Antiadhesive Sites Present in the Fibronectin Type III-Like Repeats of Human Plasma Fibronectin

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We have found that fibronectin (FN) has a functional cryptic site opposing cell adhesion to extracellular matrix (ECM): a synthetic FN peptide derived from the 14th FN type III-like (FN-III) repeat, termed peptide FNIII14, inhibits cell adhesion to the FN without binding to β1 integrins. This antiadhesive activity of peptide FNIII14 depends on its C-terminal amino acid sequence YTIYVIAL. A 50-kDa membrane protein (p50) has been detected as a specific binding protein of peptide FNIII14. Here we showed that antiadhesive activity of peptide FNIII14 was depedent upon the presence of p50 on cell surfaces. Furthermore, we found that there exists a sequence, analogous to the YTIYVIAL, in the 10th FN-III repeat of the FN molecule and that a FN peptide containing this analogous sequence, termed peptide FNIII10, inhibited cell adhesion to the FN. Peptide FNIII10 appeared to share p50 with peptide FNIII14 in expressing the antiadhesive activity. As a physiological consequence of decreased adhesion, peptides FNIII10 and FNIII14 accelerated the anoikis-like apoptosis of normal fibroblasts by down-regulating Bcl-2 expression through blocking the FAK/PI3K/Akt signaling pathway. Thus, the YTIYVIAL-related sequences of the FN molecule may be involved in cell regulation by modulating negatively cell adhesion to the ECM, in which p50 probably serves as a membrane receptor.

Key words extracellular matrix; fibronectin; fibronectin type III-like repeat; integrin; cell adhesion

Cell adhesion to the extracellular matrix (ECM) is important determinants of cellular morphology and function.[1] The ECM proteins, such as fibronectin (FN), collagen and laminin, support cell adhesion and thereby influences various cellular processes, such as proliferation, differentiation, survival and migration. Cell adhesion should be controlled reversibly *in vivo*, and it has therefore been presumed that cell adhesion-inhibiting factors as well as cell adhesion-stimulating factors are required for normal development.[2,3] From this view point, endogenous factors modulating negatively cell adhesion to the ECM, so-called "antiadhesive factors," have been explored, and some protein factors such as thrombospondine,[4] tenascin,[5,6] SPARC[7] and osteopontin[8] have been identified.

FN which is one of the essential cell adhesive ECM proteins, harbors several cell adhesive sites,[9] such as RGD central cell adhesive site recognized by integrin $\alpha 5\beta 1$ and CS-1 and -5 in alternative splicing domain recognized by integrin $\alpha 4\beta 1$. We previously found that the FN has not only these cell adhesive sites but also an additional functional site opposing cell adhesion to the ECM, so-called "antiadhesive site."[10,11] A synthetic FN peptide derived from the 14th FN type III-like (FN-III) repeat, termed peptide FNIII14 (Fig. 1), strongly inhibits $\beta 1$ integrin-mediated cell adhesion to the FN without binding to integrins,[12] whose activity depends on its C-terminal amino acid sequence YTIYVIAL.[11] The antiadhesive sequence YTIYVIAL is cryptic at least in the plasma FN molecule, but it is exposed by either FN processing with MMP-2 or FN interaction with heparin.[13] As a negative modulator of cell-ECM interaction, peptide FNIII14 influences physiological cellular processes *in vitro*, such as survival,[14,15] differentiation[12,16] and gene expression,[17] as well as pathological events including tumor metastasis.[18] Therefore, it might be possible that this cryptic antiadhesive sequence YTIYVIAL, once exposed, plays important roles in cell regulation *in vivo*. Although an intracellular signaling pathway by which peptide FNIII14 expresses the antiadhesive activity remains unclear, our following previous findings have indicated that antiadhesive activity of peptide FNIII14 is mediated by a putative membrane receptor: (i) peptide FNIII14 conjugated with polyethylenglycol (Mr. $>$10000), which is membrane impermeable hydrophilic polymer, is able to inhibit cell adhesion to FN (Miura, S. and Fukai, F., unpublished data), (ii) peptide FNIII14 has the antiadhesive effect even when coated on culture plates,[11] (iii) peptide FNIII14 specifically binds to a membrane protein with molecular mass of 50-kDa (p50), not to $\beta 1$ integrins.[12]

There are several different sequences analogous to the antiadhesive sequence YTIYVIAL in another FN-III repeat of the FN molecule and also in the FN-III repeats of other ECM proteins. They include the YTITVYAV of the 10th FN-III repeat of the FN, the YQVTVIAL in the 1st FN-III repeat of type VII collagen and the YTITVSSL and YKITVIAV in the 13th and 16th FN-III repeats, respectively, of type XII collagen (Fig. 1). These analogous sequences may be important for these ECM protein molecules in serving as scaffoldings for cell adhesion. In this study, we first investigate whether the presence of p50, a specific binding protein of peptide FNIII14, on cell surface is necessary for peptide FNIII14 in expressing its antiadhesive activity. The results show that cellular susceptibility to the antiadhesive effect of peptide FNIII14 is in parallel with the presence of p50 on cell surfaces, suggesting that this p50 serves as a receptor for peptide FNIII14. Next, we examine whether peptides derived

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from the FN and types VII and XII collagens containing analogous sequences as above also show antiadhesive effect on cell adhesion to the FN. Among the ECM peptides, only a FN peptide, termed peptide FNIII10, is shown to inhibit cell adhesion to the FN, probably through binding with p50. As a consequence of negative regulation of cell adhesion, peptide FNIII10 as well as peptide FNIII14 induces apoptotic cell death by decreasing cell adhesion to the FN. The YTIYVIAL-related matricryptic sites of the FN molecule may serve as negative modulator of cell adhesion to the ECM, probably in patho-physiological situation where ECM degradation and remodeling occur frequently.

MATERIALS AND METHODS

Cells Human umbilical vein endothelial cells (HUVEC) were grown on gelatin-coated plates in MCB105 medium supplemented with 15% fetal bovine serum (FBS) and EGM2-MV (Sanko Lab., Tokyo). Human melanoma cells A375SM and normal mouse fibroblasts NIH3T3 were cultured in DMEM containing 10% FBS and 10% calf serum, respectively. Human B lymphoma cells Ramos were culturted in RPMI1640 containing 10% FBS.

Peptides Peptides derived from human FN and collagens and their mutants (Fig. 1, Table 1) were synthesized using the solid phase strategy, in which a Cys was added to the C-terminus of each peptide to increase their activity by dimerization, as described previously.[11] Synthetic peptides were purified by reversed-phase HPLC and characterized by mass spectrometry.

Antibodies Function-blocking monoclonal antibodies (mAbs) against integrin subunits α5 (P1D6) and α4 (P1H4) and β1 subunit (DE9) were purchased from CHEMICON and UPSTATE, respectively. Anti-β1 integrin mAb, which recognizes an active conformation-specific epitope of β1 integrin,[19] was obtained from MLB (Nagoya, Japan). The following antibodies were used for western blotting: pFAK [pY(397)] (Biosource Internatioanl, Inc.), FAK (Transduction Laboratories), pAkt [pS(473)] and Akt (Cell Signaling Technology), Bcl-2 (Santa Cruz Biotechnology, Inc.), and β-actin (Sigma).

Cell Adhesion Assay Cell adhesion assay was performed in medium without serum, as described previously.[18] In the assay, cell adhesion was evaluated by counting the number of cells spread on culture plates coated with human plasma FN (2 μg/ml).

Affinity Label Affinity labeling of cells to detect a putative FNIII14 receptor was carried out as described previously.[12] Briefly, cells were incubated with biotinylated FNIII14 (3 μg/ml) in the presence or absence of 67-fold molar excess of unlabeled peptide FNIII14 or its analogous peptides at 37 °C for 1 h. After washing unbound peptides off, peptides bound to cell surface proteins were covalently linked with EDC (15 mM) (Pierce). Cell lysates with equal amount of proteins were subjected to immunoblot analysis using POD-conjugated streptavidin.

Cell Survival Assay NIH3T3 cell suspensions in serum-free DMEM were mixed with various concentrations of pep-

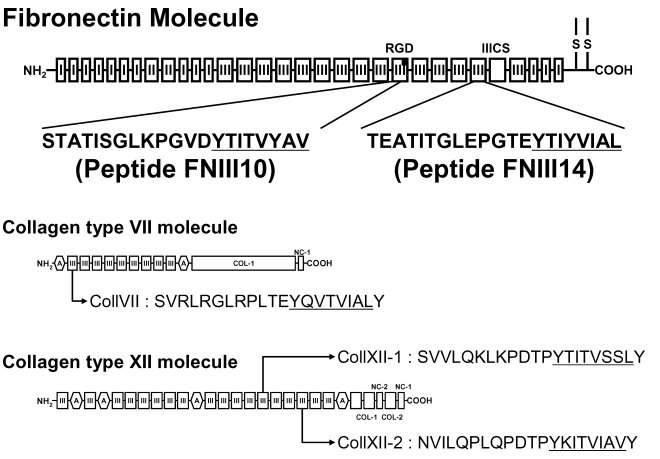

Fig. 1. Domain Structure of Human FN and Types VII and XII Collagens

Sequences analogous to the antiadhesive sequence YTIYVIAL in the 14th FN-III repeat of the FN molecule are also present in the other FN-III repeats of the FN and types VII and XII collagen molecules, as depicted.

Table 1. Scanning Mutagenesis of Peptides FNIII14 and FNIII10

Peptide	Sequence position																				FNIII14 activity[a]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
FNIII14	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	T	I	Y	V	I	A	L	+++
Y14A	T	E	A	T	I	T	G	L	E	P	G	T	E	A	T	I	Y	V	I	A	L	+++
T15A	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	A	I	Y	V	I	A	L	−
I16A	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	T	A	Y	V	I	A	L	−
Y17A	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	T	I	A	V	I	A	L	−
V18A	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	T	I	Y	A	I	A	L	−
I19A	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	T	I	Y	V	A	A	L	−
L21A	T	E	A	T	I	T	G	L	E	P	G	T	E	Y	T	I	Y	V	I	A	A	−
FNIII10	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	T	I	T	V	Y	A	V	++
Y14A	S	T	A	T	I	S	G	L	K	P	G	V	D	A	T	I	T	V	Y	A	V	+++
Y15A	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	A	I	T	V	Y	A	V	−
Y16A	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	T	A	T	V	Y	A	V	−
Y17A	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	T	I	A	V	Y	A	V	−
Y18A	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	T	I	T	A	Y	A	V	+
Y19A	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	T	I	T	V	A	A	V	−
Y21A	S	T	A	T	I	S	G	L	K	P	G	V	D	Y	T	I	T	V	Y	A	A	+

Sequence positions mutated are underlined. *a*) FNIII14 activity evaluated by the adhesion assay as in Fig. 2: −, no activity; +, weak activity; ++, moderate activity; +++, strong activity.

tides, and then seeded onto plates coated with indicated concentrations of FN. After culturing for 24 h, the number of viable cells was evaluated using Cell Counting Kit (DOJINDO laboratories) according to the manufacturer's instructions.

Western Blot Analysis NIH3T3 cells were serum-starved for 16 h, harvested with 2% EDTA in PBS(−), and then suspended with the serum-free DMEM medium. A part of this cell suspension was kept for 30 min in a microfuge tube with slow rotation at 37 °C (termed "Suspension"). Other parts of the NIH3T3 cell suspension were seeded onto a 6-well plate coated with FN (1 μg/ml) and then incubated in the presence or absence of peptides (200 μg/ml) for 30 min (for the detection of FAK, Akt) or 8 h (for Bcl-2, β-actin). Cells were harvested and lysed with Laemmli's buffer. Equal amounts of protein samples were separated on SDS-PAGE, followed by electroblotting to polyvinylidene difluoride membrane. pFAK, FAK, pAkt, Akt, Bcl-2, and β-actin were detected by using their corresponding antibodies, respectively, followed by chemiluminescence detection (Enhanced Chemiluminescence Plus; Amersham Pharmacia Biotech).

RESULTS

Antiadhesive Effect of Peptide FNIII14 We first confirmed antiadhesive effect of peptide FNIII14 on cell adhesion to the FN substrate using two different cell types that utilize different β1 integrins in adhering to the FN. HUVEC adhesion to the FN, which was mediated mainly by integrin $\alpha5\beta1$, was inhibited by peptide FNIII14 in a dose-dependent manner (Fig. 2A). Control peptide FNIII14scr (TEATITGLEPGTELIVYATYI),[11] in which the YTIYVIAL sequence is shuffled (underlined), was inactive in the antiadhesive effect (Fig. 2A). On the other hand, Ramos cells adhered only in the presence of Mn^{2+}, an integrin activator (Fig. 2B), because their β1 integrins are in an inactive status.[18] In agreement with the previous knowledge that Ramos cell line almost expresses $\alpha4\beta1$ as β1 class integrin,[20] adhesion of the cells to the FN was remarkably inhibited by anti-α4 and β1 mAbs, but not by anti-α5 mAb (Fig. 2B). Peptide FNIII14, but not peptide FNIII14scr, inhibited this Mn^{2+}-induced adhesion to the FN (Fig. 2B). These results suggested that peptide FNIII14 has the ability to inhibit cell adhesion to FN, which is mediated by at least integrins $\alpha4\beta1$ and $\alpha5\beta1$, and that this peptide activity is dependent on its C-terminal sequence YTIYVIAL.

Necessity of p50 Membrane Protein for Expression of Antiadhesive Activity of Peptide FNIII14 Next, we investigated whether only limited cell types are susceptible to the antiadhesive effect of peptide FNIII14. To solve this issue, several normal and transformed cell lines (normal mouse fibroblasts NIH3T3, SV40-transformed human embryonic lung fibroblasts WI38VA13, Mardin Darby caine kidney epithelial cells MDCK, human melanoma A375SM, mouse colon cancer cells Colon26M3.1 and human erythroleukemia cells K562) were subjected to cell adhesion assay with or without peptide FNIII14. As a result, most of cell lines tested was sensitive to peptide FNIII14. As represented by A375SM cells, their adhesion to the FN, which was blocked by GRGDSP peptide, an antagonistic peptide for integrin $\alpha5\beta1$, was inhibited dose-dependently by peptide FNIII14 (Fig. 3A). Adhesion of any other cell line, except Colon26M3.1

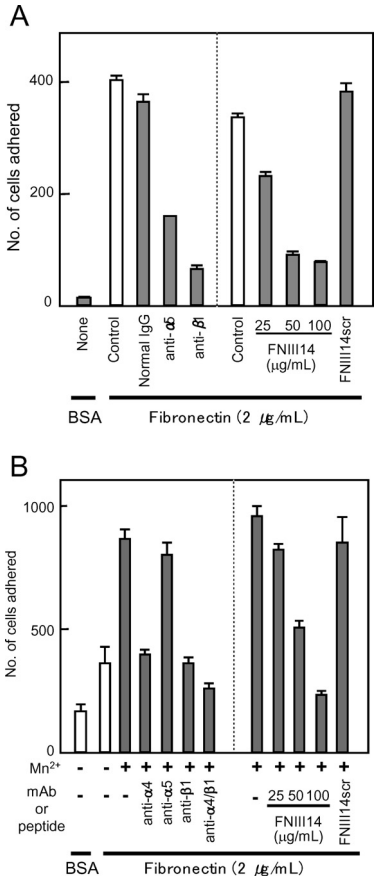

Fig. 2. Effects of Peptide FNIII14 on Adhesion of HUVEC (A) and Ramos Cells (B) to the FN Substrate

Cells suspended in the serum-free medium with or without Mn^{2+} (1 mM) were seeded on a 96-well plate coated with FN (2 μg/ml) or BSA (5 mg/ml) in the presence or absence of either function-blocking mAb [anti-α5 (P1D6), anti-α4 (P1H4), anti-β1 (DE9)] (30 μg/ml), peptide FNIII14 (100 μg/ml) or peptide FNIII14scr. After an 1 h-incubation, the number of cells spread on the FN substrate was counted as described in Materials and Methods. Data represent the means±S.D. of three determinations.

cells, to the FN was inhibited by peptide FNIII14, to an extent similar to A375SM melanoma cells. In contrast, adhesion of Colon26M3.1 cells to the FN was not influenced by peptide FNIII14 even at high dosages (Fig. 3B). This adhesion was also blocked by addition of GRGDSP peptide (Fig. 3B), indicating the $\alpha5\beta1$-mediated adhesion of Colon26M3.1 cells to the FN. Since peptide FNIII14 had the ability to inhibit $\alpha5\beta1$-mediated cell adhesion (see Fig. 2A), Colon26M3.1 cells might be lacking in a putative membrane receptor mediating the antiadhesive effect of peptide FNIII14.

We previously found the presence of a 50-kDa membrane protein (p50) that has a specific binding activity to peptide FNIII14, as detected by affinity-labeling using biotinylated FNIII14.[12] We then performed the affinity labeling to ascertain whether cellular susceptibility to the antiadhesive effect of peptide FNIII14 is involved in the presence of p50 on cell surfaces. As can be seen in Fig. 4A, a specific binding of biotinylated peptide FNIII14 with p50 was visualized as a 50-kDa band using A375SM cells that were sensitive to peptide FNIII14. This p50 band disappeared when the affinity labeling was performed in the presence of excess molar unlabeled peptide FNIII14, but not control peptide FNIII14scr, indicating that p50 specifically bound with biotinylated peptide

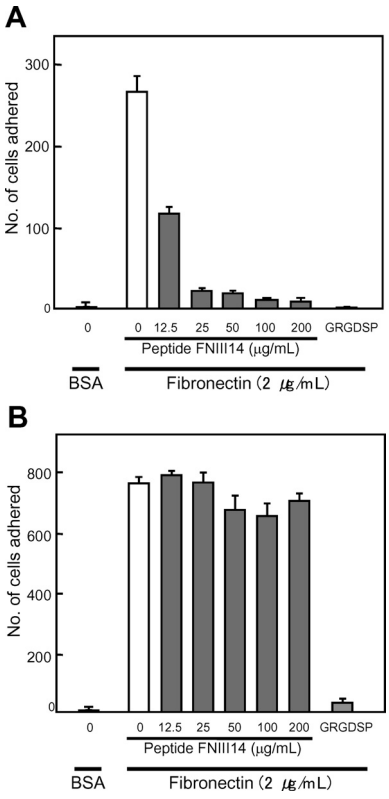

Fig. 3. Effects of Peptide FNIII14 on Adhesion of A375 SM (A) and Colon26M3.1 Cells (B) to the FN Substrate

Cell adhesion assay was carried out as described in the legend of Fig. 2. GRGDSP peptide (300 μg/ml) was used as an antagonist for integrin $\alpha5\beta1$. Data represent the means±S.D. of three determinations.

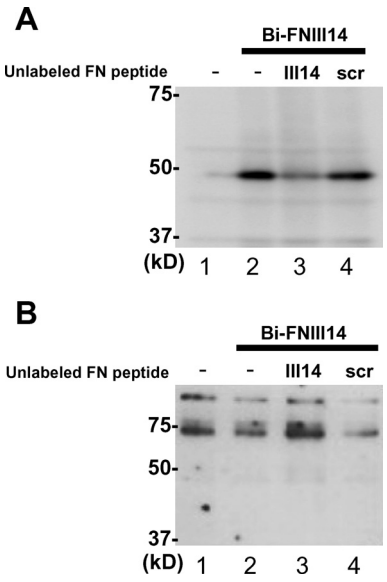

Fig. 4. Detection of p50 on A375SM and Colon26M3.1 Cell Surfaces

p50 of A375SM (A) and Colon26M3.1 (B) cells were detected by the affinity-labeling method as described in Materials and Methods. Lane 1, cell lysate; lane 2, +biotinylated FNIII14 (3 μg/ml); lane 3, +biotinylated FNIII14+unlabeled peptide FNIII14 (200 μg/ml); lane 4, +biotinylated FNIII14+unlabeled FNIII14scr (scr) (200 μg/ml).

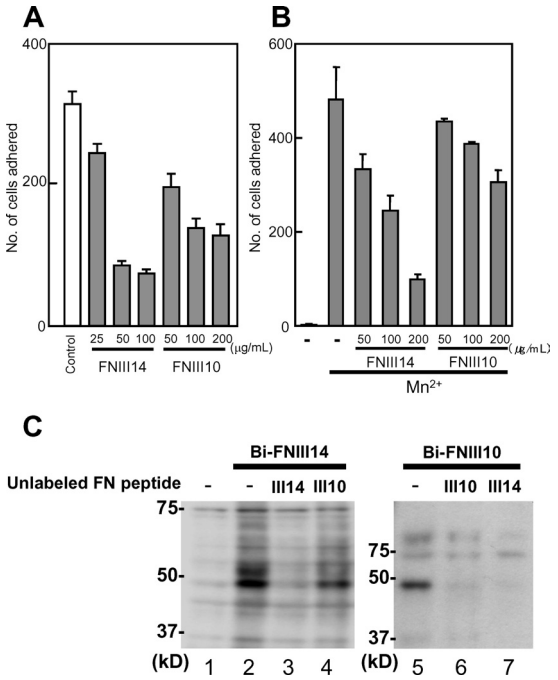

Fig. 5. Antiadhesive Effect of Peptides FNIII10

Effects of peptides FNIII14 and FNIII10 on cell adhesion were assayed using A375SM (A) or Ramos (B) cells as described in the legend of Fig. 2. Data represent the means±S.D. of three determinations. (C) Binding of peptides FNIII10 to p50, as evaluated using Ramos cells by the affinity labeling method. The affnity labeling was performed as described in the legend of Fig. 4, in which biotinylated peptide FNIII10 was also used as follows. Lane 1, cell lysate; lane 2, +biotinylated FNIII14; lane 3, +biotinylated FNIII14+unlabeled peptide FNIII14; lane 4, +biotinylated FNIII14+unlabeled FNIII10; lane 5, +biotinylated FNIII10; lane 6+biotinylated FNIII10+unlabeled peptide FNIII10; lane 7, +biotinylated FNIII10+unlabeled peptide FNIII14.

FNIII14 recognizing selectively the antiadhesive sequence YTIYVIAL. In contrast, no clear band due to specific binding of biotinylated peptide FNIII14 was detected using Colon26M3.1 cells (Fig. 4B) that were insensitive to peptide FNIII14. Presence of p50 was confirmed in all other cell types which were susceptible to the antiadhesive effect of peptide FNIII14 (data not shown). Thus, the presence of p50 on cell surfaces was in parallel with cellular susceptibility to the antiadhesive effect of peptide FNIII14, suggesting that p50 on cell surfaces might be indispensable for peptide FNIII14 in expressing its antiadhesive activity.

Antiadhesive Activity of ECM Peptides Containing Sequences Analogous to the YTIYVIAL There are several different sequences analogous to the antiadhesive sequence YTIYVIAL also in other FN-III repeats of FN molecule and collagens. They include the YTITVYAV of the 10th FN-III repeat in the FN, the YQVTVIAL of 1st FN-III repeat in type VII collagen, and the YTITVSSL and YKITVIAV of the 13th and 16th FN-III repeats, respectively, in type XII collagen (Fig. 1). We examined whether synthetic peptides containing these analogous sequences in their C-terminus[11] could exert any effect on cell adhesion to the FN. In this cell adhesion assay, we adopted A375SM and Ramos cells that adhered to the FN *via* integrin $\alpha5\beta1$ (see Fig. 3A) and $\alpha4\beta1$ (see Fig. 2B), respectively. Results showed that peptide FNIII10 was capable of suppressing the RGD-dependent cell adhesion to the FN, but to a slightly lesser extent as compared to peptide FNIII14 (Figs. 5A, B). All other peptides derived from types VII and XII collagens showed no significant effect on both types of cell adhesion to the FN (data not shown).

We then tested whether peptide FNIII10 has the ability to bind specifically to p50, as evaluated by competitive inhibition of peptide FNIII14 binding to p50 (Fig. 5C). Specific labeling of p50 with biotinylated peptide FNIII14 was inhibited by unlabeled peptide FNIII14 added excessively (lanes 2, 3). Addition of unlabeled peptide FNIII10, instead of unlabeled peptide FNIII14, also resulted in a siginificant, but to a lesser extent, inhibition of the p50 labeling with biotinylated peptide FNIII14 (lane 4). When the affinity labeling was carried out using biotinylated peptide FNIII10, a band with molecular mass of 50-kDa was also detected (lane 5). This 50-kDa band disappeared by addition of not only peptide FNIII10 (lane 6) but also peptide FNIII14 (lane 7). Addition of the collagen peptides showed no significant effect on labeling of this 50-kDa band (data not shown). These results indicated that peptide FNIII10 was capable of binding specifically to the p50 that was affinity-labeled with peptide FNIII14. The membrane protein p50 appeared to serve as a common membrane receptor mediating the antiadhesive effects of peptides FNIII14 and FNIII10.

Estimation of Amino Acids Essential for the Antiadhesive Activity To determine amino acid residues essential for the antiadhesive activity, we performed alanine scanning mutagenesis of peptides FNIII14 and FNIII10 in their C-terminal sequences (YTIYVIAL and YTITVYAV). Table 1 presents the mutant peptides tested here. In peptide FNIII14, the Tyr residue at position 14 was dispensable for the antiadhesive activity (Fig. 6A). Conversely, the antiadhesive activity was highly sensitive to single Ala replacement of the Thr, Ileu, Tyr, Val, Ileu and Leu residues at positions 15—21, respectively, and the resultant peptides (T15A, I16A, Y17A, V18A, I19A and L21A) were inactive in the antiadhesive effect. Also in peptide FNIII10, Ala replacement of Tyr at position 14 did not cause a significant decrease in the antiadhesive activity, but rather increased it (Fig. 6B). Single replacement of the Thr, Ileu, Thr and Tyr residues at positions 15—17 and 19, respectively, abolished the antiadhesive activity. Single Ala replacement of two Val residues at positions 18 and 21 caused a partial reduction in the antiadhesive activity, whereas their double replacement completely diminished the antiadhesive activity. Thus, scanning mutagenesis of peptides FNIII14 and FNIII10, taken together with lacking of the antiadhesive activity of the collagen peptides, suggested that the T-I-Y/T-V-I/A-L/V might exert as an amino acid motif for the antiadhesive activity.

Antiadhesive FN Peptides Accelerate Anoikis-Like Apoptosis as a Physiological Consequence of Cell Adhesion Suppression It is important to define whether peptides FNIII14 and FNIII10 are able to produce a reliable cellular response. Therefore, we finally investigated cellular response to the antiadhesive peptides.

It has been well-established that normal cell types, such as endothelial cells, epithelial cells and fibroblasts, undergo apoptotic cell death caused by loss of the integrin-ECM interaction, a process termed "anoikis."[21,22] When normal mouse fibroblasts NIH3T3 were kept under serum-free conditions, cells underwent apoptotic death (Fig. 7A). This cell death became more evident in cells cultured on a lower density of the FN substrate, but completely blocked in the presence of a general caspase inhibitor z-VAD fmk (Fig. 7A), suggesting that cell death observed here was a caspase-dependent, anoikis-like apoptosis due to decreased adhesion to the FN. This anoikis-like apoptosis of the NIH3T3 cells was remarkably accelerated by addition of peptide FNIII14 in a dose-dependent manner (Fig. 7A), and this was blocked by z-VAD fmk. Similarly, peptide FNIII10 significantly accelerated the NIH3T3 cell death. We then examined the activation status of the FAK/PI3K/Akt-signaling pathway, which is known to function as a main survival signaling pathway activated by integrin-mediated cell adhesion to ECM. As can be seen in Fig. 7B, both peptides FNIII10 and FNIII14 clearly reduced phosphorylation/activation of FAK and Akt. Furthermore, expression of Bcl-2, one of the well-accepted anti-apoptotic factors, was down-regulated by treatment with peptides FNIII10 and FNIII14 (Fig. 7B). Thus, peptide FNIII10 as well as FNIII14 was capable of blocking the FAK/PI3K/Akt cell survival signal generated through the integrin-mediated cell adhesion to the FN, resulting in a physiological cellular response including acceleration of the anoikis-like apoptosis of normal fibroblasts by down-regulating the Bcl-2 expression.

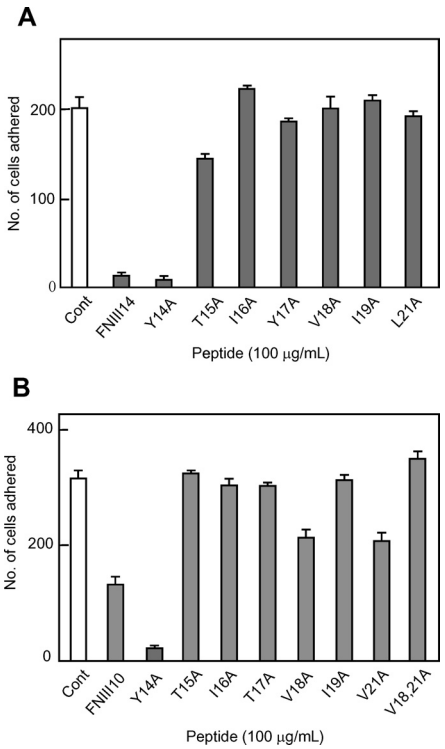

Fig. 6. Alanin Scanning Mutagenesis of Peptides FNIII14 and FNIII10

Effects of mutant peptides as presented in Table 1 on A375SM cell adhesion to the FN were carried out as described in the legend of Fig. 3.

DISCUSSION

Interaction of β1 integrins with the ECM components is important in regulation of cellular processes, such as proliferation, differentiation and survival.[23] A characteristic feature of integrins is their ability to alter their affinity for ligands in response to intracellular signaling, a process termed "inside-out signaling."[24] Several studies suggest that there are cytoplasmic signaling pathways that regulate either positively or negatively integrin activation. For example, H-Ras can suppress β1 integrin activation *via* its downstream effec-

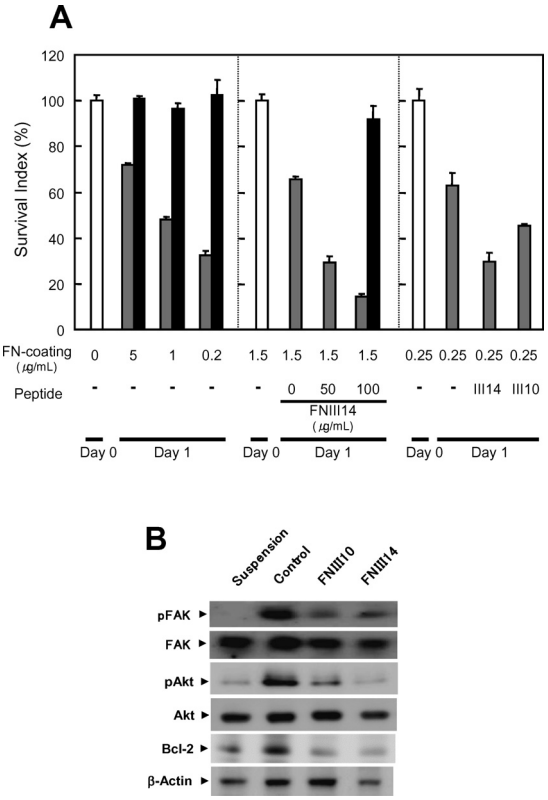

Fig. 7. Antiadhesive ECM Peptides Accelerate Apoptotic Cell Death of NIH3T3 Cells

(A) NIH3T3 cell suspension in the presence or absence of peptides FNIII14 or FNIII10 was seeded into 96-well plates coated with the FN at the indicated concentrations and cultured for 24 h under the conditions as indicated in the figure (open and shadow bars). Samples further mixed with z-VAD fmk (60 μM) were also cultured (closed bars). After the incubation, the number of viable cells was evaluated by measuring the absorbance of the formazan product with Cell Counting Kit. Data represent the means±S.D. of three determinations. (B) NIH3T3 cell suspension kept in a microfuge tube for 30 min (Suspension) or NIH3T3 cells adhered to the FN coated on a culture plate in the presence or absence (Control) of the indicated peptide (200 μg/ml) (FNIII14, FNIII10) for 30 min (FAK, Akt) or 8 h (Bcl-2, β-actin) were lysed with Laemmli's buffer, as described in Materials and Methods. Equal amount protein from each sample was subjected to Western blot analysis with antibodies to phospho-FAK, FAK, phospho-Akt, Akt, Bcl-2, or β-actin. Shown are results representative of two similar experiments.

tors,[25] in contrast, R-Ras promotes it.[26] Although their signaling cascades are incompletely understood, functions of these small G proteins are generally considered to be controlled by extracellular stimuli *via* certain membrane receptors. Thus, there have been many examples showing that integrin activation is controlled by extracellular signals *via* their membrane receptors.[27—29] Our following previous results have indicated that antiadhesive activity of peptide FNIII14 is also mediated by a putative membrane receptor: (i) peptide FNIII14 conjugated with membrane impermeable polymer, polyethylenglycol (Mr. >10-kDa), is able to inhibit cell adhesion to the FN (Miura, S. and Fukai, F., unpublished data), (ii) peptide FNIII14 has the antiadhesive effect even when coated on culture dishes,[11] (iii) peptide FNIII14 specifically binds to p50, not to β1 integrins, present on cell surface.[12]

To verify a functional significance of this p50 in expression of the antiadhesive activity of peptide FNIII14, various cell lines were examined for their susceptibility to the antiadhesive effect of peptide FNIII14. The results showed that p50 was detected in all cell lines, which were sensitive to peptide FNIII14, whereas only Colon26M3.1 cell line, which was insensitive to peptide FNIII14, did not have detectable amount of p50 on their surfaces. The results suggest that whether peptide FNIII14 is able to inhibit cell adhesion to the FN is dependent upon the presence of the p50 on cell surfaces. Furthermore, peptide FNIII10 which was found as an additional antiadhesive FN peptide, also had a specific binding affinity toward p50, as judged by the affinity labeling method. Thus, the antiadhesive peptides FNIII14 and FNIII10 appeared to utilize the p50 as a common membrane receptor in expressing their antiadhesive activity. Identification of the p50 is extremely required to define the molecular mechanism by which these antiadhesive FN peptides suppress β1 integrin-mediated cell adhesion. A large scale purification of the p50 is now in progress.

It has been well established that FN has multiple functional sites mediating cell adhesion to the ECM.[9] Cellular interactions with these adhesive sites cause switching on a variety of intracellular signaling pathways, resulting in modulation of cellular processes such as proliferation, differentiation and survival. Our previous study indicated that FN also has a functional site opposing cell adhesion to the ECM, *i.e.* antiadhesive sequence YTIYVIAL of the 14th FN-III repeat.[10,11] The present study showed that there exists one more antiadhesive site within the 10th FN-III repeat. Our previous study demonstrated that the antiadhesive sequence YTIYVIAL of the FN is buried within the 14th FN-III repeat structure at least in the plasma FN molecule, probably due to its hydrophobic nature.[11] This cryptic antiadhesive sequence YTIYVIAL is exposed by either interaction of FN with heparin or processing of intact FN molecule with MMP-2.[10,11,13] In the present study, we did not confirm whether the active sequence YTITVYAV of the 10th FN-III repeat is also buried within its surounding structure. However, because the sequence YTITVYAV is composed of hydrophobic amino acids, it may be also cryptic in the intact FN molecule. If so, this cryptic site may be exposed through the processes by which the antiadhesive sequence YTIYVIAL of the 14th FN-III repeat is done.[12] The ECM generates a variety of signals for cell regulation, some of which are originated from biologically active cryptic sites, so-called "matricryptic site," of ECM molecules.[30—32] ECM–ECM and cell-ECM interactions can lead to exposure of matricryptic sites through conformational changes of ECM proteins, and subsequent unfolding of ECM proteins renders its individual protein chains susceptible to broad-spectrum proteases that can release biologically active ECM fragments, termed matricryptins.[32] We have demonstrated that peptide FNIII14 is capable of modulating cellular processes *in vitro*, such as proliferation,[17] survival,[14,15] differentiation[13,16] and gene expression.[17] In this study, peptide FNIII10 as well as FNII14 was shown to induce anoikis-like apoptosis of normal fibroblastic cells. Therefore, two antiadhesive sequences found in the FN molecule, if exposed, would influence cellular processes by modulating negatively cell adhesion to the ECM, particularly in pathological tissues where exposure of the matricryptic sites and release of matricryptins happen frequently. It is needed to investigate whether the FNIII14-related antiadhesive sites are actually exposed and exerted as a cell adhesion modulator *in vivo*.

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