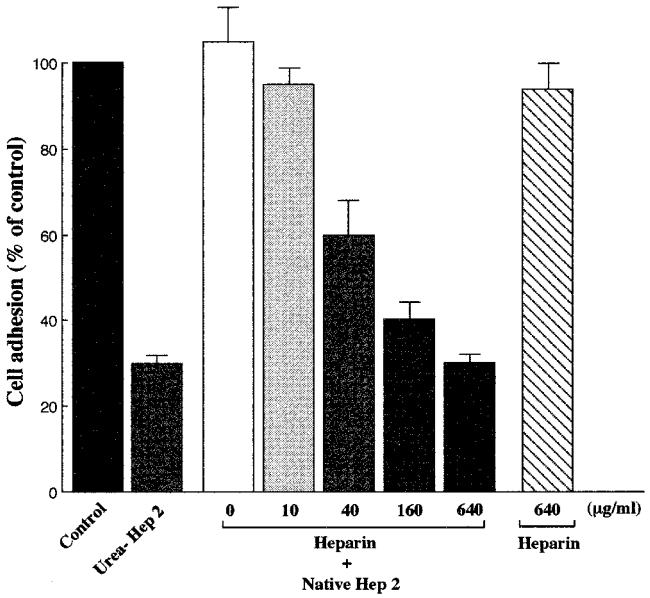

FIGURE 2: Heparin induces the anti-adhesive activity of the Hep 2 fragment. The Hep 2 fragment in PBS(−) (final concentration 300 μg/mL) was incubated (37 °C, 1 h) with 6 M urea or the indicated concentrations of heparin. Ninety-six-well plates coated with FN (control) were overcoated with the urea-treated Hep 2 fragment (20 μg of protein/mL) (urea-Hep2), each of the heparin-treated Hep 2 fragments (20 μg of protein/mL), or heparin (640 μg/mL). Assay of the anti-adhesive activity against $KN_7$8 cells was carried out as described under Materials and Methods. Data represent the means ± SE of three determinants.

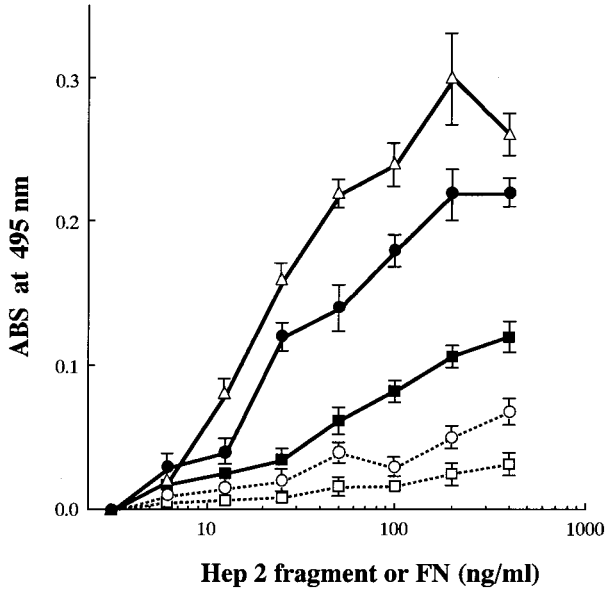

FIGURE 3: Accessibility of the Hep 2 fragment or intact FN to αIII14A antibody. The sandwich-type enzyme immunoassay was constructed as described under Materials and Methods. The Hep 2 fragment in PBS(−) (300 μg/mL) was treated in the absence (○) or presence of urea (6 M) (△) or heparin (600 μg/mL) (●). Intact FN in PBS (−) (500 μg/mL) was treated with (■) or without (□) heparin (600 μg/mL). These samples were used as antigens on EIA. Data represent the means ± SE of six determinants.

released FN fragments having biological activities (*19*). To ascertain if MMP-2 is also efficient in releasing a fragment having the anti-adhesive activity, the Hep 2 fragment without anti-adhesive activity was incubated with MMP-2, and this MMP-2 digest was examined for the anti-adhesive activity. As shown in Figure 4A, the MMP-2 digest of the Hep 2

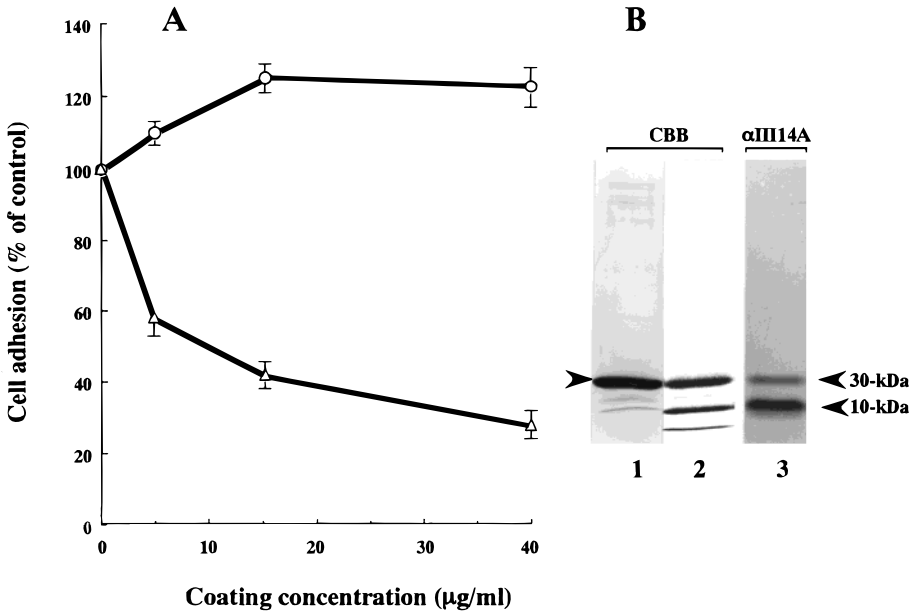

FIGURE 4: MMP-2 releases a fragment with anti-adhesive activity. (A) Dose-dependent increase in the anti-adhesive activity of MMP-2 digest of the Hep 2 fragment. The Hep 2 fragment was incubated at 37 °C for 24 h with MMP-2 (1/50 w/w) and the reaction was terminated by addition of *o*-phenanthroline. To examine the anti-adhesive activity, 96-well plates coated with FN were overcoated with the Hep 2 fragment (○) or its MMP-2 digest (△) at the indicated concentrations. $KN_7$8 cells were seeded onto each culture plate, incubated, and then fixed with formalin. Data represent the means ± SE of three determinants. (B) SDS−PAGE (lanes 1 and 2) and immunoblot analysis with αIII14A antibody (lane 3). Lane 1, native Hep 2 fragment; lanes 2 and 3, MMP-2 digest of the native Hep 2 fragment.

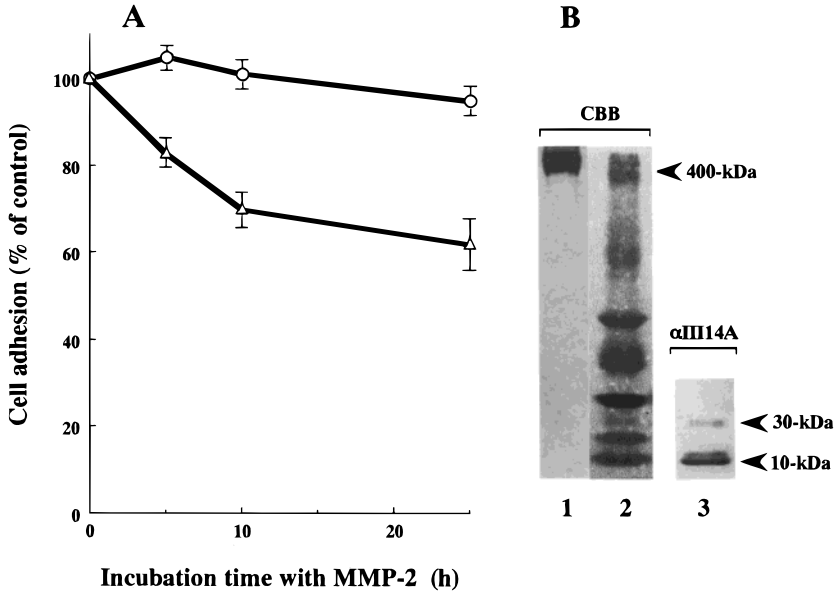

FIGURE 5: Effects of MMP-2 digestion on the cell adhesive activity of intact FN. (A) Time-dependent decrease in the cell adhesive activity of FN by MMP-2 digestion. FN was incubated with (○) or without (△) MMP-2 (1/25 w/w) for the indicated periods of time and the reaction was terminated by addition of *o*-phenanthroline. To examine the cell adhesive activity of these MMP-2 digests, 96-well plates were coated with each of the MMP-2 digests at 10 μg/mL as intact FN. $KN_7$8 cells were seeded onto each of the culture plates, incubated, and then fixed with formalin. Data represent the means ± SE of four determinants. (B) SDS−PAGE (lane 1) and the immunoblot analysis with αIII14A antibody (lanes 2 and 3). Lane 1, intact FN; lanes 2 and 3, MMP-2 digest of intact FN.

fragment suppressed the adhesion of $KN_7$8 cells to FN substrate in a dose-dependent manner. Figure 4B shows that MMP-2 released a fragment (around 10 kDa) that was recognized by αIII14A antibody, suggesting that the suppression of $KN_7$8 cell adhesion with the MMP-2 digest was due to this 10-kDa fragment. Any of the digests prepared with other MMPs such as MMP-1, -3, and -7 did not express the anti-adhesive activity (data not shown).

Furthermore, when intact FN was incubated with MMP-2, cell adhesive activity of intact FN decreased according to the degradation time periods with MMP-2 (Figure 5A). A 10-kDa fragment, which was recognized by αIII14A antibody, was also found in the MMP-2 digest of intact FN (Figure 5B). Since we previously demonstrated that MMP-2 efficiently degraded intact FN but did not destroy the cell adhesive activity of CCB domain (*19*), this decrease in cell adhesive activity following MMP-2 digestion might be due to release of the 10-kDa fragment. To examine this posibility, the MMP-2 digest of intact FN was subjected to molecular-sieve chromatography (Figure 6). Cell adhesive activity was

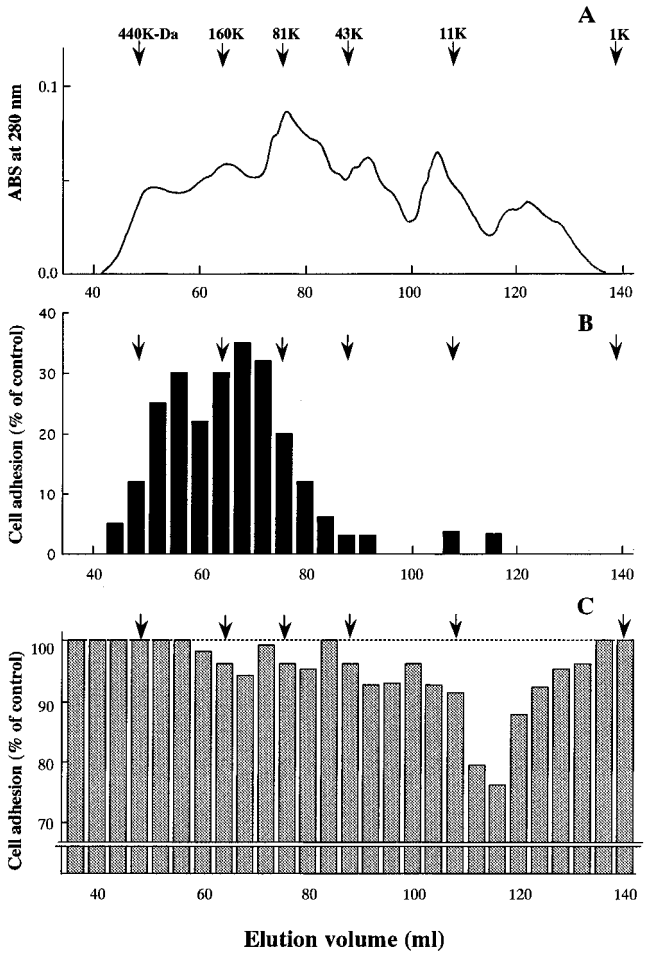

FIGURE 6: Molecular sieve chromatography of MMP-2 digest of intact FN. Intact FN was incubated (37 °C, 24 h) with MMP-2 and then subjected to gel filtration on Superdex 200 pg column (A) as described under Materials and Methods. The fractions were concentrated and examined for the cell adhesive (B) and anti-adhesive (C) activities. The ordinate values in panels B and C are presented as percent of the mean control cell adhesions which was determined on the plate coated with FN (2 μg/mL).

detected in the fractions of large molecular sizes (over 80 kDa) (Figure 6B), which was due to intact FN and large FN fragments containing the CCB domain. On the other hand, anti-adhesive activity was eluted at around 10 kDa (Figure 6C). Anti-adhesive activity of this peak was diminished by immunoprecipitation with αIII14A antibody (data not shown). The amino-terminal amino acid residues of the 10-kDa fragment were determined to be Val-Thr-Glu-Ala-Thr. The result indicates that MMP-2 cleaved between Gly (1833) and Val (1834), which is present 14 residues upstream of the anti-adhesive site (YTIYVIAL). Taking together the molecular size of the 10-kDa fragment, this fragment was expected to have the exposed anti-adhesive site on the amino-terminal side.

DISCUSSION

ECM proteins including FN have a variety of cryptic functional sites, many of which were exposed by proteolytic cleavage (*24*−*28*). Recent studies have demonstrated the importance of this mode of regulation through proteolytic processing (*29*−*32*). The major enzymes that degrade ECM proteins are the MMP family of proteinases. To evaluate the potential of MMPs to expose the cryptic anti-adhesive site, we examined effects of the MMP digests of the Hep 2 fragment on the KN_f8 cell adhesion. The results showed that only MMP-2, but not MMP-1, -3, and -7, was functional in liberating the cryptic anti-adhesive activity from FN. Nevertheless, we cannot conclude that MMP-1, -3, and -7 have no ability to expose the anti-adhesive site. Our experiment only examined the MMP actions under certain reaction conditions. In general, MMP-3 and -7 are known to degrade the FN molecule, whereas MMP-1 (i.e., collagenase) has a limited substrate specificity (*15*, *33*, *34*). In our study, MMP-3 as well as MMP-1 did not cleave the Hep 2 fragment at all. Although MMP-3 can degrade FN into smaller fragments, it may not cleave the type III structure of Hep 2 domain. On the other hand, MMP-7 cleaved the Hep 2 fragment but did not liberate the anti-adhesive activity. It might be possible that MMP-7 either did not cleave the Hep 2 fragment enough to expose the anti-adhesive site or destroyed the anti-adhesive site. In any case, at least MMP-2 was shown to release the anti-adhesive fragment from FN. Giannelli et al. (*32*) reported that specific cleavage of laminin 5 induced migration of breast epithelial cells by exposing a putative cryptic promigratory site on laminin 5. MMP-2, which cleaves at the restricted position of ECM proteins, may play an important role in generation of the biologically active ECM fragments as well as in disruption of ECM architecture (*32*−*35*). MMP-2 is expressed prominently during tumor progression and metastasis (*36*−*39*). A loss in cell-to-ECM interaction by ECM disruption with MMP-2 is a prerequisite for the development of invasive tumors and metastasis (*36*−*39*). The 10-kDa FN fragment generated by MMP-2 had the potential to induce a loss in cell-to-ECM adhesion. Generation of the anti-adhesive fragment from FN may be one of the important consequences of MMP-2 action for the promotion of tumor progression.

Whether the cryptic anti-adhesive site of FN could be expressed without proteolytic modification is important. Our data of CD and EIA indicated that the anti-adhesive site seemed to be exposed upon the interaction of heparin with the heparin-binding sites of Hep 2 domain. There have been a large number of studies on the conformation change of FN (*25*, *40*−*45*). By use of CD, sedimentation, and electron spin resonance techniques, many of them have already shown that heparin as well as urea changes the FN conformation to a more relaxed or flexible state (*43*−*45*). Particularly, Ankel et al. (*45*) reported that the heparin effect was noticeable at a heparin/FN ratio of over 20, which supports our data of the heparin effect testing at a heparin/Hep 2 fragment ratio of 40. Additionally, the EIA data were in parallel with appearance of the anti-adhesive activity from the Hep 2 fragment following heparin and urea treatments. Therefore, we concluded that the cryptic anti-adhesive site can be also exposed through the conformational changes induced by heparin.

In conclusion, the present studuy indicates that the cryptic anti-adhesive site of the FN molecule can be exposed through biological processes such as proteolytic cleavage and conformational change of the FN molecule. It remains to identify the physiological and/or pathological events that induce exposure of the cryptic anti-adhesive site of FN in the complexed ECM architecture of local tissues.

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