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Synthetic RGD-containing α-helical coiled coil peptides promote integrin-dependent cell adhesion

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Abstract: Integrin receptors are the main mediators of cell adhesion to the extracellular matrix. They bind to their ligands by interacting with short amino acid sequences, such as the RGD sequence. Soluble, small RGD-based peptides have been used to block integrin-binding to ligands, thereby interfering with cell adhesion, migration and survival, while substrate-immobilized RGD sequences have been used to enhance cell binding to artificial surfaces. This approach has several important medical applications, e.g. in suppression of tumor angiogenesis or stimulation of bone formation around implants. However, the relatively weak affinity of short RGD-containing peptides often results in incomplete integrin inhibition or ineffective ligation. In this work, we designed and synthesized several new multivalent RGD-containing molecules and tested their ability to inhibit or to promote integrin-dependent cell adhesion when used in solution or immobilized on substrates, respectively. These molecules consist of an oligomeric structure formed by α-helical coiled coil peptides fused at their amino-terminal ends with an RGD-containing fragment. When immobilized on a substrate, these peptides specifically promoted integrin αVβ3-dependent cell adhesion, but when used in solution, they blocked αVβ3-dependent cell adhesion to the natural substrates fibronectin and vitronectin. One of the peptides was nearly 10-fold more efficient than fibronectin or vitronectin in promoting cell adhesion, and almost 100-fold more efficient than a linear RGD tripeptide in blocking adhesion. These results indicate that α-helical coiled coil peptides carrying an amino-terminal RGD motif can be used as soluble antagonists or surface-immobilized agonists to efficiently inhibit or promote integrin αVβ3-mediated cell adhesion, respectively.

Keywords: coiled coil; design; fibrils; integrin; peptide synthesis

INTRODUCTION

Integrins are heterodimeric cell surface molecules consisting of two noncovalently associated transmembrane subunits, α and β [1,2]. There are 18α and 8β subunits, which combine to form at least 24 different heterodimers. Integrins are receptors for extracellular matrix (ECM) proteins, such as fibronectin, laminin, collagen and vitronectin and thereby act as the main mediators of cell adhesion to the ECM. Integrins exist in (at least) two different conformational states: a 'resting' conformation with low ligand-binding affinity and an 'active' conformation with high ligand-binding affinity [3]. Upon ligand binding, the cytoplasmic domain of the integrin β-subunit associates with structural and signaling cytosolic proteins resulting in integrin clustering, organization of the cytoskeleton, and initiation of signaling events essential for cell adhesion, spreading, migration, proliferation, survival and differentiation [2,4]. Integrin receptors bind ligands by interacting with short specific sequences exposed on the ligand surface. The tripeptide RGD motif is a paradigm of the integrin-recognition sequence [5]. It occurs on many matrix proteins that act as integrin ligands (e.g. fibronectin, vitronectin, fibrinogen), and deletion or mutation of this motif abolishes ligand recognition and cell adhesion [6]. The 3D-structures of 'resting' and 'ligand-occupied' active αVβ3 integrin have been recently published and have provided significant insights into the structural bases of integrin activation and ligand binding [7,8]. αVβ3 has a large 'head', consisting of a seven blades-'propeller' domain on the α-subunit and a seven blades-'propeller-like' domain on the β-subunit, and two 'legs' consisting of tightly packed domains forming a rigid stalk with a flexible hinge near the head. The head domain contains five cation-binding sites, one of which is on the top of the β-propeller that participates in ligand binding. The ligand-binding pocket is located between the β-propeller domain of the α-subunit and the domain of the β-subunit. During integrin activation conformational changes occur in the 'head' domain. The cation-binding sites on the top of the β-propeller from the β-subunit is repositioned toward the RGD motif, resulting in stable high-affinity ligand binding [9].

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Soluble RGD-based peptides have been used to block integrin-binding to ligands, thereby interfering with cell adhesion, migration and survival, while surface-immobilized peptides have been used to promote integrin-dependent cell adhesion [10]. Both approaches have relevant therapeutic medical applications: soluble integrin-specific antagonists have been shown to inhibit platelet aggregation and suppress tumor angiogenesis [11,12], while RGD-based peptides immobilized on surfaces are known to stimulate osteoblast adhesion and bone formation around implants [13]. The development of powerful soluble integrin antagonists, or surface-immobilized agonists, however, has been limited by the weak affinity of short RGD-containing peptides. To circumvent this limitation, nonpeptidic mimetics and multivalent RGD-ligands have been developed [14,15].

In this work, we fused short RGD-containing peptides with previously designed α-helical coiled coil peptides [16,17] that are able to form oligomers, thereby generating carriers of multiple RGD motifs. The peptide conformations and oligomeric states of their associates were characterized and their biological activities in modulating integrin functions were tested in cell-adhesion assays.

MATERIALS AND METHODS

Materials

Chemicals and solvents used for the synthesis were purchased from Fluka (Buchs, Switzerland) and Novabiochem (Laufelfinger, Switzerland). Bovine plasma fibronectin and human plasma vitronectin were purchased from Sigma Chemie (Buchs, Switzerland). Function-blocking mAbs: LM609 (anti-$\alpha V\beta 3$) was from Chemicon, Temencula, CA, USA; Sam-1 (anti-$\alpha 5$) and Lia1/2 (anti-$\beta 1$) were from Beckman Coulter (Nyon, Switzerland). Linear RGD and RGE trimeric peptides were purchased from Sigma Chemie.

Peptide Synthesis

All the peptides used in this study were synthesized at our institute using solid-phase Fmoc chemistry (Applied Biosystem 431A, Foster City, CA). Briefly, peptides were prepared on a *p*-alkoxybenzylalcohol resin (Wang resin). The amino acid side-chain protections used were trityl for glutamine, *tert*-butyl-oxy for glutamic or aspartic acid and *tert*-butyl for serine. After cleavage from the resin with a solution containing 0.5 g of phenol, 0.5 ml of water, 1.2 ml of triisopropylsilane and 8.3 ml of trifluoroacetic acid [18,19], the crude peptide, was purified by RP-HPLC [C_{18} preparative column (22 mm $\times$ 250 mm); H_2O containing 0.1% TFA/acetonitrile containing 0.1% TFA from 90/10 to 20/80 in 70 min with a flow rate of 10 ml/min]. The purity (>90%) was determined by analytical C_{18} HPLC and mass spectrometry (MALDI-TOF; Applied Biosystem, Foster City, CA; Table 1).

Circular Dichroism and Sedimentation-diffusion Measurements

Circular dichroism (CD) spectra were obtained on a Jasco-600 spectropolarimeter (Japan Spectroscopic Co; Tokyo, Japan) equipped with a temperature-controlled holder in 0.185-mm thick cells at the peptide concentration of 0.3–0.5 mg/ml. The molar ellipticity was calculated from the equation $[\theta] = [\theta]_{obs} \cdot M_{res}/(101c)$; $[\theta]_{obs}$ is the ellipticity measured in degrees at the wavelength λ, and M_{res} is the mean residue molecular weight of the peptide 1 is the optical pathlength of the cell (mm) and c is the peptide concentration (g/l). The percentage

Table 1 Sequences of peptides with self-assembling features and peptides carrying integrin-binding motif

	Peptide sequences	Average mass (Da)	$[M + H]^+$	Features
1	QLAREL(QQLAREL)$_4$	4084.7	4086.4	Long fibrils at slightly acid pH Spherical aggregates at neutral pH [16]
2	QLARSL(QQLARSL)$_4$	3874.5	3876.3	
3	QLARQL(QQLARQL)$_4$	4079.8	4083.8	α-helical conformation at $2.5 < pH < 10.0$ Fibril formation at pH = 2.8 and pH = 7.2 [17]
4	QLAQQL(QQLAQQL)$_4$	3939.5	3941.5	
5	QLAQQL(QQLARQLQQLAQQL)$_2$	3995.6	3997.4	
6	GRGDSPSGGQLAREL(QQLAREL)$_4$	4855.4	4857.8	
7	GRGDSPSGGQLARSL(QQLARSL)$_4$	4645.3	4647.9	
8	GRGDSPSGGQLARQL(QQLARQL)$_4$	4850.5	4848.9	α-helical conformation through the range of pH values ($2.5 < pH < 10.0$)
9	GRGDSPSGGQLAQQL(QQLAQQL)$_4$	4710.2	4713.1	
10	GRGESPSGGQLAQQL(QQLAQQL)$_4$	4724.3	4726.2	
11	GRGDSPSGGQLAQQL(QQLARQLQQLAQQL)$_2$	4766.4	4768.7	

of α-helicity was determined in accordance with Chen *et al.* [20].

Sedimentation-diffusion experiments were performed in buffer solution (0.1 M sodium chloride, 10 mM sodium phosphate, pH 2.8) using a Beckman model E analytical ultracentrifuge with the schliren optical system. The sedimentation coefficient was evaluated at a speed of 42 040 rpm by standard procedure [21].

Cells

The human melanoma cell lines SKMel 28 and Me315 were obtained from Dr. Agnese Mariotti, CePO laboratory, ISREC, Epalinges, Switzerland. The cells were cultured in an RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (100 IU/ml; 100 µg/ml) (all from Invitrogen, Basel, Switzerland). The cells were grown to confluence in 75 cm^2 culture flasks (Beckton Dickinson, Basel, Switzerland) at 37 °C in 5% CO_2 atmosphere. For adhesion experiments cells were detached with a solution of trypsin/ethylenediaminetetracetic acid (EDTA) (Invitrogen) for 5 min at 37 °C in 5% CO_2 atmosphere. Detached cells were washed with complete medium and counted in a Neubaeur chamber with trypan blue to assess viability.

Cell-adhesion Assays

Adhesion assays were performed using 96-well polystyrene plates (NUNC-Immuno™ Plates MaxiSorp™ Surface, NUNC, Roskilde, Denmark). Proteins or peptides were coated by incubating wells with a phosphate buffered saline (PBS) solution (100 µl/well) overnight at 4 °C. Free binding sites on the plastic were blocked with 1% bovine serum albumin (BSA)/PBS (100 µl/well) for 2 h at 37 °C. BSA only-coated wells were used as negative control. All conditions were tested in duplicate. Freshly collected cells were resuspended in a serum-free RPMI 1640 medium (5.10^5 cells/ml), and plated onto protein-coated wells (100 µl/well). In cell adhesion inhibition experiments, peptides or antibodies were added together with the cells at this step. After 1 h incubation at 37 °C in a 5% CO_2 atmosphere, unattached cells were removed by gently rinsing the wells twice with warm PBS. Attached cells were fixed with 2% paraformaldehyde in PBS (100 µl/well) and stained with 0.5% crystal violet in water. After a final washing step with water, a 50/50 solution of trisodium citrate dihydrate 100 mM and ethanol 100% were added (100 µl/well) to extract the crystal violet. Absorbance of each well was read at 570 nm in a plate reader (Packard Spectra Count, Meriden, CT). Results are expressed as mean value of duplicate determinations.

RESULTS

Design and Biochemical Characterization of RGD-containing α-helical Coiled Coil Peptides

Previously, we designed peptide **1**, which self-assembles into long soluble fibrils containing about 80 monomers at a slightly acidic pH and into spherical aggregates at a neutral pH reversibly [16] (Table 1). Then, the design of coiled coil peptide **1** was modified to obtain peptides **2**–**5** that were able to form soluble α-helical fibrils at physiological pH which opens new possibilities for biological applications [17]. In this work, we fused these fibril-forming peptides with a sequence containing RGD residues to produce oligomers that may bind integrin receptors more efficiently compared to monomeric RGD peptides. We synthesized six different RGD- or the inactive analogue RGE-containing peptides (peptides **6**–**11**; Table 1). The RGD segments

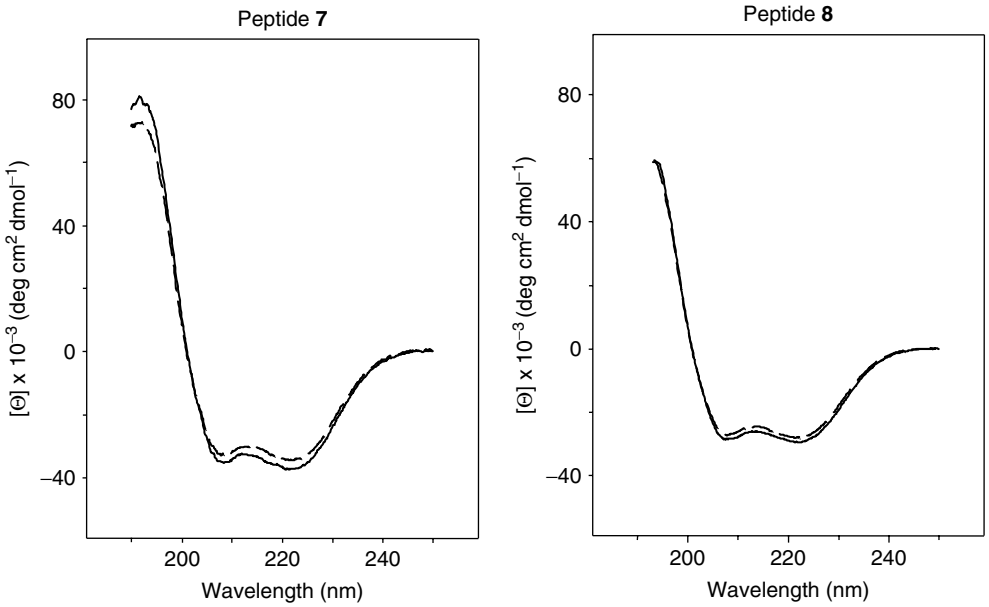

Figure 1 Far-UV CD spectra of peptides **7** and **8** at pH 2.7 (full line) and pH 7.3 (dashed line). Spectra were recorded at a peptide concentration of 0.1 mM in 10 mM sodium phosphate buffer at 20 °C. Peptides **6**, **9**, **10** and **11** have similar spectra (data not shown).

were added to the *N*-terminus of the α-helical coiled coil domain. CD spectroscopy showed that the addition of the RGD segments preserved the peptide α-helical conformation at room temperature and at pH = 2.7 and pH = 7.3 (Figure 1). Furthermore, sedimentation-diffusion experiments demonstrated that these peptides formed oligomeric complexes. However, the addition of the RGD-containing segment disrupted the head-to-tail fibril formation. The size of the oligomers was reduced from 80 to 5–6 monomers (data not shown). These results were supported by the electron microscopy data, which demonstrated that these peptides do not form long fibrils (data not shown). Thus, our biochemical analysis showed that the RGD-containing coiled coil peptides self-assembled into soluble α-helical oligomers consisting of five to six monomers.

Substrate-immobilized Peptides Promote $\alpha V\beta 3$ Integrin-dependent Adhesion

To test for the ability of the different RGD-containing peptides to act as cell adhesion substrates, we performed short-term adhesion assays using two melanoma cell lines. Peptides **2** (without RGD) and **7** (carrying RGD) or **3** (without RGD) and **8** (carrying RGD) promoted adhesion of the $\alpha V\beta 3$-expressing SKMel 28 human melanoma cells and the $\alpha 5\beta 1$-expressing Me315 human melanoma cells (Table 2). In this case, the adhesion is nonintegrin specific and is due to the positive charges present on the repetitive part of the peptides.

These chains were modified to obtain peptides **4**, **5** and **9**–**11**. The adhesion assays showed that peptide **9** promoted adhesion of the $\alpha V\beta 3$-expressing SKMel 28 human melanoma cells (Figure 2A), whereas control peptide **10**, which contains an RGE sequence instead of the RGD motif, did not. The coating concentration of peptide **9** that promoted 50% cell adhesion was 10^{-7} M (Table 2). In contrast, these peptides did not support the adhesion of another melanoma cell line, Me315, which expresses integrin $\alpha 5\beta 1$ but lacks the integrin $\alpha V\beta 3$ expression (Figure 2B). As positive control for integrin-mediated adhesion, SKMel 28 cells were plated on vitronectin, a natural ligand of $\alpha V\beta 3$, while Me315 cells were plated on fibronectin which is a ligand for $\alpha 5\beta 1$.

The result of this experiment demonstrated that Me315 cells did not attach to any of the RGD-containing oligomers tested, suggesting that RGD-containing peptides promotes specifically $\alpha V\beta 3$-dependent adhesion (Table 2).

To directly demonstrate the integrin specificity of cell adhesion to the various RGD-containing oligomers, we plated SKMel28 melanoma cells on substrate-immobilized peptides **7**–**11**, in the absence or presence of the integrin-specific inhibitory antibodies LM609 (anti-$\alpha V\beta 3$), Lia1/2 (anti-$\beta 1$) and Sam-1 (anti-$\alpha 5$) [22].

Table 2 Concentrations of substrate-immobilized peptides that promoted 50% cell adhesion

Peptide immobilized	(Peptide) (M) SKMel28	(Peptide) (M) Me315
2	1×10^{-3}	2×10^{-3}
3	5×10^{-3}	2×10^{-3}
4	—	—
5	—	—
7	1×10^{-3}	2×10^{-3}
8	1×10^{-3}	2×10^{-3}
9	1×10^{-7}	—
10	—	—
11	1×10^{-7}	—
Vitronectin	2×10^{-9}	
Fibronectin		4×10^{-9}

– indicates that no adhesion was observed.

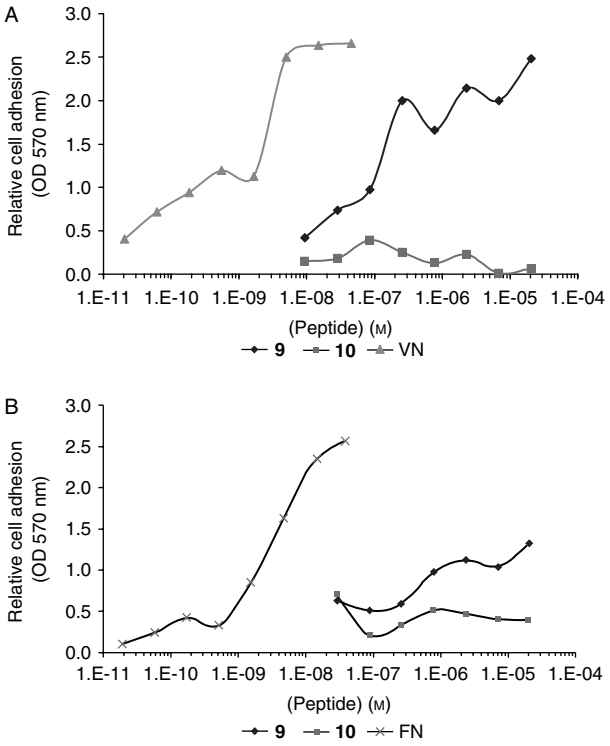

Figure 2 Promotion of cell adhesion by substrate-immobilized peptides. Plates were coated with peptide **9** (20 μM), peptide **10** (20 μM), vitronectin (VN, 46 nM) or fibronectin (FN, 42 nM) and titrated over several orders of magnitude (1 : 3 steps). (A) SKMel 28 cells (5×10^5 cells/well) or (B) Me315 cells (5×10^5 cells/well).

Antibody LM609 inhibited adhesion on SKMel28 cells on peptide **9** and **11**, whereas Lia1/2 or Sam-1 had no effect (Figure 3). Antibody LM609 did not inhibit adhesion on SKMel 28 cells on peptides **7** and **8**.

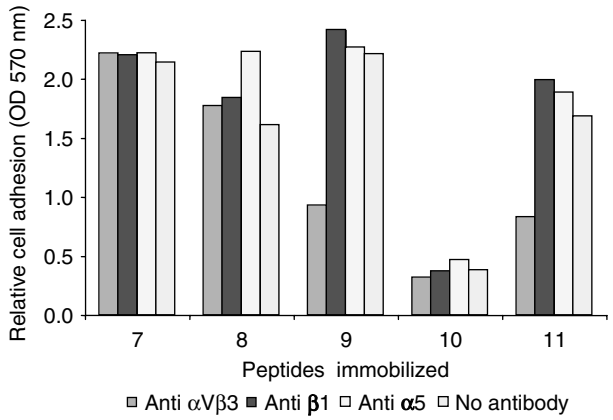

Figure 3 RGD-containing oligomer-forming peptides promote $\alpha V\beta 3$-dependent adhesion. Plates were coated with peptides **7**–**11** (4 μM). SKMel 28 cells (3×10^5 cells/well) were added to the wells in the absence or presence of function-blocking mAbs against $\alpha V\beta 3$ (LM609), $\beta 1$ (Lia1/2) or $\alpha 5$ (Sam-1) integrins.

From these results we conclude that the RGD-containing peptides **9** and **11** promote $\alpha V\beta 3$ integrin-dependent adhesion, whereas peptides **7** and **8** do not.

Soluble Peptides Inhibit $\alpha V\beta 3$ Integrin-dependent Adhesion

Next, we tested the ability of soluble peptides **7**–**11** to inhibit cell adhesion. For this purpose, SKMel 28 cells were plated on vitronectin, a ligand for integrin $\alpha V\beta 3$, in the absence or presence of increasing concentrations of peptides **7**–**11** (Table 3). Linear tripeptides RGD and RGE were used as controls. The peptide **9** inhibited 50% of the SKMel 28 cell adhesion to vitronectin at a concentration of 3.10^{-5} M (Figure 4, panel A and Table 3). The same features were noticed for peptides **7**–**11** (Table 3). Peptide **10** carrying the RGE sequence did not inhibit the adhesion (Figure 4). To test whether these peptides interfered with $\alpha 5\beta 1$-mediated adhesion, we plated Me315 cells on fibronectin in the absence or presence of the different peptides. Although Me315 adhesion to fibronectin was less efficient than adhesion to vitronectin, only peptides **7** and **8** had an inhibitory effect on Me315 cell adhesion. This observation was consistent with the Me315 nonspecific cell adhesion previously observed. As a comparison, a linear RGD tripeptide inhibited 50% of $\alpha V\beta 3$-dependent SKMel28 adhesion to vitronectin at 2.10^{-3} M, while it did not inhibit Me315 cell adhesion to fibronectin up to the highest tested concentration (3.10^{-3} M).

Thus, these experiments demonstrated that the RGD-containing and coiled coil-forming peptides **9** and **11** are more potent inhibitors of $\alpha V\beta 3$ integrin-dependent cell adhesion as compared to the linear RGD tripeptide.

Table 3 Concentrations of soluble peptides that caused 50% of inhibition of SKMel 28 adhesion to vitronectin and Me315 adhesion to fibronectin

Peptide	(Peptide) (M) SKmel28	(Peptide) (M) Me315
4	—	—
5	—	—
7	4×10^{-5}	1×10^{-5}
8	5×10^{-5}	2×10^{-4}
9	3×10^{-5}	—
10	—	—
11	3×10^{-5}	—
Linear RGD	2×10^{-3}	—
Linear RGE	—	—

– indicates that no inhibition was observed.

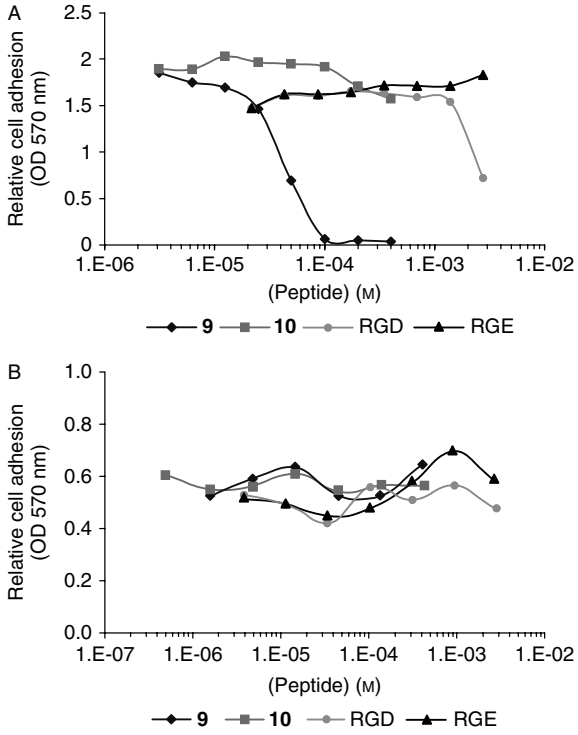

Figure 4 Inhibition of cell adhesion by soluble peptides. (A) Coating with vitronectin VN (15 nM), peptides **9**, **10** (400 μM in the first well; titration 1 : 2) and linear RGD or RGE peptides (2.8 mM in the first well; titration 1 : 2). SKMel 28 cells (5×10^5 cells/well) were added to the wells. (B) Coating with fibronectin FN (12 nM) then peptides **9**, **10** (400 μM in the first well; titration 1 : 2) and linear RGD or RGE peptides (2.8 mM in the first well; titration 1 : 2) were added, followed by Me315 cells (5×10^5 cells/well).

DISCUSSION

This work was initiated to test the possibility of generating multivalent RGD-containing peptides by

fusing a RGD sequence to α-helical coiled coil peptides that we have previously shown to form oligomers, thereby generating carriers of multiple RGD motifs. Here we report the following findings: (i) most of the peptides tested were able to support cell adhesion when immobilized on a solid surface, or did inhibit cell adhesion when added in solution; (ii) some of the tested peptides promoted or inhibited integrin $\alpha V\beta 3$-mediated adhesion, while they did not interfere with $\alpha 5\beta 1$-dependent adhesion; (iii) the sequence of the α-helical coiled coil-forming domain influenced the activity of the individual peptides; (iv) two peptides were approximately 100-fold more potent than the linear RGD tripeptide in inhibiting adhesion.

A physicochemical analysis confirmed that all the peptides have an α-helical conformation and form oligomeric structures through the range of pH values ($2.5 < pH < 10$). The addition of the RGD sequence, however, had a dramatic effect on the number of the monomers present in each complex: the original fibril-forming peptide generated a complex consisting up to over 80 monomers, while RGD-modified peptides formed complexes containing five to six peptides on an average. Thus, it appears that addition of the GRGD/ESPSGG sequence had a dramatic effect on the number of the monomers present in each complex. In view of a biomedical application, in particular as inhibitory molecules, however, this may be a positive effect, since small complexes (i.e. pentamers have a molecular mass of 22 kDa) have a better diffusion capacity. On the other hand, larger complexes (80-mer complexes have a calculated molecular mass of 360 kDa) would have a longer half-life in plasma since they would not be filtered by the kidney. A possible strategy to form larger complexes could be to mix a small number of RGD-modified peptides with a large number of unmodified peptides.

The adhesion-promoting activity of these peptides is specifically due to the integrin-binding RGD sequence, as substitution of the RGD motif with a RGE motif abolished activity. Moreover, two peptides tested promoted $\alpha V\beta 3$- but not $\alpha 5\beta 1$-mediated cell adhesion. This feature is not completely surprising, since $\alpha V\beta 3$ has a broader natural ligand-binding specificity (e.g. it binds to fibronectin, vitronectin, fibrin, thrombospondin, tenascin-C, von Willenbrand factor, denatured collagen I, osteopontin, MMP-2, Del-1, and others), as compared to $\alpha 5\beta 1$ (e.g. it binds to fibronectin). This is likely due to the ability of $\alpha V\beta 3$ to recognize RGD motifs in different conformations and within different flanking sequences, while $\alpha 5\beta 1$ seems to recognize preferentially the RGD and flanking sequence in fibronectin. Importantly, peptides **9** and **11** efficiently supported $\alpha V\beta 3$-dependent adhesion. This result opens the possibility to effectively use such peptides to coat artificial surfaces and implants to promote adhesion for bioengineering purposes. In addition, the peptides tested were 10- to 100-fold more efficient than the linear RGD tripeptide to inhibit $\alpha V\beta 3$-mediated adhesion when used in solution. This increased activity is likely due to multimerization. While the adhesion-promoting activity is consistent with *in vivo* applications, the adhesion-blocking capacity is probably too weak for *in vivo* use, and concentrations as high as 80 μg/ml are necessary for 50% inhibition. However, if increasing multimericity of these peptides leads to a further increase of their antagonist potency by 10- to 100-fold, this may open the way to *in vivo* applications. In this case, such peptides would have a potency similar to that of EMD121974 (i.e. Cilengitide), an RGD-based pentapeptide currently tested in the clinic as an anticancer agent [23]. Because of their higher molecular weight, such complexes (20–100 kDa) may allow to overcome a major limitation of small cyclic peptides, which is a short half-life in serum due to their low molecular weight (<1000 Da). In addition, because of their compact structure, coiled coil peptides are in general inaccessible to proteases, and therefore more resistant to degradation.

A further application of such peptides is their use as targeting moieties to deliver nanoparticle or liposomes to the $\alpha V\beta 3$-positive vasculature. This approach has recently been successfully attempted. Cationic nanoparticles were coupled to an integrin $\alpha V\beta 3$-targeting ligand to deliver a plasmid encoding dominant negative Raf kinase to angiogenic blood vessels in tumor-bearing mice, resulting in apoptosis of the tumor-associated endothelium and tumor regression [24].

In conclusion, we designed α-helical coiled coil-forming multivalent RGD-containing molecules and demonstrated their ability to promote or inhibit $\alpha V\beta 3$ integrin-dependent cell adhesion when immobilized on substrates or used in solution, respectively. The proadhesive activity was close to that of fibronectin or vitronectin, and the adhesion-blocking activity was 100-fold higher than that of linear monovalent RGD peptides. These results indicate the feasibility of our approach to obtain synthetic RGD-based agonists or antagonists of $\alpha V\beta 3$-mediated cell adhesion for biomedical applications. It also encouraged us to design novel RGD-containing α-helical coiled coil structures that are able to form fibril of higher multivalency and hopefully higher affinity.

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REFERENCES

1. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992; **69**: 11–25.

2. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673–687.
3. Takagi J, Springer TA. Integrin activation and structural rearrangement. *Immunol. Rev.* 2002; **186**: 141–163.
4. Longhurst CM, Jennings LK. Integrin-mediated signal transduction. *Cell. Mol. Life Sci.* 1998; **54**: 514–526.
5. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu. Rev. Cell Dev. Biol.* 1996; **12**: 697–715.
6. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science* 1987; **238**: 491–497.
7. Xiong JP, Stehle T, Diefenbach B, Zhang R, Dunker R, Scott DL, Joachimiak A, Goodman SL, Arnaout MA. Crystal structure of the extracellular segment of integrin $\alpha V\beta 3$. *Science* 2001; **294**: 339–345.
8. Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL, Arnaout MA. Crystal structure of the extracellular segment of integrin $\alpha V\beta 3$ in complex with an Arg-Gly-Asp ligand. *Science* 2002; **296**: 151–155.
9. Xiong JP, Stehle T, Goodman SL, Arnaout MA. Integrins, cations and ligands: making the connection. *J. Thromb. Haemost.* 2003; **1**: 1642–1654.
10. Craig WS, Cheng S, Mullen DG, Blevitt J, Pierschbacher MD. Concept and progress in the development of RGD-containing peptide pharmaceuticals. *Biopolymers* 1995; **37**: 157–175.
11. Nurden AT, Nurden P. GPIIb/IIIa antagonists and other anti-integrins. *Semin. Vasc. Med.* 2003; **3**: 123–130.
12. Westlin WF. Integrins as targets of angiogenesis inhibition. *Cancer J.* 2001; **7**: S139–S143.
13. Kantlehner M, Schaffner P, Finsinger D, Meyer J, Jonczyk A, Diefenbach B, Nies B, Holzemann G, Goodman SL, Kessler H. Surface coating with cyclic RGD peptides stimulates osteoblast adhesion and proliferation as well as bone formation. *ChemBioChem* 2000; **1**: 107–114.
14. Nicolaou KC, Trujillo JI, Jandeleit B, Chibale K, Rosenfeld M, Diefenbach B, Cheresh DA, Goodman SL. Design, synthesis and biological evaluation of nonpeptide integrin antagonists. *Bioorg. Med. Chem.* 1998; **6**: 1185–1208.
15. Thumshirn G, Hersel U, Goodman SL, Kessler H. Multimeric cyclic RGD peptides as potential tools for tumor targeting: solid-phase peptide synthesis and chemoselective oxime ligation. *Chem. Eur. J.* 2003; **9**: 2717–2725.
16. Potekhin SA, Melnik TN, Popov V, Lanina NF, Vazina AA, Rigler P, Verdini AS, Corradin G, Kajava AV. De novo design of fibrils made of short α-helical coiled coil peptides. *Chem. Biol.* 2001; **8**: 1025–1032.
17. Melnik TN, Villard V, Vasiliev V, Corradin G, Kajava AV, Potekhin SA. Shift of fibril-forming ability of the designed α-helical coiled-coil peptides into the physiological pH region. *Protein Eng.* 2003; **16**: 1–6.
18. Barany G, Solé N. Optimization of solid-phase synthesis of [Ala^8]-dynorphin A. *J. Org. Chem.* 1992; **57**: 5399–5403.
19. King D, Fields C, Fields G. A cleavage method which minimizes side reactions following Fmoc solid-phase peptide synthesis. *Int. J. Pept. Protein Res.* 1990; **35**: 284–286.
20. Chen YH, Yang JT, Chau KH. Determination of the helix and β-form of proteins in aqueous solution by circular dichroism. *Biochemistry* 1974; **13**: 3350–3359.
21. Bowen TJ. An introduction to ultracentrifugation. *J. Polym. Sci. B: Polym. Lett.* 1971; **9**: 636–637.
22. Zaric J, Ruegg C. Integrin-mediated adhesion and soluble ligand binding stabilize COX-2 protein levels in endothelial cells by inducing expression and preventing degradation. *J. Biol. Chem.* 2005; **280**: 1077–1085.
23. Aumailley M, Gurrath M, Muller G, Calvete J, Timpl R, Kessler H. Arg-Gly-Asp constrained within cyclic pentapeptides. *FEBS Lett.* 1991; **291**: 50–54.
24. Hood JD, Bednarski M, Frausto R, Guccione S, Reisfeld RA, Xiang R, Cheresh DA. Tumor regression by targeted gene delivery to the neovasculature. *Science* 2002; **296**: 2404–2407.

