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Coupling Tumor Necrosis Factor-α with α_V Integrin Ligands Improves Its Antineoplastic Activity

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ABSTRACT

Despite the impressive results obtained in animal models, the clinical use of tumor necrosis factor-α (TNF) as an anticancer drug is limited by severe toxicity. We have shown previously that targeted delivery of TNF to aminopeptidase N (CD13), a marker of angiogenic vessels, improved the therapeutic index of this cytokine in tumor-bearing mice. To assess whether the vascular-targeting approach could be extended to other markers of tumor blood vessels, in this work, we have fused TNF with the ACDCRGDCFCG peptide, a ligand of α_V integrins by recombinant DNA technology. We have found that subnanogram doses of this conjugate are sufficient to induce antitumor effects in tumor-bearing mice when combined with melphalan, a chemotherapeutic drug. Cell adhesion assays and competitive binding experiments with anti-integrin antibodies showed that the Arg-Gly-Asp moiety interacts with cell adhesion receptors, including $\alpha_V\beta_3$ integrin, as originally postulated. In addition, ACGDRGDCFCG-mouse TNF conjugate induced cytotoxic effects in standard cytolytic assays, implying that ACGDRGDCFCG-mouse TNF conjugate can also bind TNF receptors and trigger death signals. These results indicate that coupling TNF with α_V integrin ligands improves its antineoplastic activity and supports the concept that vascular targeting is a strategy potentially applicable to different endothelial markers, not limited to CD13.

INTRODUCTION

Tumor necrosis factor-α (TNF) is an inflammatory cytokine originally identified for its cytotoxic activity against some tumor cell lines and for its ability to induce hemorrhagic necrosis of transplanted solid tumors (1, 2). Despite the impressive results obtained with various animal models, the clinical use of TNF as an anticancer drug is limited by systemic toxicity, the maximum tolerated dose (5–10 μg/kg) being 10–50 times lower than the estimated effective dose. For this reason, TNF can be administered to patients only locoregionally. Regional administration of relatively high doses of TNF in combination with chemotherapeutic drugs, by isolated limb or hepatic perfusion, has produced high-response rates in patients with advanced tumors of the limbs (3–5) as well as regression of primary and metastatic tumors confined to the liver (6). These results are of outstanding interest because they show that, in principle, the antitumor effects of TNF can be exploited therapeutically in humans with great success if sufficient dose levels can be attained locally and if the organism can be shielded from the systemic toxic effect of TNF.

The antitumor activity of TNF depends on a variety of effects that this cytokine can trigger on neoplastic cells as well as on normal cells within the tumor microenvironment, leading to selective obliteration and damage of tumor-associated vessels, activation of inflammatory and immune mechanisms, tumor cell apoptosis, and tumor cell necrosis (7–10). Among the various cells that are affected by TNF, endothelial cells are of central importance, particularly in mediating tumor vascular damage. TNF-induced vascular damage is thought to occur because of the combination of direct toxic effects on endothelial cells (11) and because of the TNF-induced shift in the homeostatic properties of endothelial cells from anticoagulant to procoagulant (9). Unfortunately, endothelial cells of normal vessels can also be affected by TNF, and this interaction is likely at the origin of hypotension (12), the most important dose-limiting toxicity for this cytokine (5). This is an obvious reason why the therapeutic window was found to be so narrow for systemically administered TNF.

These notions provide the rational for developing vascular-targeting strategies aimed at increasing the selectivity of TNF for the endothelial lining of tumor vessels, while sparing normal vessels. For instance, the targeted delivery of TNF to vascular proliferation antigens, *e.g.*, angiogenic vessel endothelium markers, is an attractive possibility.

In vivo panning of phage libraries in tumor-bearing animals has proven useful for selecting peptides able to interact with angiogenic endothelium markers and "to home" to tumors (13). Among the various peptides identified thus far, the CNGRC and ACDCRGDCFC peptides have proven useful for delivering various antitumor compounds, like chemotherapeutic drugs and apoptotic peptides to tumor vessels (13–15). These peptides bind, respectively, to an aminopeptidase N (CD13) isoform (16) and to α_V integrins expressed by angiogenic vessels (17). In previous work, we showed that a CNGRC-mTNF conjugate (NGR-mTNF) is endowed with more potent antitumor properties than TNF (15, 18), supporting the hypothesis that targeted delivery of TNF to CD13-positive tumor vessels could be a valid strategy to increase its therapeutic index. To assess whether the vascular-targeting approach could be extended to other markers of tumor blood vessels, such as α_V integrins, we have prepared an ACDCRGDCFCG-murine TNF conjugate (RGD-mTNF), by recombinant DNA technology, and studied its *in vitro* and *in vivo* antitumor activity. We have found that this peptide, fused to the NH_2 terminus of TNF, is able to target TNF to cell membrane adhesion receptors. Moreover, we show that subnanogram doses of this conjugate are sufficient to induce antitumor effects in tumor-bearing mice.

MATERIALS AND METHODS

Cell Lines and Reagents. Mouse RMA lymphoma cells of C57BL/6 origin (19) were prepared and cultured as described previously (20). EA.hy926 cells (generated by fusing human umbilical vein endothelial cells with the human lung carcinoma A549 cells (21) were obtained from Dr. E. Ferrero (San Raffaele H. Scientific Institute, Milan, Italy). EA.hy926 cells were cultured in DMEM (Euroclone, Milan, Italy) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin-B, and 10% fetal bovine serum (DMEM complete medium) and expanded three times/week by detaching with trypsin-EDTA. L-M cells (murine fibroblasts) were cultured as described previously (22). The following antibodies were used: monoclonal antibody (mAb) LM609 (antihuman $\alpha_V\beta_3$; Chemicon, Temecula, CA), mAb 15.2 [antihuman intercellular adhesion molecule 1 (ICAM-1); Boehringer Mannheim Gmbh, Germany], mAb B4E11 (antihuman chromogranin A; Ref. 23), H9.2B8 (biotinylated antimouse α_V-integrin subunit; PharMingen, San Diego, CA), and goat antimouse-FITC secondary antibody (Sigma). Actinomycin D, crystal violet, and oxidized L-glutathione were

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obtained from Fluka Chemie (Buchs, Switzerland); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was obtained from Merk (Darmstadt, Germany); human serum albumin was supplied by Farma-Biagini SpA (Lucca, Italy); and strepavidin-phycoerythrin was obtained from Sigma. Endotoxin content was measured using the quantitative chromogenic *Limulus* Amoebocyte Lysate test (BioWhittaker Europe, Verviers, Belgium).

Peptide Synthesis. CNGRC and ACDCRGDCFCG peptides were prepared using the stepwise solid-phase Fmoc method and a 433A Applied Biosystem peptide synthesizer (Foster City, CA). Peptide folding was obtained by leaving the peptide solutions [100 μM in 20 mM Tris-HCl (pH 8)] for 2 days in open air under stirring. Oxidized peptide was then purified by reverse-phase high-performance liquid chromatography on a Jupiter 10-μm C18 300A column (250 $\times$ 21.2 mm; Phenomenex, Torrance, CA). Free sulfhydryl groups in peptides were $<$0.1%, as checked by titration with Ellman's reagent (Pierce, Pierce, Rockford, Illinois). Peptide concentrations were measured using the Protein Assay ESL kit (Roche). The molecular masses of CNGRC and ACDCRGDCFCG were 550.23 $\pm$ 1 Da and 1145.58 $\pm$ 1 Da, respectively (expected for disulfide-bridged peptide, 551.18 Da and 1145.29 Da, respectively), by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Preparation of mTNF, NGR-mTNF, and RGD-mTNF. Murine TNF (mTNF) and NGR-mTNF (mTNF fused with the COOH-terminus of CNGRCG) were prepared as described previously (15). The cDNA coding for RGD-mTNF (murine TNF fused with the COOH-terminus of ACDCRGDCFCG) was obtained by PCR on plasmid containing the mTNF cDNA sequence (15), using the following primers: TGCAGATCATATGGCTTGCGACTGCCGTGGTGACTGCTTCTGCGGTCTCAGATCATCTTCTC (5′ primer); TCAGGATCCTCACAGGGCAATGATCCCAAAGTAGAC (3′ primer). Primer sequences were designed to include the *Nde*I and *Bam*HI restriction sites (underlined) for cloning in pET11 plasmid (Novagen, Madison, WI). The *Nde*I site contains also the translation starting codon. A glycine residue was interposed between ACDCRGDCFC and mTNF as a spacer. The RGD-mTNF cDNA was expressed in BL21(DE3) *E. coli* cells and purified from cell extracts by ammonium sulfate precipitation and hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast Flow (Amersham Biosciences Europe GmbH, Freiburg, Germany), followed by ion exchange chromatography on DEAE-Sepharose Fast Flow (Amersham). Oxidized L-glutathione was added to the product of ion exchange (2 mM final concentration) and incubated for 72 h at 4°C. RGD-mTNF was then denatured by adding urea (7 M, final concentration) and gel-filtered through an HR Sephacryl S-300 column (1025 ml; Amersham) pre-equilibrated with 7 M urea, 100 mM Tris-HCl (pH 8.0). Fractions corresponding to monomeric RGD-mTNF were pooled and refolded by adding, slowly, 4 volumes of distilled water, followed by 1 volume of 20 mM Tris-HCl (pH 8.0). The diluted RGD-mTNF solution (10 μg/ml) was left to incubate overnight at 4°C. Finally, the product was concentrated by ion exchange chromatography on DEAE-Sepharose Fast Flow and gel-filtered through an HR Sephacryl S-300 column (1025 ml) pre-equilibrated with 150 mM sodium chloride, 50 mM sodium phosphate (pH 6.8). All solutions used in purification and refolding steps were prepared with sterile and endotoxin-free water (S.A.L.F. Laboratorio Farmacologico SpA, Bergamo, Italy). Protein concentration was measured using the BCA Protein Assay Reagent (Pierce, Rockford, Illinois). About 2 mg of purified protein was recovered from 1 liter of *E. coli* culture. Protein purity and identity was checked by SDS-PAGE and Western blotting. The molecular mass of RGD-mTNF monomer was 18,383.2 $\pm$ 2.4 Da as determined by electrospray ionization mass spectrometry (expected for Met-ACDCRGD-CFCG-mTNF$_{1-156}$, 18,388.8 Da; Fig. 1*A*). The endotoxin content of RGD-mTNF was 0.033 units/μg.

***In Vitro* Cytolytic Assay.** *In vitro* cytotoxicity of mTNF and RGD-mTNF was measured by cytolytic assay using L-M mouse fibroblasts cultured in 96-well flat-bottomed plates. Because TNF is known to activate protective mechanisms in these cells that require transcription and protein synthesis (24), the assay was carried out in the presence of actinomycin D, a transcription inhibitor. L-M cells, plated in DMEM complete medium (3 $\times$ 10^4 cell/well, 100 μl) were incubated overnight at 37°C, 5% CO_2. Actinomycin D (50 μl/well, 2 μg/ml final concentration) and mTNF or RGD-mTNF solutions (50 μl/well, 0.003–3 ng/ml) were added to each well and incubated for 20 h at 37°C, 5% CO_2. Viable cells were quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described previously (22). Alternatively, L-M cells were detached with trypsin-EDTA, washed, and immediately incubated with mTNF or RGD-mTNF (0.3–300 ng/ml, 6 $\times$ 10^4 cells/100 μl) in test tubes, in the presence or absence of 5 μg/ml ACDCRGDCFCG peptide, and diluted in DMEM complete medium for 1.5 h on ice. After washing three times with 0.9% sodium chloride solution, the cells were resuspended in DMEM complete medium containing 2 μg/ml actinomycin D and plated (200 μl/well, 96-well plate). After 20 h at 37°C, 5% CO_2, the cytotoxic activity was quantified by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

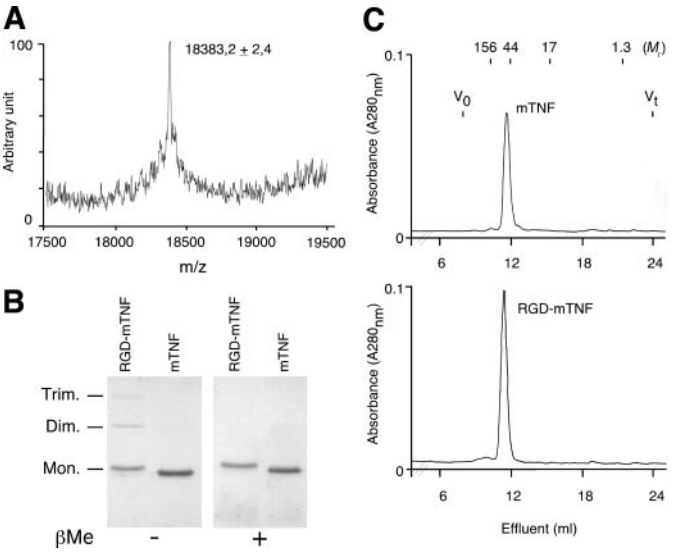

Fig. 1. Electrospray mass spectrometry analysis (ESI-MS; *A*), SDS-PAGE (*B*) and analytical gel-filtration chromatography (*C*) of ACGDRGDCFCG-murine tumor necrosis factor-α conjugate (RGD-mTNF) and mTNF. RGD-mTNF and mTNF were analyzed under nonreducing ($-\beta$Me) and reducing ($+\beta$Me) conditions. Analytical gel filtration chromatography of RGD-mTNF and mTNF on Superdex 75 HR column; void volume (Vo), total volume (Vt); *bars* represent the elution volume of molecular weight markers.

ICAM-1 and Integrin Flow Cytometry Analysis. Detection of ICAM-1 and $\alpha_V\beta_3$ integrin on the membrane of human EA.hy926 cells was carried out as follows. The cells were resuspended in cold 138 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate [(pH 7.3) PBS] containing 2% FCS and 1 μg/ml mAb 15.2 (anti human-ICAM-1) or mAb LM609 (antihuman $\alpha_V\beta_3$ integrin), and incubated for 1 h on ice. After washing with PBS, the cells were incubated with goat antimouse-FITC secondary antibody, diluted (1:100) in PBS containing 2% FCS (30 min, on ice), washed again, and fixed with 4% formaldehyde in PBS. The cells were then analyzed by fluorescence-activated cell sorter. Expression of α_V integrin subunits on murine L-M cells was quantified using 3 μg/ml biotinylated mAb H9.2B8 (rat anti-murine α_V integrin) and streptavidin-phycoerythrin, essentially as described above.

EA.hy926 Cell Adhesion Assay. Polyvinyl chloride microtiter plates (Falcon code no. 3912; Becton Dickinson, Franklin Lakes, NJ) were coated with RGD-mTNF or mTNF solutions at various concentrations [50 μl/well in 150 mM sodium chloride, 50 mM sodium phosphate (pH 7.3) at 4°C overnight]; after washing with 0.9% sodium chloride, each well was filled with DMEM containing 2% BSA (45 min at 37°C) and washed again. EA.hy926 cells were then detached, washed three times with 0.9% sodium chloride, resuspended in incomplete DMEM and added to mTNF- or RGD-mTNF-coated plates (3 $\times$ 10^4 cells/100-μl well). After incubation (1–1.5 h) at 37°C, 5% CO_2, unbound cells were removed by washing with incomplete DMEM. Adherent cells were fixed with 3% paraformaldehyde, 2% sucrose in PBS (pH 7.3) and stained with 0.5% crystal violet. Adherent cells were quantified by reading the absorbance of each well at 540 nm, using a microplate reader.

Radio-Binding Assay. ^{125}I-mTNF (13.91 μCi/μg) and ^{125}I-RGD-mTNF (11.64 μCi/μg) were prepared using Iodo-GenR precoated tubes (Pierce, Rockford, IL) according to the manufacturer's instructions. Binding of radiolabeled mTNF or RGD-mTNF to EA.hy926 cell suspensions (10^6 cell/tube) was carried out in the presence or absence of 10 μg/ml of unlabeled competitors (mTNF or RGD-mTNF) diluted in DMEM containing 1 mg/ml human serum albumin and 0.02% sodium azide. The mixtures (100 μl) were incubated

in tubes containing 100 μl of 20% mineral oil and 80% melting point bath oil (Sigma). After incubation (2 h) at 37°C, the tubes were centrifuged and cut at the bottom level. The bound radioactivity was measured using a gamma counter.

***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/6 mice (Harlan, Italy) 8 weeks old were challenged with s.c. injection in the left flank of 7×10^4 RMA living cells; 10 days later, mice were treated with RGD-mTNF or NGR-mTNF solutions (100 μl) followed 2 h later by administration of melphalan (100 μl; Glaxo Wellcome Operations, Dartford, United Kingdom). All drugs, diluted with 0.9% sodium chloride containing 100 μg/ml endotoxin-free human serum albumin, were administered i.p. Tumor growth was monitored daily by measuring tumor volumes with calipers as described previously (7). Animals were sacrificed before tumors reached 1.0–1.3 cm in diameter. Tumor sizes are shown as mean ± SE (5 animals/group).

RESULTS

Production and Characterization of RGD-mTNF. RGD-mTNF was produced by recombinant DNA technology and purified by a series of chromatographic steps including hydrophobic interaction and ion exchange chromatography, denaturing and nondenaturing gel-filtration chromatography. Because mTNF is a compact homotrimeric protein (25), only fractions corresponding to trimeric species were collected during the final nondenaturing, gel filtration chromatography. Reducing SDS-PAGE of RGD-mTNF showed a single band of 17000–18000 M_r, as expected for mTNF monomers (Fig. 1*B*). In contrast, nonreducing SDS-PAGE showed three bands of 18000, 36000, and 55000 M_r. These bands were immunoreactive with anti-mTNF antibodies by Western blot analysis (data not shown) suggesting that they correspond to RGD-mTNF monomers, dimers, and trimers. Under reducing conditions, the 55000- and 36000-M_r bands were converted into the 18000-M_r form, suggesting the presence of subunits with intra- and inter-chain disulfide bridges. The 18000-M_r subunits with intra-chain disulfide bridges accounted for about two-thirds of the total material. Analytical gel-filtration under nondenaturing conditions showed that RGD-mTNF was homogeneous and characterized by a hydrodynamic volume of about 45000–50000 M_r, which corresponds to trimers (Fig. 1*C*). These electrophoretic and chromatographic patterns suggest that RGD-mTNF was a mixture of trimers made up by subunits with intra-chain disulfides (at least 60%), with the remainder being mostly trimers with one or more inter-chain disulfides.

RGD-mTNF Promotes Cell Adhesion and Spreading. To assess whether the Arg-Gly-Asp (RGD) domain of RGD-mTNF is functional and accessible to integrins, we compared the cell pro-adhesive properties of RGD-mTNF and mTNF in a cell adhesion assay. This assay takes advantage of the fact that RGD is the minimal recognition sequence of many integrins and serves as an adhesion motif. To this aim, microtiter wells were coated with various amounts of RGD-mTNF or mTNF and seeded with EA.hy926 cells. Cell adhesion was then measured 1.5 h later. We observed adhesion and spreading of EA.hy926 cells on plates coated with RGD-mTNF but not with mTNF

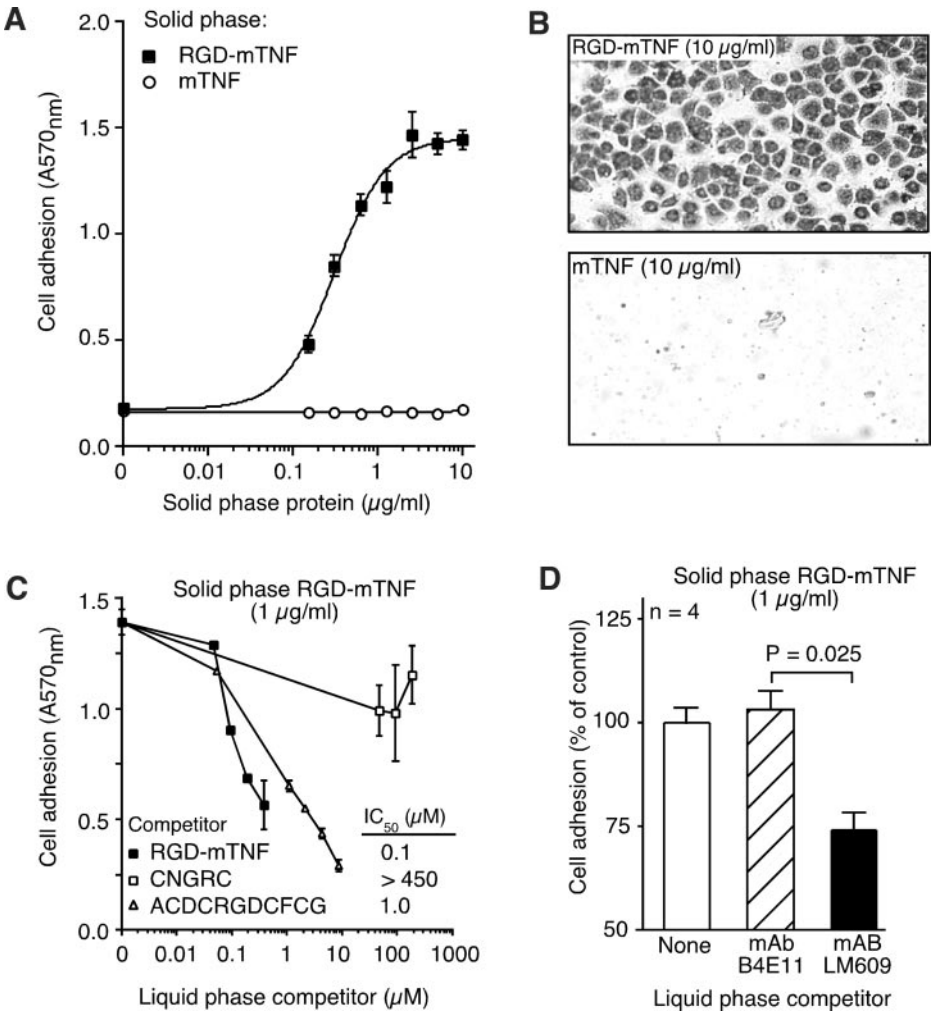

Fig. 2. Adhesion of EA.hy926 cells to solid phases coated with ACGDRGDCFCG-murine tumor necrosis factor-α conjugate (RGD-mTNF) or mTNF in the absence (*A*) and presence (*B*) of competitors in the liquid phase. Cell adhesion assay was performed as described in "Materials and Methods." Microphotograph of wells coated with 10 μg/ml RGD-mTNF or mTNF, ×200 (*B*). For the competitive cell adhesion assay, EA.hy926 cells were plated in the presence of various concentrations of RGD-mTNF, ACDCRGDCFCG, CNGRC (*C*), or 10 μg/ml anti-$\alpha_V\beta_3$ integrin monoclonal antibody (mAb) LM609 or mAb B4E11 (isotype-matched control mAb; *D*) on microplates coated with 1 μg/ml RGD-mTNF.

(Fig. 2, *A* and *B*). These results strongly suggest that the RGD domain of RGD-mTNF was properly folded and able to interact with adhesion receptors, very likely integrins, on cell membrane. Cell adhesion was completely abrogated when the cells were plated in DMEM containing 5 mM EDTA (data not shown), as expected for divalent ion-dependent integrin binding.

To compare the binding properties of the RGD domain of RGD-mTNF with those of free-ACDCRGDCFCG peptide, we performed cell adhesion inhibition experiments using solid-phase RGD-mTNF and liquid-phase competitors. Various competitors were used in this assay, as follows: RGD-mTNF, ACDCRGDCFCG peptide, and CNGRC peptide. Both RGD-mTNF and ACDCRGDCFCG peptide were able to compete cell adhesion, albeit to a different extent (Fig. 2*C*). These results indicate that both products, added to the liquid phase, can bind adhesion receptors. However, 10-fold more peptide was necessary to inhibit cell adhesion compared with RGD-mTNF, suggesting that multivalent interactions, potentially occurring with trimeric RGD-mTNF, increase the binding avidity. Interestingly, the CNGRC peptide inhibited little or no cell-adhesion to solid-phase RGD-mTNF. This suggests that the RGD- and NGR-targeting domains recognize different receptors on these cells.

To further analyze the binding specificity of RGD-mTNF, we performed cell adhesion inhibition experiments with an anti-$\alpha_V\beta_3$ integrin antibody. mAb LM609 (antihuman $\alpha_V\beta_3$) partially inhibited EA.hy926 cell adhesion to RGD-mTNF-coated plates (Fig. 2*D*), whereas the isotype-matched control mAb B4E11 (antihuman chromogranin A) did not affect cell adhesion. These results suggest that $\alpha_V\beta_3$ integrin is a receptor for RGD-mTNF.

Determination of RGD-mTNF-Binding Constants. The binding of radiolabeled ^{125}I-RGD-mTNF and ^{125}I-mTNF to EA.hy926 cells was then investigated. Scatchard plot analysis of binding data suggests that more than one receptor is involved in the binding (Fig. 3). The results of dissociation constant (Kd) and binding site measurements (Table 1) suggest that RGD-mTNF binds a small fraction of EA.hy926 receptors with a 10-fold higher affinity (Kd_1) than mTNF and a large fraction of receptors with an affinity similar to that of mTNF.

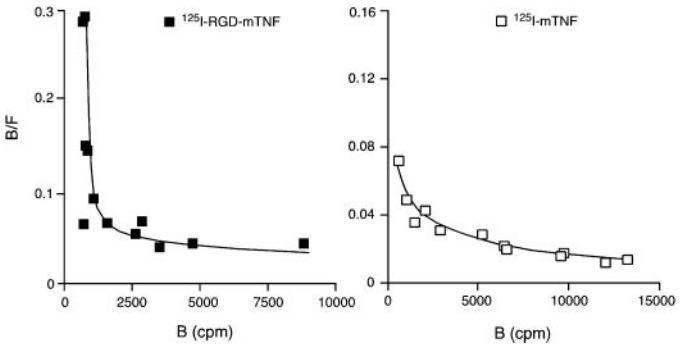

Fig. 3. Scatchard plot of ^{125}I-murine tumor necrosis factor-α conjugate (^{125}I-mTNF) or ^{125}I-ACGDRGDCFCG-murine tumor necrosis factor-α conjugate (^{125}I-RGD-mTNF) binding to EA.hy926 cells. The radio-binding assay was performed as described in "Materials and Methods." *B*, specific binding; *B/F*, specific binding divided by free radioligand concentration.

Table 1 *Dissociation constant (Kd) and number of binding sites/cell for ^{125}I-mTNF[a] and ^{125}I-RGD-mTNF on EA.hy926 cells*

Product	Kd_1 (nM)	Binding sites/cell with Kd_1	Kd_2 (nM)	Binding sites/cell with Kd_2
^{125}I-mTNF	0.43 ± 0.12	2026 ± 783	3.01 ± 2.00	4263 ± 2345
^{125}I-RGD-mTNF	0.03 ± 0.01	516 ± 9	1.72 ± 0.54	4376 ± 2115

[a] mTNF, mouse tumor necrosis factor; RGD-mTNF, ACGDRGDCFCG-mTNF conjugate.

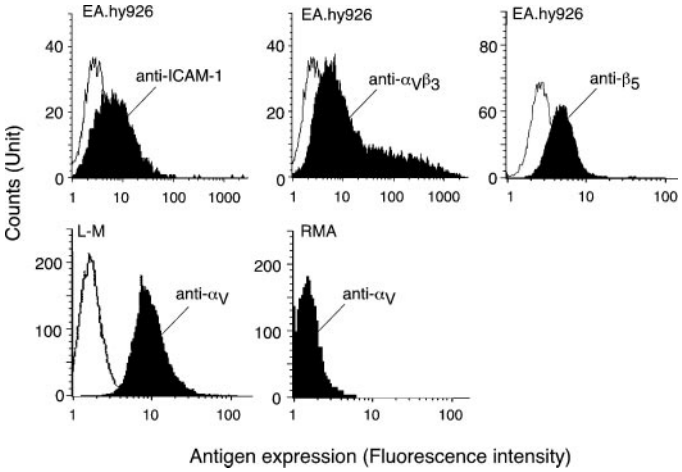

Fig. 4. Expression of integrins and intercellular adhesion molecule 1 (ICAM-1) on L-M, RMA, and EA.hy926 cells. Fluorescence-activated cell sorter (FACS) analysis of α_V subunits on L-M and RMA cells was carried out using biotinylated monoclonal antibody (mAb) H9.2B8 (antimurine α_V) and streptavidin-phycoerythrin. FACS analyses of β_5 integrin, $\alpha_V\beta_3$ integrin, and ICAM-1 on EA.hy926 cells were carried out using mAb IG8 (antihuman β_5), mAb LM609 (antihuman $\alpha_V\beta_3$), and mAb 15.2 (antihuman ICAM-1) respectively, followed by goat antimouse FITC. Controls cells (*white histograms*) were incubated with streptavidin-phycoerythrin or goat antimouse-FITC alone, respectively.

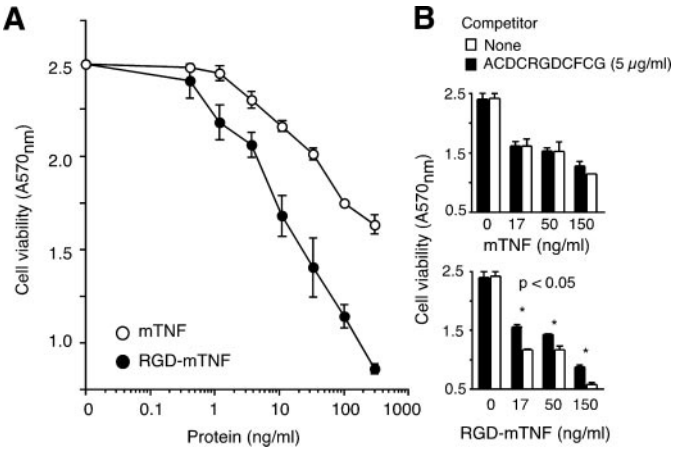

Fig. 5. Cytolytic activity of murine tumor necrosis factor-α (mTNF) and ACGDRGDCFCG-mTNF conjugate (RGD-mTNF) against L-M cells. L-M cells were detached with trypsin-EDTA, washed, and immediately incubated with mTNF or RGD-mTNF solutions at various concentrations, for 1.5 h on ice. After washing, the cells were resuspended in DMEM complete medium containing 2 μg/ml actinomycin D and plated (200 μl/well, 96-well plate). After 20 h at 37°C, 5% CO_2, cell viability was quantified by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (*A*). Effect of ACDCRGDCFCG peptide (5 μg/ml) on the cytotoxic activity of mTNF and RGD-mTNF (*B*).

Biological Activity of RGD-mTNF *in Vitro*. To characterize the *in vitro* biological activity of RGD-mTNF, we used murine L-M fibroblasts, murine RMA lymphoma cells, and human endothelial/epithelia EA.hy926 hybrid cells. Because ACDCRGDCFCG is a ligand of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins (26), the expression of integrin subunits on these cells was first characterized. Fluorescence-activated cell sorter analysis with specific antibodies showed that EA.hy926 cells express $\alpha_V\beta_3$ and β_5-subunits (Fig. 4). L-M cells, but not RMA cells, express α_V-subunits (Fig. 4).

The cytolytic activities of RGD-mTNF and mTNF against L-M cell and EA.hy926 cells, after incubation for 20 h with adherent cell, in presence of actinomycin D, were similar (8.9×10^7 units/mg and 7.6×10^7 units/mg, respectively). However, when L-M cell suspensions were incubated with RGD-mTNF or mTNF for 1.5 h, washed, and further incubated for 20 h in the presence of actinomycin D, their cytolytic effects were markedly different (Fig. 5*A*). These results

suggest that RGD-mTNF binds more efficiently than mTNF to these cells or persists for a longer time on their surface.

To assess whether the improved cytotoxic activity of RGD-mTNF depends on binding via the ACDCRGDCFCG-targeting domain, we performed a competition experiment using an excess of ACDCRGDCFCG peptide (5 μg/ml) during the 1.5-h preincubation. As expected, the ACDCRGDCFCG peptide significantly inhibited the activity of RGD-mTNF but not that of mTNF (Fig. 5*B*). These results suggest that this peptide completes the binding of RGD-mTNF to ACDCRGDCFCG receptors, likely α_V integrins, and the results also support the hypothesis that the improved activity of RGD-mTNF is related to targeting mechanisms depending on the ACDCRGDCFCG domain. Also noteworthy is that the addition of 5 mM EDTA to the binding buffer inhibited the cytolytic activity of RGD-mTNF to the level of mTNF (not shown). Given that divalent cations are critical for integrin structure and function, these results are in accordance with the hypothesis of a role of integrins as receptors for RGD-mTNF.

It is well known that TNF can induce the expression of ICAM-1 on endothelial cells (27). To provide additional evidence that RGD-mTNF is more active than mTNF, the effect of RGD-mTNF and mTNF on ICAM-1 expression by EA.hy926 cells was then investigated. To this aim, EA.hy926 cells were detached and immediately incubated with various doses of mTNF or RGD-mTNF for 1.5 h, washed, further incubated for 20 h, and then analyzed by fluorescence-activated cell sorter. The dose-response curves of mTNF and RGD-mTNF were different, the latter inducing more efficiently ICAM-1 expression at doses of 200 and 20 ng/ml (Fig. 6*A*). Also in this assay, the ACDCRGDCFCG peptide (40 μg/ml) partially inhibited the activity of RGD-mTNF but not that of mTNF (Fig. 6*B*), suggesting again that the improved activity of RGD-mTNF depends on the ACDCRGDCFCG domain.

***In Vivo* Antitumor Activity.** In previous work, we showed that picogram doses of CNGRC-mTNF (NGR-mTNF) administered to RMA tumor-bearing mice are sufficient to increase the antitumor activity of melphalan, whereas 10^4-10^5 greater doses of mTNF are necessary to achieve comparable results. In other studies, we showed that the mechanism of action of NGR-mTNF is based on vascular targeting and that a CD13 isoform expressed in tumor vessels is a critical receptor for the CNGRC domain of NGR-mTNF. To compare the activity of CNGRC and ACDCRGDCFCG peptides as TNF-targeting ligands, we investigated the antitumor activity of RGD-mTNF and NGR-mTNF in combination with melphalan, using the RMA mouse lymphoma model. A single administration of melphalan (50 μg) induced no effects (Fig. 7*A*). When melphalan was administered to mice in combination with 0.1 ng of NGR-mTNF, we observed a synergistic effect, as expected (Fig. 7*A*). RGD-mTNF (0.1 ng) alone or in combination with melphalan induced little or no effect. However, significant antitumor effects were observed when animals were treated with melphalan and 0.3 ng of RGD-mTNF. These results indicate that RGD-mTNF, although less active than NGR-mTNF on a molar basis, is capable of inducing antitumor effects in the picogram range. Because comparable doses of mTNF are inactive in this model (28), these results suggest that conjugation of mTNF to ACDCRGDCFCG improves its antitumor activity.

To estimate the efficacy/toxicity ratio of each treatment, we monitored the animal body weight, daily, after treatment. Melphalan induced a transient loss of body weight 1 day after treatment (Fig. 7*B*). However, the combination of melphalan with 0.3 ng of RGD-mTNF did not increase the loss of body weight, suggesting that low doses of this derivative can promote antitumor effects without causing major toxic reactions.

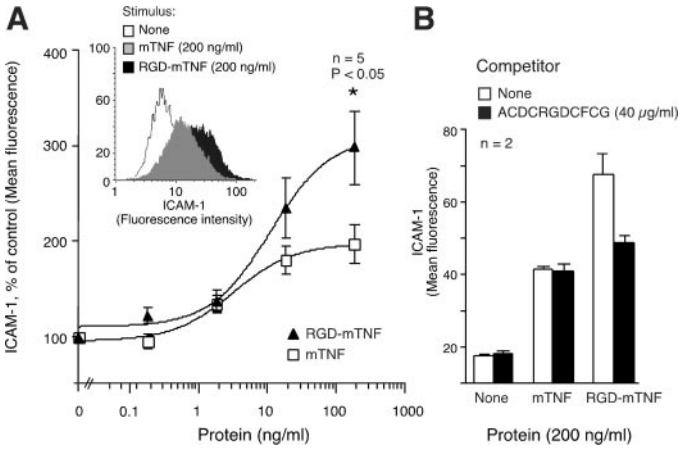

Fig. 6. Effect of murine tumor necrosis factor-α (mTNF) or ACGDRGDCFCG-mTNF conjugate (RGD-mTNF) on intercellular adhesion molecule 1 (ICAM-1) expression by EA.hy926 cells. EA.hy926 cells were detached with trypsin-EDTA, washed, and immediately incubated with mTNF or RGD-mTNF solutions at various concentrations, for 1.5 h on ice. After washing, the cells were resuspended in DMEM complete medium, incubated for 16 h at 37°C, 5% CO_2, and analyzed by fluorescence-activated cell sorter. Dose-response curves obtained with RGD-mTNF or mTNF (*A*). Representative fluorescence-activated cell sorter analysis on EA.hy926 cells incubated with 200 ng/ml of RGD-mTNF or mTNF (*inset A*). Effect of ACDCRGDCFCG peptide (40 μg/ml) on RGD-mTNF and mTNF-induced expression of ICAM-1 (*B*).

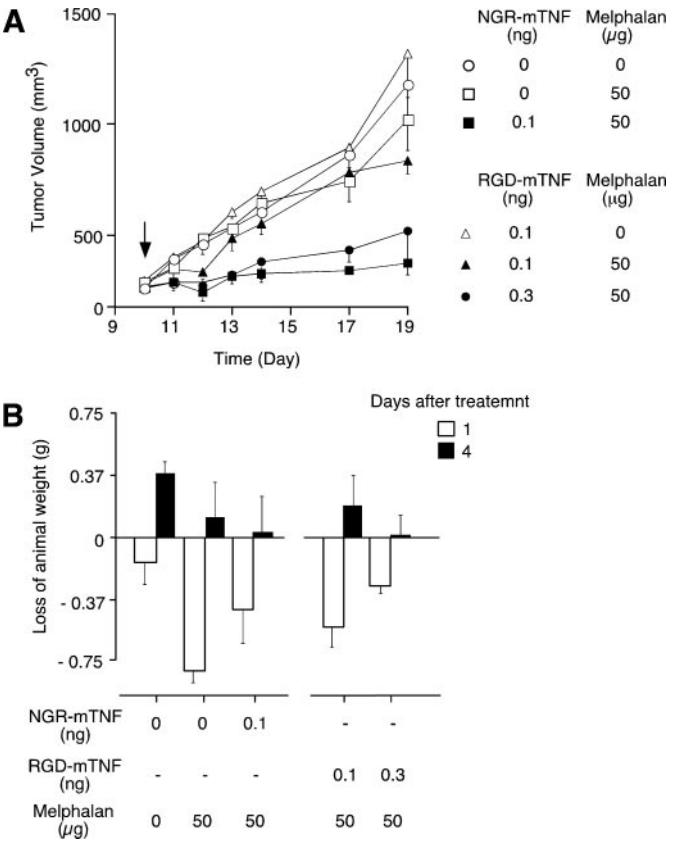

Fig. 7. Effect of ACGDRGDCFCG-murine tumor necrosis factor-α conjugate (RGD-mTNF) on tumor growth and body weight of animal-bearing RMA tumors. Animals (5 mice/group) were treated i.p. at day 10 with melphalan alone or in combination with RGD-mTNF or CNGRCG-mTNF conjugate (NGR-mTNF) at the indicated doses (*A*). Loss of animal weight at day 1 and day 4 after treatment (*B*).

DISCUSSION

We have shown previously that administration of >5–10 ng of TNF to tumor-bearing mice induces the release of soluble TNF receptors (28). Although these soluble inhibitors may protect the animals from

the harmful effects of this cytokine, they may also prevent its antitumor activity and explain, in part, the need for high doses (micrograms) for effective therapy. On the other hand, it is also known that systemic administration of low doses (subnanogram) of TNF does not elicit soluble receptor shedding nor induce significant antitumor effects (28). The main finding of this work is that subnanogram doses of TNF fused with ACDCRGDCFCG (RGD-mTNF), a tumor homing ligand, are sufficient to induce significant antitumor responses in an animal model. These results support the concept that targeted delivery of low doses of TNF to tumors could be a strategy for circumventing the problem of negative feedback mechanisms.

It has been shown recently that the CDCRGDCFC peptide binds $\alpha_V\beta_3$ integrin expressed by both tumor cell and tumor endothelial cells (17). Because RMA tumor cells used in our *in vivo* experimental model do not express α_V integrins, the improved activity of RGD-mTNF is likely related to endothelial cell targeting. We have also shown previously that NGR-mTNF, another conjugate prepared by fusing TNF with CNGRC peptide, can induce significant antitumor effects when administered at very low doses to tumor-bearing mice. Given that this peptide is known to target different receptors, *e.g.*, CD13, in angiogenic vessels (16), the results obtained with both RGD-mTNF and NGR-mTNF support the concept that vascular targeting is a strategy potentially applicable to a broader spectrum of endothelial markers, not limited to CD13.

The results of *in vitro* experiments suggest that the RGD moiety of RGD-mTNF improves the amount and/or the persistence of this cytokine on targeted cells. Interestingly, cell adhesion assays and competitive binding experiments with anti-integrin antibodies showed that the RGD moiety interacts with cell adhesion receptors, including $\alpha_V\beta_3$ integrin, as originally postulated. On the other hand, the strong cytolytic activity of RGD-mTNF on L-M cells implies that RGD-mTNF can also bind TNF receptors and trigger death signals. The finding that the *in vitro* biological activity of RGD-mTNF is decreased by coincubation with an excess of free RGD peptide suggests that the improved activity of RGD-mTNF is related to targeting. Also of note is that the antitumor activity of NGR-mTNF can be inhibited by an excess of free-NGR peptide or by an anti-CD13 antibody (15). These findings suggest that the improved properties of RGD-mTNF or NGR-mTNF depend on targeted delivery of TNF to RGD or NGR receptors and not to unspecific mechanisms related to NH_2-terminal extension.

TNF binds p55- and p75-TNF receptors with Kds of 0.3–0.6 pM and 0.07–0.2 pM, respectively (29–34). However, depending on assay conditions and cell species, other values have been reported in the literature (35–38). For instance, mouse L-929 and L-M cells express on their surface 5000 receptors/cell with Kd of 3–5 nM (35). Accordingly, we found that mTNF binds EA.hy926 cells with $Kd_1 = 0.43$ nM and $Kd_2 = 3$ nM. Scatchard analysis of RGD-mTNF binding to EA.hy926 cells ($\alpha_V\beta_3$ positive) showed the presence of a few high-affinity binding sites (Kd = 0.03 nM), potentially related to high-avidity multimeric complex interactions. Possibly, these sites are related to simultaneous double interaction of RGD-mTNF with integrins and mTNF receptors, leading to formation of high-avidity trimolecular complexes. However, a high proportion of binding sites able to bind RGD-mTNF with an affinity comparable with that of mTNF was also observed. Thus, a clear conclusion on the mechanism of interaction occurring among RGD-mTNF, integrins, and TNF receptors is difficult to draw from these data. The time of exposure of these cells to RGD-mTNF or mTNF also seems to be very important for activity. For instance, the *in vitro* cytotoxic activity of RGD-mTNF was stronger than that of mTNF when the cells were treated for 1.5 h, washed, and further incubated for 20 h, whereas their activities were similar in a standard 20 h-incubation assay. One possibility is that the targeting mechanism increases the binding and/or the persistence of TNF on endothelial cells of tumor vessels. The results obtained with the 1.5 h-incubation assay, followed by washing, are likely to simulate in a better manner the *in vivo* conditions, given that TNF is rapidly cleared from circulation after injection (39).

The addition of TNF to regional isolated limb perfusion with melphalan has produced response rates in patients with extremity soft-tissue sarcomas or melanomas higher than those obtained with chemotherapeutic drugs alone (3–5, 40, 41). TNF-induced alteration of endothelial barrier function, reduction of tumor interstitial pressure, and increased chemotherapeutic drug penetration are believed to be important mechanisms of the synergy between TNF and chemotherapy (4, 40, 42–45). We proposed previously that targeted delivery of minute amounts of NGR-mTNF to tumor vessels can also increase the penetration of chemotherapeutic drugs in tumors in animal models. It is therefore possible that the synergistic activity observed with low doses of RGD-mTNF and melphalan is also related to alteration of the vessel wall barrier function and, consequently, to increased penetration of the chemotherapeutic drug in tumors. However, other mechanisms are possible. For instance killing of both endothelial and cancer cells cannot be excluded.

Molecules containing the ACDCRGDCFCG motif are expected to target murine as well as human angiogenic vessels (13, 46). Thus, RGD-mTNF might be expected to have better antitumor properties than TNF in patients. Also noteworthy is that when we compared the activity of RGD-mTNF with that of NGR-mTNF in the RMA lymphoma model, we observed that >3 times higher doses of RGD-mTNF were necessary to achieve comparable responses. Thus, apparently, the antitumor activity of NGR-mTNF is superior to that of RGD-mTNF. However, because it is difficult to predict the level of expression of functional CD13 and α_V integrin in patients, RGD-mTNF could be a valid alternative to NGR-mTNF that deserves to be investigated further. Moreover, because it is possible that the expression of CD13 and α_V integrin does not entirely overlap in tumor vasculature, the combination of NGR-mTNF and RGD-mTNF could target different vessels and induce stronger antitumor effects than the single agents.

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Coupling Tumor Necrosis Factor-α with α_V Integrin Ligands Improves Its Antineoplastic Activity

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