Coupling Tumor Necrosis Factor-α with αν 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 antitumor drug is limited by severe toxicity. We have shown previously that targeted delivery of TNF to angiogenic vessels (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 αν 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 αβ3 integrin, as originally postulated. In addition, ACGRDGRDCFCG-mouse TNF conjugate induced cytotoxic effects in standard cytolytic assays, implying that ACGRDGRDCFCG-mouse TNF conjugate can also bind TNF receptors and trigger death signals. These results indicate that coupling TNF with αν 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 ACDCRGDCFCG 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) isofrom (16) and to αν 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 αν 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 NH2 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) 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 detachment with trypsin-EDETA. L-M cells (murine fibroblasts) were cultured as described previously (22). The following antibodies were used: monoclonal antibody (mAb) LM609 (antihuman αvβ3; Chemicon, Temecula, CA), mAb 15.2 [antihuman intercellular adhesion molecule 1 (ICAM-1); Boehringer Mannheim GmbH, Germany], mAb B4E11 (antihuman chomatogranin A; Ref. 23), H9.288 (bionylated antimonise αv integrin subunit; Pharmingen, San Diego, CA), and goat antimonise-FITC secondary antibody (Sigma). Actinomycin D, crystal violet, and oxidized 1-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 streptavidin-phycocerythrin 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 × 21.2 mm; Phenomenex, Torrance, CA). Free sulhydryl 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 ECL kit (Roche). The molecular masses of CNGRC and ACDCRGDCFCG were 550.23 ± 1 Da and 1145.58 ± 1 Da, respectively (expected for disulfide-brided 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: TGCAGATCATATGGCTTGCGACGTCCGGTTGCTTGCTTCGGCTTCGATCTATCCTTC (5′ primer); TCAGGATCTTACAGGCAATTGATCCAAATGAC (3′ primer). Primer sequences were designed to include the Ndel and BamHI restriction sites (underlined) for cloning in pET11 plasmid (Novagen, Madison, WI). The Ndel site contains also the translation start codon. A glycine residue was inserted between ACDCRGDCFCG 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 C-terminal glutathione was added to the product of ion exchange (2 μM 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 quantitative chromogenic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**ICAM-1 and Integrin Flow Cytometry Analysis.** Detection of ICAM-1 and α5β1 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 α5β1 integrin), and incubated for 1 h on ice. After washing with PBS, the cells were incubated with goat anti-mouse-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 α5 integrin subunits on murine L-M cells was quantified using 3 μg/ml biotinylated mAb H9.28B8 (rat anti-mouse α5 integrin) and streptavidin-phycocerythrin, 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 × 104 cells/100 μl well). After incubation (1–1.5 h) at 37°C, 5% CO2, 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.** 125I-mTNF (13.91 μCi/μg) and 125I-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 (106 cell/tube) was carried out in the presence or absence of 10 μCi/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 by 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 × 10^5 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 melphanal (100 µl; Glaxo Wellcome Operations, Dartford, United Kingdom). All drugs, diluted with 0.9% sodium chloride containing 0.1% 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 170,000–180,000 Mr, as expected for mTNF monomers (Fig. 1B). In contrast, nonreducing SDS-PAGE showed three bands of 180,000, 360,000, and 550,000 Mr. These bands were immunoreactive with antimTNF antibodies by Western blot analysis (data not shown) suggesting that they correspond to RGD-mTNF monomers, dimers, and trimers. Under reducing conditions, the 550,000- and 360,000-Mr bands were converted into the 180,000-Mr form, suggesting the presence of subunits with intra- and inter-chain disulfide bridges. The 180,000-Mr 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 45,000–50,000 Mr, which corresponds to trimers (Fig. 1C). 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, ×200 (B). For the competitive cell adhesion assay, EA.hy926 cells were plated in the presence of various concentrations of RGD-mTNF, ACDCRGDCFGC, CNGRC (C), or 10 µg/ml anti-αvβ3 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-ACDGRGDCFCG 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. 2C). 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-α3β1 integrin antibody. mAb LM609 (antihuman α3β1) partially inhibited EA.hy926 cell adhesion to RGD-mTNF-coated plates (Fig. 2D), whereas the isotype-matched control mAb B4E11 (antihuman chromogranin A) did not affect cell adhesion. These results suggest that α3β1 integrin is a receptor for RGD-mTNF.

**Determination of RGD-mTNF-Binding Constants.** The binding of radiolabeled 125I-RGD-mTNF and 125I-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 (Kd1) than mTNF and a large fraction of receptors with an affinity similar to that of mTNF.

**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/epithelial EA.hy926 hybrid cells. Because ACDCRGDCFCG is a ligand of αvβ3 and αvβ5 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 αvβ5 and β3-subunits (Fig. 4). L-M cells, but not RMA cells, express αv-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 × 107 units/mg and 7.6 × 107 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. 5A). These results

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

<table>
<thead>
<tr>
<th>Product</th>
<th>Kd (nM)</th>
<th>Binding sites/cell with Kd</th>
<th>Binding sites/cell with Kd</th>
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<tbody>
<tr>
<td>125I-mTNF</td>
<td>0.43 ± 0.12</td>
<td>2026 ± 783</td>
<td>3.01 ± 2.00</td>
</tr>
<tr>
<td>125I-RGD-mTNF</td>
<td>0.03 ± 0.01</td>
<td>516 ± 9</td>
<td>1.72 ± 0.54</td>
</tr>
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mTNF, mouse tumor necrosis factor; RGD-mTNF, ACDCRGDCFCG-mTNF conjugate.
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. 5B). These results suggest that this peptide completely blocks the binding of RGD-mTNF to ACDCRGDCFCG receptors, likely αv 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. 6A). Also in this assay, the ACDCRGDCFCG peptide (40 µg/ml) partially inhibited the activity of RGD-mTNF but not that of mTNF (Fig. 6B), 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 10^5-10^6 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 isofrom 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. 7A). When melphalan was administered to mice in combination with 0.1 µg of NGR-mTNF, we observed a synergistic effect, as expected (Fig. 7A). 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. 7B). 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.

**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 toxic reactions, we have shown that TNF does not elicit a detectable biological response in vivo. The dose-dependent antitumor effects observed in our study are consistent with the dose-response curves obtained with RGD-mTNF or mTNF. The effect of RGD-mTNF on ICAM-1 expression at doses of 200 and 20 ng/ml (Fig. 6A). Also in this assay, the ACDCRGDCFCG peptide (40 µg/ml) partially inhibited the activity of RGD-mTNF but not that of mTNF (Fig. 6B), suggesting again that the improved activity of RGD-mTNF depends 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.

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the harmful effects of this cytokine, they may also prevent its antitu-
mor 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 ACDCRGDCCFG (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 CD13, in angiogenic vessels (16), the results obtained with both
RGD-mTNF and NGR-mTNF support the concept that vascular tar-
geting 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
αvβ3 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 NH2-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). Accord-
ingly, we found that mTNF binds EA.hy926 cells with Kd1 = 0.43 nm
and Kd2 = 3 nm. Scatchard analysis of RGD-mTNF binding to
EA.hy926 cells (αvβ3 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 inte-
grins and mTNF receptors, leading to formation of high-avidity tri-
molecular 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 persist-
ence of TNF on endothelial cells of tumor vessels. The results ob-
tained 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 chemother-
apy (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 penetra-
tion of the chemotherapeutic drug in tumors. However, other mech-
anisms are possible. For instance killing of both endothelial and
cancer cells cannot be excluded.

Molecules containing the ACDCRGDCCFG 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 lym-
phoma model, we observed that >3 times higher doses of RGD-
mTNF were necessary to achieve comparable responses. Thus, appar-
ently, 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 αv 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 expres-
sion of CD13 and αv 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 αv Integrin Ligands Improves Its Antineoplastic Activity

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