Spontaneous Formation of L-Isospartate and Gain of Function in Fibronectin*

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Isospartate formation in extracellular matrix proteins, by aspartate isomerization or asparagine deamidation, is generally viewed as a degradation reaction occurring in vivo during tissue aging. For instance, non-enzymatic isospartate formation at RGD-integrin binding sites causes loss of cell adhesion sites, which in turn can be enzymatically “repaired” to RGD by protein-L-isoAsp-O-methyltransferase. We show here that isospartate formation is also a mechanism for extracellular matrix activation. In particular, we show that deamidation of Asn263 at the Asn-Gly-Arg (NGR) site in fibronectin N-terminal region generates an αβ3-integrin binding site containing the l-isoDGR sequence, which is enzymatically “deactivated” to DGR by protein-l-isoAsp-O-methyltransferase. Furthermore, rapid NGR-to-isoDGR sequence transition in fibronectin fragments generates αβ3 antagonists (named “isonectons”) that competitively bind RGD binding sites and inhibit endothelial cell adhesion, proliferation, and tumor growth. Time-dependent generation of isoDGR may represent a sort of molecular clock for activating latent integrin binding sites in proteins.

Fibronectins are adhesive proteins that mediate a variety of cellular interactions with extracellular matrix and play important roles in hemostasis, thrombosis, inflammation, wound repair, angiogenesis, and embryogenesis (1, 2). About 20 isoforms of human fibronectin can be generated as a result of alternative splicing of the primary transcript (1, 3). Fibronectins are large glycoproteins (~450 kDa) composed of two nearly identical disulfide-bonded subunits present in most body fluids and extracellular matrix of many tissues. Each subunit consists of three types of repeating homologous modules termed FN-I, FN-II, and FN-III repeats. Alternatively spliced modules, called EDA, EDB, and IIICS, can also be present (1, 3). Single modules or groups of modules may contain binding sites for different molecules, including sulfated glycosaminoglycans, DNA, gelatin, heparin, and fibrin (1, 3, 4). Furthermore, fibronectins contain binding sites for about half of the known cell surface integrin receptors (5, 6). In particular, the FN-III10 repeat contains an RGD site that can bind α3β1, α5β1, α6β1, αβ3, αβ5, α5β2, α6β3, and αIIbβ3 integrins, while the FN-III1 repeat contains the so-called “synergy site” PHSRN that cooperates with RGD in the binding of α3β1 and αIIbβ3 (1, 7).

Primary and tertiary structure analysis of human fibronectin showed that this protein contains two GNGRG loops, located in FN-I5 and FN-I7 modules, that are conserved in bovine, murine, rat, amphibian, and fish (8). Two additional NGR sites, less conserved, are also present in human FN-II1 and FN-III4 (see Fig. 1). Recent experimental work showed that peptides containing the NGR motif can inhibit α3β1 and αβ3-mediated cell adhesion to fibronectin (9).

These notions prompted us to investigate the functional role of NGR in fibronectin. We observed that the NGR sequence of FN-I5 (residues 263–265) promotes endothelial cell adhesion via an unusual mechanism based on non-enzymatic deamidation of Asn263 to l-isoAsp, generating isoDGR, a new cell adhesion motif. Furthermore, we show that αβ3 integrin is an important receptor of isoDGR and that the deamidated FN-I5 module (named “isonecton-1”) regulates endothelial cell adhesion and inhibits tumor growth. Finally, we show that this motif is regulated in a negative manner by protein-l-isoAsp-O-methyltransferase (PIMT). Based on these findings we propose a new “activation/deactivation” model for isoAsp “formation/removal” in fibronectin.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Mouse RMA lymphoma cells, B16 melanoma, and EA.hy926 cells (human endothelial cells fused with human lung carcinoma A549 cells) were cultured as described previously (10, 11). Human microvascular endothelial cells, HMEC-1, were provided by Dr. A. Manfredi (San Raffaele H Scientific Institute, Milan, Italy) and cultured in MCDB131 medium supplemented with 2 mm l-glutamine, 10 units/ml penicillin, 10 units/ml streptomycin, 25 ng/ml amphotericin B, 10% fetal bovine serum, 10 ng/ml human recombinant human growth hormone, and 1% anticoagulant. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion of umbilical cords and cultured as described previously (12). Primary human umbilical vein endothelial cells (HUVECs) were cultured as described previously (12). HMEC-1 cells were cultured as described previously (12).

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‡ The abbreviations used are: PIMT, protein-L-isoAspO-methyltransferase; TNFα, tumor necrosis factor α; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; RP-HPLC, reverse phase high performance liquid chromatography; HMEC, human microvascular endothelial cell.
nant epidermal growth factor (R&D Systems, Inc.), and 1 μg/ml bovine hydrocortisone (complete medium). Crystal violet (Fluka Chemicals), bovine serum albumin (BSA), goat anti-rabbit IgG horseradish peroxidase conjugate, FN-70 kDa, FN-45 kDa, and FN-30 kDa fragments (Sigma), RetroNectin (Takara Biomedicals), human αβ2α3β4 integrins (Immunological Sciences), and streptavidin peroxidase (Società Prodotti Antibiotici) were used. Human fibronectin was freshly isolated from plasma by affinity chromatography on gelatin-Sepharose as described (12).

**Preparation and Characterization of Recombinant FN-I4–5 and FN-I4–5SGS**—The cDNA coding for human fibronectin fourth-fifth type I repeats (FN-I4–5), residues 184–273 of fibronectin, was synthesized using the following primers: 5'-TATATTAAGCTTTCAGTGCCTCTCACACTTCC (forward) and 5'-TATATTAAAGCTTTCAGTGCCTCTCACACTTCCACTCTCCACTGCCGCTG. Amplified fragments were cloned into a pRSET-A plasmid (Invitrogen), and the following reverse primer, 5'-TGCCTCTCACACTTCCACTCTCCACTGCCGCTG. Amplified fragments were cloned into a pRSET-A plasmid (Invitrogen), expressed in BL21(DE3)pLysS cells (Novagen), and purified from cell extracts by affinity chromatography on soluble p75-TNF receptor-Sepharose essentially as described previously for CNGRC-TNF (16). Both CNGRC-TNF and CDGRC-TNF were subjected to a refolding procedure as described previously (14). Protein purity and identity were checked by SDS-PAGE, electrospray mass spectrometry, and gel filtration chromatography. The in vitro cytolytic activity of CNGRC-TNF and CDGRC-TNF, measured by standard cytolytic assay with L-M mouse fibroblasts (17), was 2.96 (± 0.56) × 10^8 and 2.59 (± 0.69) × 10^8 units/mg, respectively. CNGRC-TNF1–11, corresponding to the N-terminal sequence of NGR-TNF (lacking TNF activity) and CARAC-TNF1–11 peptide, were prepared by chemical synthesis as described above.

**Isoaspartate (isoAsp) Quantiﬁcation—isoAsp content in proteins and peptides was quantified using the IsoQuant isoaspartate detection kit (Promega). The isoAsp content in ﬁbronectin (freshly isolated from human plasma) and FN-30 kDa fragment was 0.048 and 0.026 pmol/pmol of protein, respectively.**

**Cell Adhesion Assay and PIMT Treatment**—Untreated and heat-treated ﬁbronectin fragments, peptides, and peptide-TNF conjugates were diluted at 0.1 μm ammonium bicarbonate buffer, pH 8.5, incubated for 16 h at 37 °C, and stored at −20 °C until analysis. These products are hereinafter referred to as “heat treated.”

**Isoaspartate Formation in Fibronectin**—The cDNA coding for human fibronectin fourth-fifth type I repeats (FN-I4–5), residues 184–273 of fibronectin, was prepared by reverse transcriptase PCR on MSR-3-mel cells total RNA (13) using the following primers: 5'-CTGGATCCGAGAAGTGTTTTGATCATGCTGCTGGG (forward) and 5'-TATATTAAGCTTTCAGTGCCCTCTCACACTTCC (reverse). A control fragment with NGR replaced with SGS (FN-I4–5SGS), was generated by PCR on FN-I4–5 plasmid using the above forward primer and the following reverse primer, 5'-TATATTAAGCTTTCAGTGCCCTCTCACACTTCCACTCTCCACTGCCGCTG. Amplified fragments were cloned into a pRSET-A plasmid (Invitrogen), expressed in BL21(DE3) E. coli cells (Novagen), and purified from cell extracts by affinity chromatography on soluble p75-TNF receptor-Sepharose essentially as described previously for CNGRC-TNF (16). Both CNGRC-TNF and CDGRC-TNF were subjected to a refolding procedure as described previously (14). Protein purity and identity were checked by SDS-PAGE, electrospray mass spectrometry, and gel filtration chromatography. The in vitro cytolytic activity of CNGRC-TNF and CDGRC-TNF, measured by standard cytolytic assay with L-M mouse fibroblasts (17), was 2.96 (± 0.56) × 10^8 and 2.59 (± 0.69) × 10^8 units/mg, respectively. CNGRC-TNF1–11, corresponding to the N-terminal sequence of NGR-TNF (lacking TNF activity) and CARAC-TNF1–11 peptide, were prepared by chemical synthesis as described above.
bation the plates were washed with 0.9% sodium chloride, and cell adhesion assay was performed as described above.

Binding of Peptides and Proteins to Integrins—Human α5β1, α5β3, and α1β1 integrin solutions, 0.5–2 g/ml in phosphate-buffered saline with Ca2+ and Mg2+ (DPBS; Cambrex), were added to 96-well polystyrene microtiter plates (50 μl/well) and left to incubate overnight at 4 °C. All subsequent steps were carried out at room temperature. The plates were washed with DPBS and further incubated with DPBS containing 3% BSA (200 μl/well, 1 h). The plates were washed and filled with CNGRC-TNF or TNF solutions (5 μg/ml, 50 μl/well in 3% BSA-DPBS) and left to incubate for 2 h. After washing with DPBS, each well was incubated with purified rabbit anti-murine TNF IgGs in 3% BSA-DPBS containing 1% normal goat serum (10 μg/ml, 50 μl/well, 1 h) followed by a goat anti-rabbit peroxidase conjugate in the same buffer (50 μl/well, 1 h). Bound peroxidase was detected by adding o-phenylendiamine chromogenic substrate. Binding of biotinylated peptides (CNGRC-TNF1–11, CARAC-TNF1–11, and FN-I5) to purified integrins was studied using streptavidin peroxidase conjugate com-

FIGURE 1. Schematic representation, three-dimensional structure, and primary sequence of fibronectin fragments. A, type I repeats (rectangles), type II repeats (ovals), type III repeats (squares). Modules containing NGR and RGD sequences are indicated. Natural fragments (FN-70, FN-45, and FN-30 kDa), retronectin, FN-I4–5, and FN-I5 are represented. B, three-dimensional PDB structure of FN-I4–5 (Protein Data Bank code 1FBR). The side chains of the NGR motif (Asn263, Gly264, and Arg265) are shown. C, primary sequence of synthetic FN-I5, FN-Loop-GNGRG, and FN-Loop-RGGNG peptides.
plexes. Complexes were prepared by mixing various quantities (0.5–1 μg) of heat-treated biotinylated peptides in DPBS containing 3% BSA with 0.03 units of streptavidin peroxidase (binding capacity 1 μg of biotin/unit of streptavidin peroxidase) (final volume 15 μl). Complexes were diluted in 3% BSA-DPBS (1:500), added to microtiter plates coated with integrins as described above, and incubated for 2 h at room temperature. After washing with DPBS, bound peroxidase was detected by chromogenic reaction as described above. Each assay was carried out in triplicate.

Inhibition of Endothelial Cell Proliferation—HMEC-1 cells (8 × 10^3) in complete medium were seeded in 96-well plate (100 μl/well). After 2 h of incubation at 37 °C, 5% CO_2, 100 μl of peptide solution in complete medium (100 μM) was added to each well. Cells were further incubated for 72 h at 37 °C, 5% CO_2. The number of viable cells present within each well was then determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using a calibration curve generated by plating different amounts of cells.

In Vivo Studies—Studies on animal models were approved by the Ethical Committee of the San Raffaele H Scientific Institute and performed according to the prescribed guidelines. C57BL/6N mice (Charles River Laboratories, Calco, Italy) weighing 16–18 g were challenged with subcutaneous injection in the left flank of 7 × 10^4 RMA living cells; 4 days later, mice were treated daily with 200 μg of heat-treated FN-I5 (100 μl) in 0.9% sodium chloride (intraperitoneal). Tumor growth was monitored by measuring tumors with calipers as previously described (18). Animals were sacrificed before tumors reached 1.0–1.3 cm in diameter. Tumor sizes are shown as mean ± S.E. (five animals/group).

RESULTS

Accelerated Aging of Fibronectin Fragments Generates NGR-dependent Adhesion Sites—The adhesion of EA.hy926 cells to microtiter plates coated with various peptides and proteins before (±20 °C) and after (+37 °C) accelerated aging, A, adhesion to natural proteolytic fragments FN-70 kDa, FN-45 kDa, and FN-30 kDa. B, recombinant FN-I4–5 or control FN-I5–SGS fragment. C, synthetic FN-I5. D, recombinant CNGRC-TNF, CNGRC-TNF-I1–11, and control CARAC-TNF-I1–11 peptide; E, CGRC-TNF. Microphotograph of wells coated with 30 μg/ml of heat-treated FN-I5 or BSA (C, right), × 200; bar indicates 50-μm scale. Cell adhesion assays were carried out as described under “Experimental Procedures.” Mean ± S.E. (n = 2).

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treatment will be called “heat treatment”), we observed an increase of cell adhesion to FN-I_{4–5}, but not to FN-I_{4–5}SGS (Fig. 2B). This suggests that a cell adhesion site, somehow related to the NGR sequence, was generated by accelerated aging. Similar results were obtained with synthetic FN-I_{5} (Fig. 2C).

To assess whether the NGR motif was sufficient for mediating this phenomenon, we investigated the pro-adhesive properties of short peptides containing the NGR motif before and after heat treatment. Because the NGR tripeptide is unlikely to fold and to bind to microtiter plates, we introduced two flanking cysteines. The rationale for using flanking cysteines was based on the fact that the predicted conformation of CNGRC, by molecular dynamic simulation, is similar to that of the GNGRG loop of FN-I_{3} repeat (8). Furthermore, we fused this peptide to TNF (CNGRC-TNF) or to the first eleven residues of TNF (CNGRC-TNF_{1–11}, devoid of TNF activity) to enable adsorption to microtiter plates. As expected, heat treatment of CNGRC-TNF increased cell adhesion (Fig. 2D). No adhesion was observed to TNF alone either before or after heat treatment (data not shown). Similarly, heat treatment of CNGRC-TNF_{1–11}, but not of CARAC-TNF_{1–11} (a control peptide), increased cell adhesion (Fig. 2D, right).

These results support the hypothesis that the NGR motif is sufficient for promoting cell adhesion after heat treatment. Interestingly, a CDGRC-TNF conjugate, prepared by recombinant DNA technology, was completely inactive (Fig. 2E), suggesting that Asn is a crucial residue for the enhanced activity. In conclusion, these results suggest that structural changes related to the Asn residue of NGR motif in FN-I_{3} lead to generation of a pro-adhesive site.

Deamidation of the NGR Motif Is Associated with Increased Cell Adhesion—It is well known that the Asn residues, particularly when followed by Gly, can undergo non-enzymatic deamidation via succinimide intermediate at physiological pH (19–23). This reaction leads to formation of Asp and isoAsp, predominantly in L-configuration (24). Accordingly, heat treatment of FN-I_{3}, CNGRC-TNF, and CNGRC peptides increased their molecular mass by ~1 Da as measured by mass spectrometry analysis (data not shown). Furthermore, >0.5 mol isoAsp/mol of heat-treated CNGRC-TNF subunit was detected using the IsoQuant kit.

To assess whether the enhanced adhesion properties of FN-I_{3} after heat treatment depended on NGR deamidation, we incubated the heat-treated FN-I_{3} and CNGRC-TNF with PIMT, an enzyme that converts L-isoAsp and D-Asp residues to L-Asp (25–29). PIMT almost completely inhibited the pro-adhesive activity of heat-treated FN-I_{3} and CNGRC-TNF (Fig. 3A). Moreover, this enzymatic treatment partially inhibited the pro-adhesive properties of natural FN-30 kDa (Fig. 3B) and FN-45 kDa fragments (data not shown), suggesting that Asn deamidation can occur also in natural fibronectin fragments.

To assess the specificity of this reaction we evaluated the effect of PIMT on retronectin, an FN fragment that is known to promote cell adhesion via RGD (see Fig. 1). As expected, in this case no inhibition was observed after enzymatic treatment (Fig. 3B). Conversely, in this case we observed a modest but significant increase. This was not totally unexpected as also RGD can undergo isomerization of Asp residues, with formation of isoAsp and loss of function (20, 24, 30, 31). Thus, in this case PIMT is expected to “repair” RGD and increase cell adhesion. In conclusion, these results suggest that NGR deamidation, in contrast to RGD isomerization, is associated with a “gain of function” in cell adhesion assays.

Mechanism of NGR Deamidation—The mechanism and the kinetics of peptide deamidation in DMEM, pH 7.53, and in 0.1 M ammonium bicarbonate, pH 8.5, were then investigated. To this aim the CNGRCGVRY peptide (called NGR-2C) was synthesized and analyzed by reverse-phase HPLC before and after incubation at 37 °C. Residues GVRY were added to the C terminus of NGR to enable detection and column adsorption. In addition, two peptides called CNGRCGVRY (DGR-2C) and CisoDGRCGVRY (isoDGR-2C) corresponding to the same sequence of NGR-2C except for the presence of L-Asp and L-isoAsp in place of L-Asn, respectively, were prepared and analyzed by HPLC. The half-life of NGR-2C at pH 7.53 and pH 8.5, estimated from the height of the main chromatographic peak (Fig. 4A, peak 1), was ~4 and 2 h, respectively (Fig. 4A, inset). In contrast, DGR-2C and isoDGR-2C under the same conditions were considerably more stable than NGR-2C (not shown).

Further studies on NGR-2C stability in 150 mM sodium chloride, 25 mM Hepes, pH 7.4, and in 150 mM sodium chloride, 50 mM sodium phosphate, pH 7.3, showed that 1 and 5% of peptide, respectively, was degraded after 1 day at 4 °C. These values increased to 27 and 86%, respectively, after 1 day at 37 °C. Of note, the peptide was stable for more than 1 week at 4 and 37 °C when stored in water (pH 5.6). Thus, the degradation reaction strongly depends on buffer composition.
To identify the degradation products corresponding to peaks 2 and 3, formed in a 1:3 ratio, we next analyzed heat-treated NGR-2C before and after spiking with NGR-2C or DGR-2C or isodGGR-2C. The results showed that peaks 2 and 3 correspond to DGR-2C and isodGGR-2C, respectively (Fig. 4B), suggesting that both degradation products are deamidated forms.

To verify this hypothesis and to assess whether the mechanism of deamidation occurs via succinimide intermediate we monitored the NGR-2C deamidation reaction by MALDI-TOF. This analytical method showed, as expected, a progressive decrease during incubation of the molecular species corresponding to NGR-2C (expected monoisotopic mass, $M_{H}^+$ 1025.43) and an increase of species characterized by +1 Da. Noteworthy, a peak of −17 Da was also observed during the first 80 min of treatment, likely corresponding to the succinimide intermediate, which disappeared at later time points (Fig. 4C). These results strongly support the hypothesis that deamidation of Asn in the NGR motif occurs via loss of ammonia (−17 Da) followed by hydrolysis of the succinimide ring (+18 Da) with a global gain of ~1 Da, as has been demonstrated for other Asn-containing peptides (19–23).
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**FIGURE 5. FN-I₅ and peptides containing the NGR motif bind to α₀β₃ integrin after accelerated aging.**

A, binding of heat-treated CNGRC-TNF and TNF to solid phases coated with purified human integrins. Single concentration (5 μg/ml) or various concentrations (inset) of CNGRC-TNF and TNF were used. Binding was detected with anti-TNF antibodies as described under "Experimental Procedures." Mean ± S.E. (n = 2). B, binding of biotinylated CNGRC-TNF₁₋₁₁, CARAC-TNF₁₋₁₁, and FN-I₅ (complexed with streptavidin peroxidase) to purified human integrins. Binding was detected by chromogenic reaction with o-phenylenediamine. Mean ± S.E. (n = 2). C, competitive binding of biotinylated FN-I₅-streptavidin peroxidase complexes to α₀β₃-coated plates with various amounts of FN-I₄₋₅ or FN-I₄₋₅SGS (left panel) or peptides corresponding to the NGR loop (residues 258–271) of FN-I₅ before and after heat treatment (right panel). Mean ± S.E. (n = 4).

α₀β₃ Is a Receptor for the l-isoDGR Motif—In an attempt to identify the receptors of deamidated fibronectin fragments we analyzed the binding of heat-treated FN-I₅, as well as that of CNGRC-TNF and various peptides, to purified α₀β₃, α₀β₁, and α₀β₁ integrins. To this aim, direct and competitive ELISAs with integrins adsorbed on microtiter plates were performed. Direct ELISA showed binding of all NGR-containing molecules (heat treated) to α₀β₃ but little, or not at all, to the other integrins (Fig. 5, A and B). No binding was observed with heat-treated TNF or with a control CARAC-TNF₁₋₁₁ peptide (Fig. 5, A and B), suggesting that NGR was critical. Of note, we observed binding of CNGRC-TNF to α₀β₃ even before heat treatment, although to a lower extent (data not shown), possibly due to deamidation occurring during preparation and/or assay incubation. Accordingly, pretreatment with PIMT decreased the binding of both “heat-treated” and “untreated” CNGRC-TNF to α₀β₃ (data not shown).

To verify the importance of the NGR loop of FN-I₅ for integrin binding we performed competitive binding experiments with recombinant FN-I₄₋₅ and FN-I₄₋₅SGS, the latter lacking NGR. As expected, only the fragment with NGR competed the binding of FN-I₅ to α₀β₃ (Fig. 5C, left). Binding competition was observed also with a peptide corresponding to the entire 256–271 loop of FN-I₅ (see Fig. 1C), but not with a control peptide with a scrambled sequence at the GNGR site (Fig. 5, right). Similarly, the binding of CNGRC-TNF to α₀β₃ was efficiently competed with heat-treated FN-I₅ (EC₅₀, 0.4 μM) (Fig. 6A).

To identify the NGR deamidation product responsible for integrin binding, other competitive ELISAs were performed using synthetic peptides with Asn replaced with Asp or isoAsp or Ser. The results showed that isoDGR-2C can compete 600-fold more efficiently than DGR-2C (EC₅₀, 0.1 versus 60 μM), whereas little or no competition occurred with the control peptide SGR-2C (EC₅₀ >1000 μM) (Fig. 6A). Furthermore, low efficiency was observed with D-isoDGR-2C, a peptide with D-isoAsp in place of l-isoAsp, indicating that the binding was stereospecific. Considering that the isoDGR-2C is stable under these assay conditions (see above), these results suggest that l-isoDGR is an α₀β₃ binding motif.

Kinetics of Integrin Binding Site Formation in Fibronectin Fragments—To investigate the kinetics of integrin binding site formation in fibronectin fragments, we tested, by ELISA, the capability of FN-I₅ to compete the binding of CNGRC-TNF to α₀β₃ after incubation for different times at 37 °C in DMEM. Interestingly, when we plotted the inhibitory concentrations (IC₅₀) versus incubation time we observed that maximal competitive binding activity was reached after 24–48 h of incubation (half-life 3.4 h) (Fig. 6B). This value corresponds very well to the time course of deamidation reaction of NGR-2C described above and further supports the concept that the integrin binding properties of heat-treated FN-I₅ were related to deamidation of the NGR site. Because the structural basis of heat-treated FN-I₅ function in cell adhesion is related to the presence of isoAsp we have named this product “isonectin-1” and we propose to call “isonectins” all bioactive fragments containing the deamidated FN-I₅ module.

l-isoDGR Is a Competitive Antagonist of RGD Ligands of α₀β₃ and α₀β₁ Integrins—To assess whether l-isoDGR binds the RGD binding site of α₀β₃-integrin we next performed competitive binding studies with various doses of ACDCRGDCFC-TNF, a known high affinity ligand of α₀β₃-integrin (15), and
various doses of isoDGR-2C and RGD-2C (CRGDCGVRY).

The binding curves and Schild plot of binding data showed that both peptides efficiently antagonize, in a competitive manner, the binding of ACDCRGDCFC-TNF to α3β3-integrin (Fig. 7). This strongly suggests that the isoDGR motif binds within, or close to, the RGD binding site of α3β3 with an affinity similar to that of the RGD motif ($K_I$ 0.57 and 0.41 μM, respectively).

Because peptides containing CRGDC can also bind the α5β1 integrin (32), we next performed competitive binding experiments with this integrin. As shown in Fig. 7C, the binding of ACDCRGDCFC-TNF to α5β1 was weaker than that to α3β3. The binding was competed by RGD-2C and isoDGR-2C with similar potency (Fig. 7C), suggesting that the isoDGR motif can also bind the RGD binding site of α5β1. However, the IC50 was 4–5-fold higher than that required for α3β3, suggesting that the affinity for this integrin was lower.

Deamidated FN-15 (Isonectin-1) Inhibits In Vitro Endothelial Cell Adhesion and Proliferation—Compounds able to inhibit the interaction of α5β1 integrin, the vitronectin receptor, with ECM proteins are known to affect endothelial cell function and inhibit tumor growth (33–35). To assess whether isonectins can inhibit integrin-ECM protein interactions we have investigated the effect of soluble isonectin-1 on EA.hy926 endothelial cell adhesion to vitronectin-coated microtiter plates. These cells expressed CD31, α5β1, α5β3, α5β4, and α5β2 integrin subunits as checked by fluorescence-activated cell sorter analysis (data not shown). Isonectin-1 inhibited in a dose-dependent manner ~60% of cell adhesion to vitronectin (Fig. 8A), a protein that can interact with α5β1, α5β3, α5β4, α5β2, and α5β3 (36). Similarly, isoDGR-2C, but not SGR-2C, inhibited cell adhesion (Fig. 8A), strongly suggesting that isoDGR compounds may indeed work as antagonists of integrin-ECM interactions. Furthermore, both isoDGR-2C and RGD-2C, but not SGR-2C, inhibited the proliferation of human microvascular endothelial cells (HMEC-1) in vitro (Fig. 8B).

Isonectin-1 Inhibits Tumor Growth In Vivo—Because α5β3-mediated endothelial cell adhesion is critical for endothelial cell survival and proliferation in tumors, we tested the hypothesis that isonectin-1 and isoDGR-2C peptide could affect tumor
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FIGURE 8. Effect of deamidated FN-I5 (isonectin-1), isoDGR-2C on endothelial cell adhesion, proliferation, and tumor growth. A, inhibition of EA.hy926 cell adhesion to vitronectin by soluble isonectin-1 (prepared by heat treatment of FN-I5), isoDGR-2C, and SGR-2C. Vitronectin (3 μg/ml) was adsorbed to microtiter plate, and cell adhesion assay was performed as described under "Experimental Procedures," using cell culture medium containing the soluble competitors. Mean ± S.E. (n = 3). B, inhibition of HMEC-1 cell proliferation by soluble isoDGR-2C, RGD-2C, and SGR-2C peptide. The assay was performed as described under "Experimental Procedures" using cell culture medium containing the soluble competitors. Mean ± S.E. (n = 6). C, anti-tumor effect of repeated administration of isonectin-1 (200 μg, intraperitoneal) to RMA tumor-bearing mice or isoDGR-2C or RGD-2C or SGR-2C peptide (100 μg, intraperitoneal) to B16F1 tumor-bearing mice as indicated. Animals (five/group) were treated at the indicated times (arrows). **, p < 0.0005; *, p < 0.05; statistical analysis by two-tailed t test.

growth. As expected, daily administration of isonectin-1 (200 μg, intraperitoneal) or isoDGR-2C (100 μg, intraperitoneal) to RMA lymphoma- or B16F1 melanoma-bearing mice, respectively, significantly inhibited tumor growth (Fig. 8C, upper panels). The effect was similar to that induced by RGD-2C peptide (Fig. 8C, lower panel), a known ligand of α5β3 (32). Of note, the SGR-2C peptide, with isoAsp changed with Ser, was completely inactive, suggesting that the isoDGR motif was responsible for the observed anti-tumor activity.

DISCUSSION

It is well known that proteins may contain isoAsp residues due to post-translational Asn deamidation or Asp isomerization reactions (21, 31, 37–39). These reactions can occur in vivo, e.g. in extracellular matrix proteins with slow turnover (30, 31, 37), and in vitro during protein isolation and storage (22, 40, 41). In general these phenomena are associated with protein “loss of function” and, for this reason, are generally viewed as deleterious events associated with protein aging. In this work, for the first time we have shown that Asn deamidation of the NGR site of the fifth fibronectin type I repeat (FN-I5) is associated with a gain of function, because deamidated fragments containing this module (named isonectins) are able to affect endothelial cell adhesion and proliferation in different assays.

This view is supported by the observation that (a) accelerated aging of synthetic FN-I5 or recombinant FN-I5–5, but not of FN-I5–3, SGS (a mutant with the NGR sequence replaced with SGS), increased their cell adhesion properties; (b) accelerated aging was associated with Asn deamidation, as shown by mass spectrometry and isoAsp analysis of products; (c) treatment of aged fragments and peptides with PIMT, an enzyme that converts L-isoAsp and D-Asp residues to L-Asp (25, 27), completely inhibited their pro-adhesive activity.

We have obtained evidence to suggest that Asn deamidation at NGR sites occurs via succinimide intermediate, which upon hydrolytic cleavage leads to formation of Asp and isoAsp in a 1:3 ratio (see Fig. 9 for a schematic representation). Racemization and hydrolysis of the succinimide intermediate leading to the formation of D-Asp is also possible, but this typically occurs with a much lower efficiency (19, 20, 24). Thus, deamidated products with L-configuration are likely quantitatively more relevant. Deamidation reactions can take a few hours, days, or years to occur depending on neighboring amino acid sequence, temperature, buffer composition, and ionic strength (19, 21). For instance the presence of a Gly residue after Asn has a dramatic destabilizing effect (21, 22, 24). Accordingly, we have found that the kinetics of NGR deamidation in synthetic fibronectin fragments and peptides are surprisingly rapid (half-life 3–4 h), likely due to favorable conformation.

Three-dimensional structure analysis of FN-I5 showed that the GNGRG motif forms an exposed loop, likely accessible to water and to receptors (see Fig. 1B). Of note, molecular dynamic simulation of an NGR peptide with flanking cysteines...
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The question arises as to whether the receptor binding site in deamidated fibronectin is DGR or isoDGR. The following observations suggest that isoDGR is the biologically relevant motif. First, cell adhesion to deamidated FN-I5 (named isonec-tn-1) was competed by peptides containing isoDGR but not the DGR motif. Noteworthy, no competition was observed with D-isoDGR-containing peptides, pointing to stereospecific interactions. Second, enzymatic conversion of L-isoAsp into L-Asp residues by PIMT completely inhibited the pro-adhesive activity of deamidated fibronectin fragments. This view is further supported by the observation that the CDGRC-TNF conjugate was completely inactive in direct cell adhesion assays. Thus, L-isoDGR is the bioactive motif.

Studies aimed at identifying the cellular binding sites showed that α,β3 integrin efficiently binds deamidated FN-I5 (isonec-tn-1) as well as peptides containing L-isoDGR, suggesting that this integrin is an important L-isoDGR receptor. A weaker binding was observed also with α,β1. Noteworthy, D-isoDGR was 60-fold less efficient in α,β3 binding. Furthermore, L-isoDGR was 600-fold less efficient in α,β3 recognition, supporting the concept that L-isoDGR, and not L-DGR, is the bioactive motif. Furthermore, studies aimed at characterizing the binding site on α,β3 and α,β5 showed that L-isoDGR acts as a competitive inhibitor of the RGD binding site of these integrins.

Can Asn deamidation also occur in natural fibronectins? We have found that natural FN-30 kDa fragment as well as intact fibronectin freshly isolated from human plasma contain 0.026–0.048 pmol of isoAsp/pmol of protein. Furthermore, treatment of FN-30 kDa with PIMT partially inhibited its pro-adhesive properties. This suggests that formation of isoAsp can occur also in natural fibronectins in sufficient quantity to affect cell adhesion. We cannot exclude, however, that the kinetics of Asn deamidation in fibronectin might be slower than that measured with peptides or with FN-I5, considering the strong influence that the Asn molecular microenvironment might have on deamidation kinetics. One interesting possibility that deserves to be investigated is that conformational changes occurring after deposition in tissues or local changes in the microenvironment composition or the presence of specific proteases might affect the kinetics of this reaction. Considering the low turnover of fibronectin after deposition in tissues (42) and the abundance of this protein in plasma and in tissues, it is very likely that a significant amount of deamidated fibronectin is formed also in vivo. Another question is whether generation/removal of isoDGR in fibronectin could play a role in normal or pathological conditions. We observed that isonec-tn-1 can inhibit the adhesion of endothelial cells to vironectin, a ligand of α,β3; furthermore, peptides containing the isoDGR motif inhibited the proliferation of microvascular endothelial cells in vitro. We have also observed that daily administration of isonec-tn-1 to RMA lymphoma-bearing mice significantly inhibits tumor growth in vivo. These findings suggest that deamidated fragments may play a role in endothelial cell adhesion and tumor biology. Several investigators implicated α,β3 as a receptor for various proteolytic fragments of ECM proteins that can act as anti-angiogenic factors (43–45). An interesting possibility is that deamidated fibronectin fragments contribute, together with other ECM protein fragments, to regulate angiogenesis in normal and pathological conditions.

How can cells control isoDGR generation in fibronectin? Asn deamidation is a thermodynamically spontaneous reaction independent from enzymatic regulation. Whether specific deamidases further accelerating this process exist or not is presently unknown. However, the finding that enzymatic removal of isoAsp by PIMT inhibits isonec-tn-1 pro-adhesive activity suggests a potential enzymatic mechanism for negative regulation of this site. Formation of β-linked isopeptide bond (isoAsp) in proteins and subsequent conversion in α-linked peptide bond (Asp) by PIMT are generally regarded as a sort of “damage repair” mechanism of aged proteins (31). It has also been hypothesized that in some proteins these damage repair reactions may have some useful function, e.g. as a sort of molecular clock for protein degradation or intracellular localization (21, 31, 46). Based on our findings, a new “activation-deactivation” model may also be envisaged for isoAsp formation-removal occurring at certain sites in extracellular proteins (selected by evolution), such as fibronectin. Thus, whereas PIMT may be considered a sort of “repairing” enzyme for Asp residues undergoing isomerization, e.g. to rescue RGD in aged fibronectin and collagen (30, 31), it may also be viewed as an enzyme that “destroys” the function of isoDGR, pointing to a new function for this enzyme. Of note, increased amounts of extracellular PIMT have been observed in injured tissues and wound healing (47, 48).

Finally, considering that α,β3 integrin is a good marker of angiogenic vessels, exogenous fibronectin fragments or short peptides containing the L-isoDGR motif may be exploited, in principle, as ligand for targeted delivery of drugs, cytokines, toxins, apopotic peptides, radionuclides, viral particles, genes, or imaging compounds to angiogenic vessels in tumors or in other angiogenesis-related diseases.

In conclusion, spontaneous conversion of NGR to isoDGR in fibronectin fragments represents a novel mechanism for gener-
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aturing $\alpha,\beta_3$ ligands that may regulate endothelial cell functions and tumor growth. Generation of isoDGR sites in proteins by NGR deamidation (or DGR isomerization) may represent a novel mechanism for regulating their function.

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