Articles

Structural Analysis of Integrin Recognition and the Inhibition of Integrin-Mediated Cell Functions by Novel Nonpeptidic Surrogates of the Arg-Gly-Asp Sequence†

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ABSTRACT: The pivotal role of the Arg-Gly-Asp (RGD) peptide motif in integrin-mediated cell adhesive interactions with extracellular matrix and plasma proteins stimulated the present design of nonpeptidic mimetics of this sequence. To probe the structural requirements for RGD recognition by integrins, we designed various structural mimetics of the tripeptide sequence, which consist of differentially spaced guanidinium and carboxylic groups. We now report that structures which contain guanidinium and carboxylic groups separated by an 11-carbon atom backbone mimic the distal configuration of functional RGD sequence. These compounds acquire a considerable affinity for the RGD-dependent platelet $\alpha_{IIb}\beta_3$ integrin. As a result, these mimetics specifically inhibited platelet aggregation with an IC_{50} at the submillimolar range and interfered with RGD-dependent adhesion of $CD4^+$ T-lymphocytes and metastatic tumor cells to immobilized fibronectin and vitronectin. A structural mimetic of the Arg-Gly-Glu (RGE) sequence, and structures with incorrect spacing between the functional groups, failed to inhibit these adhesive interactions. Furthermore, substitution of the guanidinium group by a primary amine abrogated the RGD-mediated biological effects. In vivo, an RGD surrogate effectively inhibited the elicitation of a delayed-type hypersensitivity reaction mediated by $CD4^+$ T-cells, while the RGE mimetic did not. This interference suggests for a central role for RGD recognition in the regulation of immune responses. These proteolytically stable RGD mimetics may thus serve as useful therapeutic agents in versatile pathologic processes which depend on RGD recognition.

The ability of various cell types to adhere to and interact with other cells or with components of the extracellular matrix (ECM)[1] is essential for maintaining cell functions and tissue integrity via signaling between and within the communicating cells (Springer, 1990; Hynes, 1992; Shimizu et al., 1991). The interactions of various cells with soluble or insoluble components of plasma, the interstitial matrix, or the ECM are carried out primarily via a family of cell-surface heterodimeric receptors called integrins that are present on most cell types, including lymphocytes and platelets (Ruoslahti, 1991; Hynes, 1990). The target epitope of several integrin receptors is the RGD sequence, a cell adhesion motif shared by several matrix-associated adhesive glycoproteins, such as fibronectin (FN), vitronectin (VN), fibrinogen, thrombospondin, and von-Willebrand factor (Yamada & Kennedy, 1984; Hynes, 1992; Rouslahti, 1988; D'souza et al., 1991a,b). The RGD sequence is recognized by several receptors, including the $\alpha_{IIb}\beta_3$ (also designated GPIIb–IIIa), $\alpha3\beta1$, $\alpha5\beta1$ (also designated VLA-3 and VLA-5 integrins, respectively), and most of the α_V-containing integrins (Shimizu et al., 1990; Elices et al., 1991; Hynes, 1992). Following activation, these receptors mediate RGD-dependent cell-matrix adhesion or platelet aggregation (Philips et al., 1991; D'Souza et al., 1991a,b; Adler et al., 1991). When present in solutions, peptides containing the RGD sequence compete with RGD-containing matrix proteins for binding to their respective integrin receptors and thus prevent cell adhesion (Rouslahti & Giancotti, 1989; Springer, 1990). Nevertheless, the affinity of the short synthetic RGD peptides to their corresponding integrins is about 2–3 orders of magnitude lower than that of the native ligands (Humphries et al., 1986). This is due, in part, to a more favorable binding conformation imposed on the RGD sequences within the intact protein. This conformational rigidity also accounts for the selectivity of integrin recognition by the various RGD-containing ECM ligands. To study the structural parameters determining RGD specificity and affinity for integrins, we prepared a series of novel nonpeptidic analogues of the RGD and RGE sequences and

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[1] Abbreviations: ADP, adenosine diphosphate; cpm, counts per minute; DTH, delayed-type hypersensitivity; ECM, extracellular matrix; FN, fibronectin; LIBS, ligand-induced binding site; LN, laminin; PHA, phytohemaglutinin; TNF, tumor necrosis factor; VLA, very late antigen(s); VN; vitronectin.

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studied their interactions with a prototype RGD-dependent integrin, the platelet $\alpha_{IIb}\beta_3$ receptor, and their interference with human CD4+ T-cells and murine tumor cell adhesion to immobilized ECM glycoproteins, mediated by RGD-dependent integrins.

In designing the nonpeptidic surrogates of RGD, we took into consideration the fact that the RGE sequence is not recognized by integrins (D'Souza et al., 1991a,b; Shimizu & Shaw, 1991). Since the differences between the two peptide sequences is in the spacing of their ionic functional groups, we hypothesized that an adequate atomic spacing between the guanidinium and the carboxylate groups is necessary for RGD recognition by integrin(s). Thus, we propose that compounds in which the ionic functional groups are separated by various chain lengths could serve as a tool to study the structural requirements for RGD recognition.

We also assumed that the nonpeptidic analogues would be resistant to proteolytic digestion and thus could be useful reagents for inhibiting RGD-specific adhesive interactions in vivo. It has been suggested that extravasation of lymphoid cells through blood vessel walls to sites of inflammation is regulated by interactions of their VLA integrins with matrix proteins such as LN and FN (Ferguson et al., 1991). Since CD4+ T-cells, the major regulators of inflammatory reactions, interact with FN primarily via their RGD-dependent VLA-5 integrin, we hypothesized that the RGD mimetics could also inhibit this immune response in vivo.

EXPERIMENTAL PROCEDURES

Design and Preparation of Nonpeptidic Analogues of RGD. Compounds SF-6,5 and SFN-70 were prepared by coupling of methyl-5-aminovaleric acid with *N*-(butyloxycarbonyl)-6-aminohexanoic acid. The reaction was carried out using the 1,3-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in tetrahydrofuran procedure. The butyloxycarbonyl protecting group was then removed by 50% trifluoroacetic acid in dichloromethane. Removal of the methyl ester protecting group was carried out with sodium hydroxide at pH 9.5, yielding SFN-70. The amine was converted to a guanidium using 3,5-dimethylpyrazole-1-carboxamidine nitrate at pH 9.5 to produce the compound designated SF-6,5. Compound SF-6,6 was prepared from methyl-6-aminohexanoic acid and *N*-(butyloxycarbonyl)-6-aminohexanoic acid under the same conditions as used for SF-6,5. GK-5,5 was prepared from methyl-5-aminovaleric acid and *N*-(butyloxycarbonyl)-5-aminovaleric acid. Compounds AC-4 and AC-14 were prepared by stepwise synthesis either in solution or on a Merrifield resin (Barany & Merrifield, 1980), followed by deprotection and conversion of the amine to the guanidinium by the above-described methods. The final compounds were purified on preparative RP-18 columns and were judged pure by thin-layer chromatography (single spot) and ^{1}H NMR spectroscopy. Compounds were characterized by ^{1}H NMR and FAB-MS spectroscopy. The structures deduced from spectroscopy were consistent with the assigned structure (Figure 1).

Analysis of the Effect of RGD Surrogates on Platelet Activation and Aggregation. Platelet-rich plasma was prepared from sodium citrate anticoagulated fresh human blood by differential centrifugation (Frelinger et al., 1988). Platelet aggregation was induced by 5 μM ADP and monitored at 695 nm by a four-channel aggregometer (Bio-Data). To evaluate the effect of the various RGD peptides and peptide analogues, the platelet-rich plasma was preincubated with various inhibitors for 5 min at 37 °C, prior to the induction of aggregation. To examine RGD peptides and related analogues as competitors for PAC-1 mAb binding (the PAC-1 mAb was kindly donated by Dr. S. J. Shattil, The University of Pennsylvania), we used the procedure described by Taub et al. (1989). Gel-filtered platelets were incubated in Tyrode's/BSA solution supplemented with 1 mM $CaCl_2$ in the presence of 0–500 μM peptide or peptide analogues, along with 10 μg/mL FITC labeled PAC-1. The platelets were activated with ADP and epinephrine (10 μM each), and the incubation proceeded for 30 min in 25 °C. The reaction was terminated by diluting the system with buffer. PAC-1 binding was immediately analyzed by FACScan (Beckton Dickenson) at 488 nm.

Detection of LIBS epitopes induced in the platelet–$\alpha_{IIb}\beta_3$ upon RGD binding was performed as previously described by Frelinger et al. (1988). Briefly, gel-filtered platelets were incubated with GRGDSPK peptide SF-6,5 or SF-6,5 mimetics (0.5 mM each) in the presence of 10 μg/mL of the anti-LIBS mAb, PMI-1 (a generous gift of Dr. M. H. Ginsberg, Scripps Clinic, La Jolla, CA), for 30 min at 25 °C. The platelets were washed once and resuspended in Tyrode's buffer, and platelet-bound mAb was probed by FITC-labeled secondary antibody and analyzed by FACScan at 488 nm.

T-Cell Adhesion to ECM Proteins and Analysis of Inhibition. To examine the adhesive properties of the human CD4+ T-cells, 1 μg/50 μL/well of the 120-kDa cell-attachment fragment of FN (1 μg/well; Telios Pharmaceuticals Inc.) or LN (1 μg/well; Sigma) was added to 96 flat-bottom microtiter-well plates (Costar) for 12 h. Unbound proteins were removed by washing with PBS, and remaining binding sites were blocked by 1% BSA in PBS. CD4+ T-cells were purified from peripheral blood mononuclear leukocytes obtained from healthy human donors. The mononuclear cells were isolated using a Ficoll gradient, washed, and incubated in RPMI supplemented with 10% heat-inactivated FCS and antibiotics in Petri dishes at 37 °C in a humidified 10% CO_2 incubator. After 2 h, the nonadherent cells were isolated and eluted from nylon–wool columns (Fenwall; after 1.5 h of incubation at 22 °C). CD4+ T-cells were then negatively selected by exposure of the cells to a mixture of anti-CD8, anti-CD19, and anti-CD14 mAb conjugated to magnetic beads (Advanced Magnetics). The magnet-bound cells were removed, and the unbound cells were recovered and their phenotypes examined. The resultant purified cells were predominantly (>90%) CD3+ CD4+, as determined by FACScan analysis. T-cell adhesion assays, following T-cell activation by 10 ng/mL PMA, were performed as previously described (Shimizu et al., 1990). The level of activated T-cell adhesion to uncoated or BSA-coated wells was 2–5%, and the level of adhesion of nonactivated T-cells was below 5%. Where indicated, 1/200 diluted anti-CD29 mAb (anti-β1; Serotec), 1/200 diluted anti-CD44 mAb (Serotec), 1/400 diluted anti-VLA-5 mAb (clone P1D6, specific for $\alpha5\beta1$ integrin; Telios Pharmaceuticals), or 1 mM RGD, GRGDSPK (Sigma), or GRGESP (Boehringer-Mannheim) was employed. Various nonpeptidic surrogates, 1 mM in PBS, as well as the RGD- or RGE-containing peptides, were used to pretreat the T-cells for 30 min at 37 °C before seeding the ^{51}Cr-labeled CD4+ T-cells into the wells (2×10^5/well) for 30 min at 37 °C in the presence of 20 ng/mL PMA (Sigma).

Metastatic Cell Adhesion to ECM Protein Components. Analysis of tumor cell attachment to ECM proteins was performed essentially as described by Akiyama et al. (1989). To examine the adhesive properties of tumor cells, B16 melanoma F10 cells were metabolically labeled with 10 μCi/

mL [^{35}S]methionine (New England Nuclear) for 2 h, chased for 18 h, and extensively washed with serum-free medium. The cells were dissociated by 2 mM EDTA in prewarmed PBS and collected. The various nonpeptidic surrogates, 0.2 mM in PBS, were used to pretreat the tumor cells for 60 min before they were seeded into the coated wells for 40 min, as described for the T-cell adhesion studies. To examine cell adhesion to VN, 0.3 μg/well of the protein (a kind gift from Dr. S. Shaltiel, The Weizmann Institute of Science) was used to coat the wells.

Proliferation Assay. To examine the effects of the nonpeptidic surrogates on the proliferative responses of T-cells, 10^5 cells were cultured in 200 μL of RPMI/well (Gibco; supplemented with 2% heat-inactivated FCS, 1% HEPES buffer, antibiotics, and glutamine). The surrogates were added together with the cells at the beginning of the assay, which was conducted in a 96-microtiter-well plate (Costar). T-cells were activated by 2.5 μg/mL phytohemaglutinin (PHA; Wellcome UK). After 2 days, 1 μCi/well [^{3}H]thymidine was added for the final 4 h. The proliferating cells were then lysed by water and harvested, and their radiolabeled DNA was measured by a β-scintillation analyzer (1600 TR, Packard).

TNF Production and Secretion Assays. Two hundred and fifty thousand CD4$^+$ T-cells were activated with PHA in a flat-bottom 96-well plate. To determine TNF secretion, the plates were incubated at 37 °C in a humidified incubator for 3–hours. Subsequenty, the contents of the wells (three to six wells per experimental group) were collected and centrifuged, and the media were assayed for TNF secretion as previously described (Wallach, 1984). Briefly, supernatants of cultured lymphocytes were added to cultures of mouse fibrosarcoma cells, clone L-929.7. Fibrosarcoma cell death was examined by the release of neutral red dye from the preincubated fibrosarcoma cells. TNF was identified by examining the neutralizing effect of anti-murine TNFα mAb (diluted 1/400; Genzyme).

Trypsin Digestion. Compounds SF-6,5 and SF-6,6 and the GRGDSPK peptide were treated as follows: 50 μg of the various molecules was suspended in 100 μL of PBS, incubated at 37 °C, and exposed to 0.25% trypsin (Gibco; 50 μL in modified Puck's buffer). Aliquots were taken after 2.5, 5, 30, and 60 min and monitored by HPLC at 220 nm.

Delayed-Type Hypersensitivity (DTH). BALB/c mice (five mice per group) were sensitized on their shaved abdomens with 3% 4-(ethoxymethylene)-2-phenyl-oxazolone (OX; BDH Chemicals) in acetone/olive oil, as previously described (Lider et al., 1990). DTH sensitivity was elicited 5 days later as follows: mice were challenged on their ears with 0.5% OX in acetone/olive oil and the amount of ear swelling was quantified as the increase of ear swelling. The ears were measured immediately before challenge and 24 h later with a Mitutoyo micrometer. The individual who measured ear swelling was unaware of the group identity of the mice. To achieve blocking of the DTH response, GRGDSPK, compound SF-6,5 and compound SF-6,6 were administered intravenously (iv) into the tail vein at 10 μg in 200 μL of PBS. Alternatively, various concentrations of the indicated compounds in 200 μL of PBS were administered orally using a 18-gauge stainless steel animal feeding needle (Thomas Scientific). Treated mice were inspected during and after (≥2 months) treatment, and no major clinical side effects were observed.

RESULTS

Design and Synthesis of Nonpeptidic Analogues of RGD and Related Control Compounds. We synthesized three RGD nonpeptidic surrogates, compounds SF-6,5, AC-4, and AC-14, shown schematically in Figure 1. These water-soluble molecules, which have no branching or chiral elements in their chains, are conceptually perhaps the simplest possible RGD surrogates. The control RGE surrogate SF-6,6 was designed with an extra methylene group in its linking chain. Moreover, to examine whether a spacer shorter than 11 atoms allows integrins to recognize the two functional groups, we designed another control compound designated GK-5,5 in which the guanidinium and carboxylic functional groups are separated by a 10-atom spacer chain. A second control compound, designated SFN-70, in which the guanidinium group of SF-6,5 was replaced by a primary amine, was prepared to study the functional selectivity of integrin binding.

Analysis of Platelet Aggregation Inhibition. To investigate the possible inhibitory role of RGD analogues on cell adhesion, we used as a model the platelet $\alpha_{IIb}\beta_3$ (GPIIb–IIIa) receptor, which mediates the aggregation of activated platelets (Hynes, 1992). The results are shown in Figure 2.

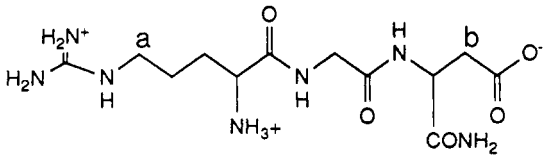
As seen in Figure 2A, compound SF-6,5, but not SF-6,6, was found to inhibit the aggregation of activated platelets in a dose-dependent manner, with an IC_{50} of 0.3 mM.

To examine the specificity of this inhibitory effect on platelet aggregation, platelets were treated with various peptidic and nonpeptidic mimetics of RGD or RGE, which were used at the fixed concentration of 0.5 mM, and activated to induce aggregation. The results are shown in Figure 2B. The tripeptide RGD was a negative control since amidation of its carboxy terminus has been shown to be required for its activity. As previously demonstrated (D'Souza et al., 1991a,b), the larger peptide GRGDSPK did exert a marked inhibitory effect (Figure 2B). The IC_{50} values of AC-4 and AC-14 were in the range of 0.35 and 0.42, respectively (data not shown). However, GK-5,5, which bears a shorter spacer than the 11-atom chain of SF-6,5, did not affect platelet aggregation. The control surrogate SF-6,6 exerted a limited inhibitory effect, reflecting the inability of RGE to inhibit platelet aggregation (Frelinger et al., 1988; Taub et al., 1989).

Direct Interaction of RGD Surrogate SF-6,5 with Integrin $\alpha_{IIb}\beta_3$. Do the RGD surrogates specifically bind to the RGD recognition site of $\alpha_{IIb}\beta_3$? To answer this question, we performed two independent binding assays.

(*I*). PAC-1 is a mAb specific for the activated platelet–$\alpha_{IIb}\beta_3$ integrin. This mAb binds to the RGD recognition site induced in $\alpha_{IIb}\beta_3$ of activated platelets. Since it binds to the activation-dependent epitope with moderate affinity ($K_i \sim 10^{-6}$ M), RGD-containing peptides interfere with PAC-1 binding since they bind $\alpha_{IIb}\beta_3$ with similar affinities (D'Souza et al., 1991b) and will therefore compete with RGD-containing peptides. To study the ability of the RGD surrogates to compete with PAC-1 binding, ADP-activated platelets were incubated with FITC-conjugated PAC-1 in the presence of various peptides (GRGDSPK and GRGESP) and the corresponding nonpeptidic compounds SF-6,5 and SF-6,6, respectively. Immunofluorescence was analyzed by flow cytometry (Abrams and Shattil, 1991). The results, shown in Figure 2C, indicate that GRGDSPK and the RGD surrogate SF-6,5 inhibit cell staining by PAC-1 in a dose-dependent manner. Neither the RGE-containing peptide nor the RGE analogue SF-6,6 affected PAC-1 binding.

(*II*). To further support the notion of direct interaction of the RGD mimetics with $\alpha_{IIb}\beta_3$, we examined the possibility that the RGD surrogate SF-6,5 alters the conformation of its RGD-binding site upon binding. It is known that the binding of RGD-containing ligands to $\alpha_{IIb}\beta_3$ leads to conformational



RGD-NH_2

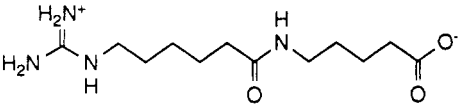
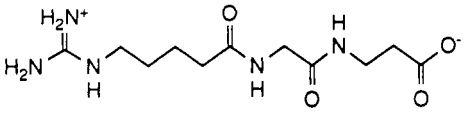
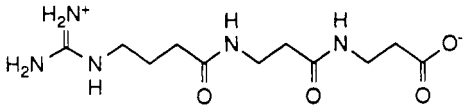
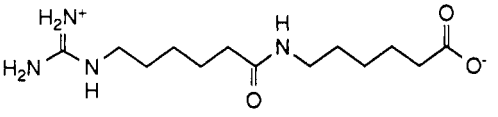
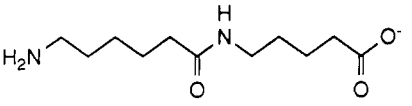
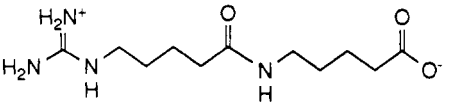
Compound		No. of atoms in the spacer
SF-6,5		11
AC-4		11
AC-14		11
SF-6,6		12
SFN-70		11
GK-5,5		10

FIGURE 1: Schematic presentations of the RGD–NH_2 peptide and the corresponding nonpeptidic surrogates. (Top) Schematic representation of the RGD–NH_2 peptide molecule. Note that the distance between positions a and b is 11 atoms. Compounds SF-6,5, AC-4, and AC-14 are nonpeptidic mimetics of RGD. Compound SF-6,6 was designed to serve as a nonpeptidic mimetic of the RGE sequence. Compounds SFN-70 and GK-5,5 were designed and synthesized to serve as controls.

changes in this integrin that result in the exposure of new antibody-binding sites, known as ligand-induced binding sites (LIBS; D'Souza et al., 1991a,b). Thus, platelets were pretreated with 0.5 mM GRGDSPK or SF-6,5 and SF-6,6. The results shown in Figure 2D indicate that both the GRGDSPK peptide and the SF-6,5 compound induced the expression of LIBS epitopes, probed with the anti-LIBS mAb PMI-1 (Frelinger et al., 1988) and stained by a secondary FITC-conjugated antibody. The effect of SF-6,5 was specific: platelets treated with RGE mimetic SF-6,6 did not express LIBS epitopes. These experiments indicate that the inhibition of platelet aggregation is a result of the direct binding of SF-6,5 to the RGD-binding site of the $\alpha_{IIb}\beta_3$ integrin.

Use of RGD Peptides and RGD Analogues To Interfere with CD4⁺ T-Lymphocyte–FN Interaction. In view of the ability of RGD surrogates to interfere with platelet aggregation, we examined the ability of these molecules to competitively inhibit $CD4^+$ human T-lymphocyte binding to fibronectin (FN). Purified human $CD4^+$ T-cells were radioactively labeled with ^{51}Cr, treated with various molecules or mAb, and then seeded in wells coated with the cell-attachment site of FN (120 kDa). Laminin (LN), a major component of the ECM, served as a control ECM-adhesive glycoprotein. The T-lymphocytes were then activated with PMA, and following incubation, the percent of ^{51}Cr-labeled $CD4^+$ T-cells attached to the protein substrates was measured (Shimizu et al., 1990). The results are shown in Table I. Activation of the T-cells resulted in their adhesion to the ECM glycoproteins in an integrin- and RGD-dependent manner. T-cell adhesion to the immobilized substrates was markedly inhibited by a mAb specific for the CD-29 molecule (the common β1-chain of integrin), as well as by a mAb specific for the $\alpha 5\beta 1$ (VLA-5) integrin. As expected, a control anti-CD44 mAb, directed against another cell-surface adhesion molecule expressed on lymphocytes, did not inhibit T-cell–FN or –LN adhesiveness.

The RGD-containing peptide inhibited T-cell adhesion to FN. The four RGD surrogates, but not the RGE surrogate, SF-6,6, or SFN-70, inhibited T-cell adhesion to FN but not to LN. Note that there are only minor differences in the

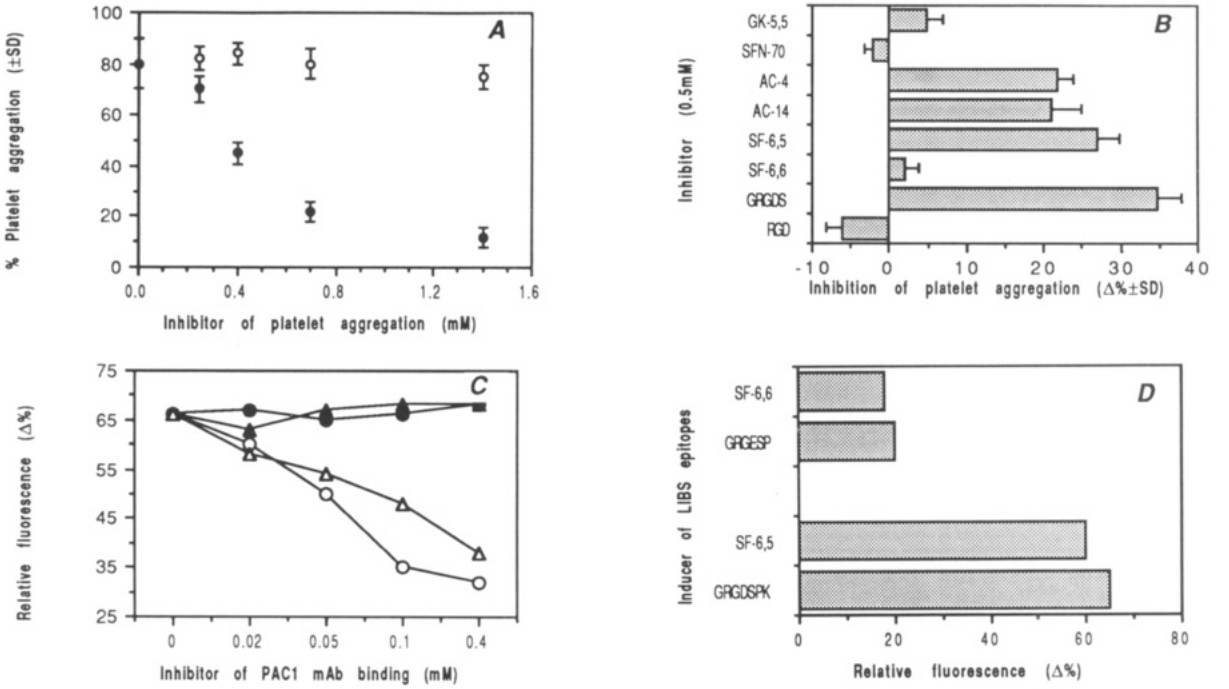


FIGURE 2: Specific inhibition of platelet aggregation by the RGD mimetics. Platelet aggregation was induced by ADP and monitored at 695 nm by an aggregometer. To evaluate the effect of the various RGD peptides and peptide analogues, platelet-rich plasma was preincubated with the various inhibitors, before induction of aggregation. (A) Dose-dependent inhibition of platelet aggregation by the RGD surrogate SF-6,5 (●) and the RGE surrogate SF-6,6 (○). Values ± SD are shown. (B) Analysis of inhibition of platelet aggregation by various peptides and nonpeptidic analogues added at the fixed concentration of 0.5 mM. (C) GRGDSPK and compound SF-6,5 inhibit PAC-1 binding to activated platelet–GPIIb–IIIa integrin in a dose-dependent manner. The activated platelets were incubated with the peptides or peptide analogues at the indicated concentrations in the presence of FITC-labeled PAC-1. The fluorescence profile of the cells was determined using FACScan. (○) GRGDSPK; (▲) GRGESP; (△) compound SF-6,5; (●) compound SF-6,6. The results shown here (A–C) are representative of one experiment of four identical experiments. (D) Analysis of LIBS epitope formation on GPIIb–IIIa integrin by the RGD- and RGE-containing peptides and the SF-6,5 and SF-6,6 mimetics. Samples of gel-filtered platelet-rich plasma were incubated with 0.5 mM each of SF-6,6 or SF-6,5 mimetics or GRGDSPK or GRGESP peptides. The formation of LIBS epitope conformers was probed by staining the platelets with the LIBS-specific PMI-1 mAb. Platelet-bound mAb binding was probed with FITC-labeled secondary antibody and analyzed by FACScan. The results shown here present data obtained from one representative experiment of three identical experiments.

Table I: Specific Inhibition of T-Cell Adhesion to FN by RGD Surrogates[a]

test molecule or mAb	% adhesion of activated CD4+ T-cell to (% inhibition)[b]	
	120 kDa of FN	LN
none	43 ± 4	55 ± 5
mAb to		
CD29	10 ± 2[c] (83)	11 ± 2[c] (80)
VLA-5	8 ± 2[c] (82)	52 ± 4 (0)
CD44	45 ± 3 (0)	52 ± 8 (0)
peptide		
RGD	38 ± 3 (12)	57 ± 3 (0)
GRGDSPK	20 ± 2[c] (54)	53 ± 4 (0)
GRGESP	46 ± 3 (0)	52 ± 3 (0)
peptide analogues		
AC-4	30 ± 2[c] (31)	57 ± 3 (0)
AC-14	25 ± 4[c] (42)	55 ± 8 (0)
SF-6,5	19 ± 2[c] (56)	55 ± 6 (0)
GK-5,5	47 ± 3 (0)	58 ± 5 (0)
SF-6,6	44 ± 2 (0)	52 ± 6 (0)
SFN-70	42 ± 5 (0)	59 ± 6 (0)

[a] Adhesion of activated CD4+ human T-cells to ECM glycoproteins in the presence of various mAb, peptides, and nonpeptidic surrogates (100 μg/mL). [b] Percent inhibition of cell adhesion was calculated as follows: 1 – [experimental/control group (without inhibitors)] × 100. Number of experiments: 12. [c] $P < 0.01$: experimental vs control groups.

Table II: Nonpeptidic Mimetics of RGD and RGE Do Not Inhibit T-Cell Proliferative Responses and TNFα Secretion[a]

test molecule	T-cell proliferative response (cpm × 10^{-3} ± SD)		TNFa secretion (pg/mL)	
	control	PHA	control	PHA
none	5 ± 1	75 ± 10	20 ± 2	650 ± 50
SF-6,6	3 ± 2	77 ± 4	25 ± 4	690 ± 40
SF-6,5	3 ± 2	82 ± 7	22 ± 4	680 ± 30
GK-5,5	4 ± 1	71 ± 6	20 ± 4	670 ± 20
GRGDSPK	3 ± 1	80 ± 9	18 ± 3	660 ± 60

[a] The proliferative responses of freshly isolated CD4+ human T-cells and their ability to secrete TNFα are not affected by the nonpeptidic surrogates of RGD and RGE (1 mM). The proliferative responses of the CD4+ T-cells were measured for 48 h, and TNF secretion was measured in the supernatant of the PHA-activated or the nonactivated cells 6 h following seeding. The results shown here represent data obtained from one experiment of a total of three experiments that produced essentially similar results.

inhibitory effect of the various RGD surrogates. This supports our working hypothesis that the peptide backbone is less critical to RGD recognition by its specific integrin(s) than are the two functional groups and the correct atom spacing between them.

The inhibitory effect of the RGD analogues on T-cell adhesion cannot be attributed to a toxic effect exerted by these molecules because these compounds did not inhibit T-cell adhesion to LN (Table I). Moreoever, none of the surrogates inhibited PHA-induced T-cell proliferative responses and TNFα secretion, conducted for 48 or 6 h, respectively. Note that none of the tested molecules were themselves immunogenic, as these molecules failed to induce cell proliferation or TNFα secretion (Table II). The GRGDSPK peptide, which served as a control in these experiments, had no effect on these immune-cell reactions.

Inhibition of Tumor Cell–FN and –VN Adhesiveness. To metastasize, tumor cells must penetrate blood vessel walls. It has been demonstrated that human melanoma cells interact

Table III: Inhibition f Tumor Cell Adhesion to FN and VN Using RGD Surrogates[a]

inhibitor of cell adhesion	% adhesion of B16 melanoma cell to (% inhibition)	
	FN	VN
none	75 ± 5	68 ± 5
RGD	70 ± 3 (7)	66 ± 6 (0)
GRGDSPK	15 ± 4[b] (80)	25 ± 2[b] (64)
GRGESP	73 ± 5 (0)	66 ± 8 (0)
SF-6,5	34 ± 4[b] (55)	40 ± 5[b] (42)
AC-4	47 ± 5[b] (38)	55 ± 5[b] (20)
SF-6,6	75 ± 9 (0)	70 ± 7 (0)

[a] To examine the adhesive properties of tumor cells, 1 μg/50 μL/well of FN or 0.3 μg/well of VN was immobilized on the plastic surfaces. Various nonpeptidic surrogates, 0.2 mM in PBS, were used to pretreat the tumor cells before seeding into the wells (2×10^5/well) for 30 min at 37 °C. The results shown here represent data obtained from one experiment of a total of five experiments that produced essentially the same results. [b] $P < 0.05$: experimental vs control groups.

with VN primarily by their $\alpha_V\beta_3$ integrin (Seftor et al., 1992) and that RGD-containing peptides inhibited in-vivo metastasis of murine B16–F10 melanoma cells in the lungs (Humphries et al., 1986). Thus, we chose to analyze the effect of the RGD surrogates on tumor-cell adhesion to the FN and VN components of the ECM. The results are shown in Table III. Similar to the results obtained with T-cells, adhesion of B16 murine melanoma cells to FN, as well as to VN, was inhibited by GRGDSPK (80 and 64%, respectively) but not by RGD or GRGESP peptides, or by the RGE surrogate SF-6,6. The RGD analogues SF-6,5 and AC-4 significantly inhibited the adhesion of these tumor cells to the immobilized substrates (20–55%).

RGD Surrogate SF-6,5 Suppresses Delayed-Type Hypersensitivity Response in Vivo. To extravasate, circulating lymphocytes must be activated and interact with the vascular endothelial cells and then adhere, interact, and penetrate the ECM lining beneath the endothelium. These processes are mediated primarily by specific β1-integrin receptors (Springer, 1989; Hynes, 1992). Recent studies indicated the pivotal role of VLA (of the β1 subfamily) integrins in cellular infiltration of blood vessel walls (Isekutz, 1991, 1991; Weeks et al., 1991; van Dinther-Janssen et al., 1991). In view of their ability to selectively inhibit RGD-dependent integrin-mediated cell adhesion in vitro, we tested whether RGD analogues could interfere with in-vivo T-cell extravasation and localization of these cells in inflammatory sites. This is based on our assumption that nonpeptidic molecules are resistant to proteolysis and fairly nonimmunogenic (Table II; Saragovi et al., 1991).

To examine a possible regulatory effect of SF-6,5 on T-cell immunity in vivo, we chose an experimental system in which RGD-dependent reactions should play a major role. We performed a delayed-type hypersensitivity (DTH) experiment in which groups of BALB/c mice were primed with the skin allergen 4-(ethoxymethylene)-2-phenyloxazolone (OX) and challenged 5 days later by applying OX to their ears. The increment in ear swelling was recorded 24 h later as a measure of DTH (Lider et al., 1990; Ferguson et al., 1991). The mice were treated followed OX priming with intravenous (iv) injections of 10 μg of the GRGDSP peptide, of compound SF-6,5 or SF-6,6, or of PBS (no treatment) at the indicated times. Alternatively, different concentrations of the compounds were administered orally from day 1 to day 6. The results, shown in Table IV, indicate that only compound SF-6,5 inhibited the DTH response, with the best results obtained when the mice were injected daily for 6 days. Furthermore, oral administration of SF-6,5, at a dose of 200 μg/day, resulted in marked inhibition of the DTH response. The RGD surrogate, when administered iv or orally, was a better inhibitor than the GRGDSPK peptide, most probably due to a shorter biological retention time of the latter. As a model to analyze the susceptibility of SF-6,5 to proteolysis, its resistance to trypsin digestion was tested. Serum is known to contain trypsin-like proteases (Saragovi et al., 1991). Indeed, HPLC analysis of trypsin-treated compounds revealed that while the GRGDSPK was completely degraded following 5 min of incubation in vitro, the SF-6,5 surrogate was left intact even following 60 min (Figure 3).

Table IV: Specific Inhibition of DTH Response to OX in Vivo by Treatment of Mice with the Nonpeptidic Surrogate of RGD, Compound SF-6,5[a]

compd	treatment of mice: dose per day (μg) and route of administration	treatment of mice: administered on days	measurement of OX-mediated DTH response: Δ ear swelling[b] ($\times 10^{-2}$ mm ± SD)	measurement of OX-mediated DTH response: % inhibition
none			21 ± 2	
GRGDSPK	10; iv	1–6	18 ± 3	14
SF-6,5	10; iv	1	16 ± 3	20
	10; iv	1, 3	13 ± 2	34
	10; iv	1, 3, 5	8 ± 2[c]	62
	10; iv	1–6	4 ± 1[c]	81
GK-5,5	10; iv	1–6	19 ± 3	0
SF-6,6	10; iv	1–6	20 ± 4	0
GRGDSPK	200; orally	1–6	22 ± 4	0
SF-6,5	10; orally	1–6	18 ± 3	15
SF-6,5	50; orally	1–6	14 ± 2	34
SF-6,5	200; orally	1–6	9 ± 1[c]	60

[a] To examine the effect of GRGDSPK, SF-6,5, and SF-6,6 on DTH responses of BALB/c mice, the mice were sensitized with OX on day 0. On day 5 they were challenged with OX in the ears and the ear swelling was measured 24 h later. The various compounds were injected intravenously (iv) or administered orally in PBS. [b] Local administration of PBS into the ear (as a negative control) resulted in an increment of $(2 \pm 2) \times 10^{-2}$ mm. [c] $P < 0.01$; P values were measured in relation to the positive control group. This experiment was repeated three times, and the results shown here represent one of these experiments.

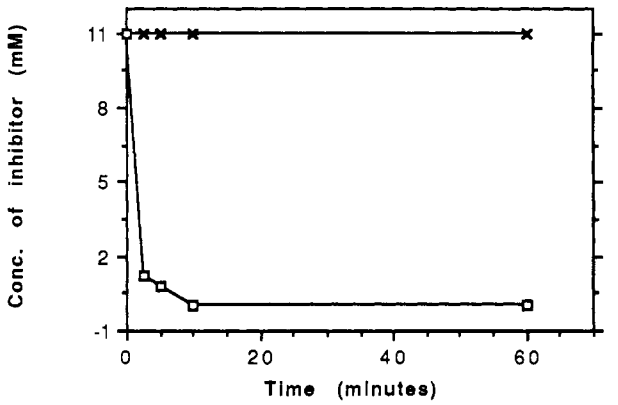


FIGURE 3: Compound SF-6,5 is resistant to trypsin hydrolysis. Compound SF-6,5 and the GRGDSPK peptide (50 μg in 100 μL of PBS) were incubated at 37 °C and exposed to 0.25% trypsin in modified Puck's buffer. Aliquots were taken after 2.5, 5, 30, and 60 min and monitored by HPLC at 220 nm for the detection of the integrity of the tested molecules. (×) compound SF-6,5; (□) GRGDSPK. The results shown here are representative of one of three identical experiments which yielded essentially the same results.

DISCUSSION

In studying the structural requirements for proper RGD recognition by integrins, we hypothesized that the major

contribution to the binding affinity of RGD-containing ligands depends on the following: (a) the guanidinium and carboxylate groups of the Arg and Asp amino acid residues, respectively [on the basis of structure–function studies demonstrating the indispensable role of these residues for integrin recognition (Pierschbacher and Rouslahti, 1987; D'Souza et al., 1991a,b)]; (b) 11-atom spacing between the two ionic functional groups [on the evidence that analogous Arg-Gly-Glu (RGE)-containing peptides, which have a 12-atom spacing chain between the two ionic groups, lack integrin specificity (Yamada and Kennedy, 1984; Elices et al., 1991)]. Thus, we assumed that a relatively flexible molecule with appropriately spaced functional moieties can acquire an RGD-like configuration.

To investigate the biological properties and possible inhibitory role of RGD surrogates on integrin binding and cell adhesion, we chose as a model the prototype platelet glycoprotein $\alpha_{IIb}\beta_3$ receptor, the most prominent integrin of platelets, which mediates aggregation by binding the RGD-containing sequences within fibrinogen. The structure of this receptor, its epitope specificity, and its functional responses following ligand occupancy are well-defined and characterized (Abrams and Shattil, 1991).

The results shown in Figure 2 indicate that (a) surrogates SF-6,5, AC-4, and AC-14, all with 11-atom spacing between the two functional groups, inhibit platelet aggregation; (b) the IC_{50} of this inhibition is around 0.3–0.5 mM; and (c) inhibition of platelet function is conceivably achieved by way of direct interaction of the RGD surrogate SF-6,5 with the $\alpha_{IIb}\beta_3$ integrin as demonstrated by direct interference with the RGD-dependent binding of PAC-1 mAb to $\alpha_{IIb}\beta_3$.

The observation that the RGD mimetics, lacking peptide backbone, still confer an RGD-like configuration suggests that the role of the backbone of the RGD sequence is mainly to dictate the correct spacing between the two ionic groups involved in interaction with the receptor(s). As expected, the same RGD analogues also interfere with $CD4^+$ T-cell interactions with FN and tumor cell adhesion to FN and VN; such interactions have been postulated to play a role in integrin-mediated cell migration (Sundquist et al., 1991).

We have found in vivo that treatment of mice with the RGD surrogate compound SF-6,5 suppresses the DTH response, while the RGE surrogate SF-6,6, as well as the RGD-containing peptide, had no effect. This modulation of $CD4^+$ T-cell-mediated immune reaction was achieved by relatively low does of the RGD analogues, i.e., 20 μM at maximum, assuming a blood volume of 2 mL. It is reasonable to speculate that this inhibition is achieved by means of blocking integrin–ECM interactions crucial for lymphocyte activation, migration, and subsequent tissue localization (Shimizu & Shaw, 1991). This hypothesis is supported by the findings of Ferguson et al. (1991) that preincubation of antigen-specific $CD4^+$ T-cells with RGD-containing ligands abrogated the ability of these cells to adoptively transfer DTH in mice. We are currently conducting in-vivo studies aimed at the delineation of this prospect. However, we cannot exclude alternative explanations for the interference with DTH: peptides containing RXD sequences were postulated to interfere with immune functions unrelated to integrins. RADS, RFDS, and RYDS sequences have been suggested to constitute functional adhesiotopes of the CD4 or MHC class I and II molecules, respectively (Mazerolles et al., 1990). At any rate, the present study suggests a defined structural requirement for an RXD motif rather than an RXE motif for interference with DTH. To the best of our knowledge, this is the first example of interference with inflammatory reaction by continuous in-vivo administration of a nonpeptidic RGD surrogate.

The structure–function analysis of the various surrogates examined herein suggests that RGD-specific integrin receptors have two binding pockets, one that binds to the carboxyl group and the other to the guanidinium group. To gain maximal binding energy, the receptor must recognize both ionic groups separated by the correct atomic spacing. Surrogates with correct spacing chain can attain the conformation required for high-affinity binding to the integrin. Thus, compounds SF-6,5, AC-4, and AC-14, all having 11-atom linear spacing, are effective inhibitors of cell adhesion, indicating that the relative location and the number of amide bonds along the chain have limited effect on the binding efficiency. Interestingly, any minute alteration in this spacing such as in SF-6,6 (with 12-atom spacing) or GK-5,5 (with the 10-atom spacer backbone) leads to inability of the model compound to acquire the RGD-active configuration. Compound SFN-70, albeit of proper spacing between its ionic groups, has a primary amine instead of a guanidinium group, does not bind integrins, and does not inhibit cell adhesion, confirming the critical role of the guanidinium group in RGD recognition. Thus, these structure–function studies of RGD and RXD-like molecules serve as probes to study integrin binding and their defined RGD specificity.

Thus, in instances in which the peptide backbone has a minor direct contribution to the binding efficacy of a given ligand, but serves mainly to dictate the spacing and the orientation of the functional groups, nonpeptidic surrogates may be constructed. Such constructs could provide novel insights into the fundamental mechanisms of receptor–ligand interactions and serve as competitive antagonists of potential therapeutic value.

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