

Enhancement of antitumor properties of TRAIL by targeted delivery to the tumor neovasculature

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent with tumor-selective apoptotic activity. TRAIL plays a role in the innate and adaptive immune response and autoimmune disease and may also be involved in hepatic cell death and inflammation. For these reasons, chronic exposure to TRAIL may have deleterious side effects in patients as a cancer therapeutic. In this study, we have improved the antitumor activity of TRAIL by targeted delivery to the tumor vasculature, leading to dramatic enhancement of its therapeutic properties. TRAIL was fused to the ACDCRGDCFC peptide (named RGD-L-TRAIL), a ligand of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Biological activity was evaluated *in vitro* and antitumor efficacy was investigated *in vivo* as a single agent and in combination with irinotecan hydrochloride (CPT-11). The fusion protein RGD-L-TRAIL, but not TRAIL or RGE-L-TRAIL, specifically bound to microvascular endothelial cells in a dose-dependent manner and showed enhanced apoptosis-inducing activity (caspase-3 and caspase-8 activation) in $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-positive cancer cells. In addition, RGD-L-TRAIL was more effective in suppressing tumor growth of COLO-205 tumor-bearing mice than an equivalent dose of TRAIL. The antitumor effect of RGD-L-TRAIL was further enhanced by combination with CPT-11 in both TRAIL-sensitive COLO-205 and TRAIL-resistant HT-29 tumor xenograft models. Our findings suggest that the novel fusion protein RGD-L-TRAIL can directly target tumor endothelial cells as well as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-positive tumor cells. The tumor-targeted delivery

of TRAIL derivatives, such as RGD-L-TRAIL, may prove to be a promising lead candidate for cancer therapy. [Mol Cancer Ther 2008;7(4):851–61]

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is an apoptosis-inducing member of the TNF gene family (1). Similar to other TNF family members, soluble TRAIL forms a homotrimer, which cross-links three receptor molecules on the surface of target cells. To date, five human TRAIL receptors have been identified: DR4/TRAIL-R1, DR5/TRAIL-R2/TRICK2, TRID/DcR1/TRAIL-R3, TRAIL-R4/DcR2, and osteoprotegerin (2–5). TRAIL triggers apoptotic signals by interaction with DR4 and DR5 via COOH-terminal death domains. DcR1 and DcR2, two decoy receptors that fail to signal cell death, were initially believed to compete with death receptors for ligand binding and protect normal tissues and TRAIL-resistant tumors from apoptosis. More recently, several *in vitro* studies suggest that TRAIL-induced cell death may be regulated by other intracellular factors (6, 7).

Although systemic toxicity has limited the therapeutic use of many members of the TNF superfamily, TRAIL appears to be a relatively safe and promising agent for cancer-specific therapy. TRAIL induces apoptosis in a wide range of cell lines and shows substantial antitumor activity in rodent xenograft models, such as colon (8), breast (8), multiple myeloma (9, 10), glioma (11), and prostate cancers (12). Systemic administration of TRAIL exhibited little or no toxicity in mice or nonhuman primates (13). Given the effectiveness of rhTRAIL in preclinical oncology studies, it is currently in clinical trials.

The success of rhTRAIL as a cancer therapy will depend on a better understanding of TRAIL biology. First, the physiologic role in innate and adaptive immune responses is not fully understood (14–17). Recent studies have shown that TRAIL regulates negative selection or apoptosis during thymocytic development and is important in the induction of autoimmune diseases, such as autoimmune type 1 diabetes (18, 19). Additionally, TRAIL has been recently shown to play a crucial role in hepatic cell death and hepatic inflammation (20). Therefore, repeated administration of high doses of rhTRAIL may lead to unforeseen immunologic effects or hepatic dysfunction. Second, TRAIL receptors are expressed in many normal tissue types besides tumors. The potential for adverse side effects needs to be carefully studied given that the mechanism of tumor selectivity by rhTRAIL is not fully understood. Third, to observe antitumor effects in mouse tumor models, a very high dose of rhTRAIL is required; this is probably due to its fast clearance. Pharmacokinetic studies have shown the half-life of rhTRAIL is only 23 to 31 min in nonhuman primates (21).

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TRAIL-FITC, or BSA-FITC (control) in 100 μ L PBS-FCS solution for 1 h at 4°C. Cells were washed and analyzed by flow cytometry using appropriate filter sets (Becton Dickinson).

Integrin Expression Analysis

The expression of cell surface adhesion molecules was determined by antibody staining and flow cytometry. Briefly, cells were harvested and washed in PBS plus 2% FCS. Cells were then incubated with primary monoclonal antibodies: anti-human $\alpha_v\beta_3$ integrin (MAB 23C6; eBioscience) or anti-human $\alpha_v\beta_5$ integrin (MAB 1961; Chemicon International) in PBS with 2% FCS for 1 h at 4°C. A purified mouse immunoglobulin G isotype (eBioscience) was used as a negative control. The cells were then washed twice and labeled with goat anti-mouse immunoglobulin G1 (γ)-FITC (Caltag Laboratories) as secondary antibody at 4°C for 30 min in the dark. After three additional washes, cells were fixed with 4% formaldehyde in PBS and analyzed by flow cytometry using appropriate filter sets. The cell staining experiments were repeated at least three times with each cell line.

Apoptosis Assessment by Annexin V-FITC and Propidium Iodide Staining

Cells were treated with recombinant TRAIL and its derivative proteins and labeled with FITC-conjugated Annexin V and propidium iodide (PI) using the Annexin V-FITC apoptosis detection kit (BD PharMingen) according to the manufacturer's instructions.

Caspase-3 and Caspase-8 Activity Assays

The activity of caspase-3 and caspase-8 was determined by caspase activity fluorometric kits (Oncogene) using the supplied protocol. Fluorescence recordings were obtained with Safire Fluorescence Absorbance and Luminescence Reader (TECAN) adjusted to 400 nm excitation and 505 nm emission.

In vivo Antitumor Efficacy Assay

Female BALB/c *nu/nu* mice (5-6 weeks old) were obtained from Shanghai Laboratory Animal Center. Mice were pretreated for 24 h before tumor challenge with a single injection (100 μ L i.p.) of purified asialo GM-1 antibody (Wako Chemicals). Next, mice were challenged with 1×10^6 COLO-205 or HT-29 cells by s.c. injection. Treatment was initiated when the tumors reached a mean volume of 70 mm³. Treatment with RGD-L-TRAIL or native TRAIL was administered by i.p. injection. CPT-11 (Pharmacia and Upjohn) was administered i.v. All dilutions of RGD-L-TRAIL, native TRAIL, and CPT-11 were made in PBS. Tumor volumes were measured with calipers and determined as mm³ using the equation: $A \times B^2 / 2$, where A is the length (mm) and B is the width (mm). The suppression rate of tumor growth expressed as $(1 - T / C) \times 100\%$, where T represents the treated volume and C is the control tumor volume.

Biodistribution Assay of RGD-L-TRAIL in Tumor Tissue

Purified recombinant RGD-L-TRAIL and TRAIL were labeled with ¹²⁵I by using Iodo-GenR precoated tubes (Pierce) according to the manufacturer's instruction. The

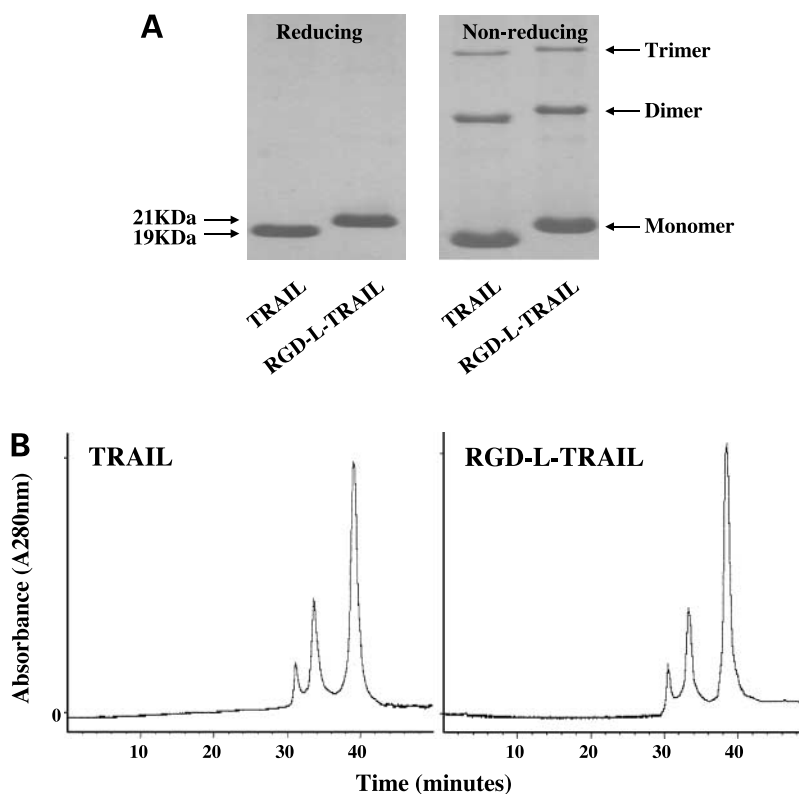


Figure 1. SDS-PAGE (**A**) and gel filtration chromatography (**B**) analysis of recombinant RGD-L-TRAIL and TRAIL. Purified RGD-L-TRAIL and TRAIL proteins were analyzed by 13.5% SDS-PAGE under nonreducing (-DTT) and reducing (+DTT) conditions. Protein (5 μ g each) was loaded and stained with Coomassie blue. Gel filtration chromatography analysis of RGD-L-TRAIL and TRAIL were done on TSK gel-3000 column. Both proteins showed three forms, which consist of monomer, dimer, and trimer.

specific activities of the ^{125}I -TRAIL and ^{125}I -RGD-L-TRAIL were 7.86 and 7.49 $\mu\text{Ci}/\mu\text{g}$, respectively. COLO-205 tumor-bearing mice were tail vein injected with radio-iodinated recombinant proteins (5 μCi) in 100 μL PBS. Three animals were used for each time point. Mice were analyzed at 5, 30, 60, 120, and 240 min postinjection. The different organs, including tumor and plasma, were taken and weighed and the radioactivity was counted to determine the %ID/g. All the results are given as the average of three independent %ID/g (SD) measurements.

In vivo Tumor Binding Comparison for TRAIL and RGD-L-TRAIL

RGD-L-TRAIL, TRAIL, and BSA were labeled with FITC as discussed above. Protein (~ 500 μg) was injected in the tail vein of COLO-205 tumor-bearing mice in 200 μL PBS. The tumor tissues were surgically separated from mice 30 min postinjection. Tumor cells ($\sim 6 \times 10^5$) were washed and analyzed by flow cytometry as described above.

Statistical Analysis

Statistical comparisons were done using Statistical Package for Social Sciences. All experiments were repeated at least three times. Data of apoptosis and cell adhesion are expressed as mean \pm SD and data of tumor sizes are expressed as the mean \pm SE. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

Results

Expression, Purification, and Characterization of TRAIL and Derivatives

The RGD-L-TRAIL and TRAIL recombinant proteins were expressed in soluble form and yielded ~ 60 mg/L for each protein. RGD-L-TRAIL and TRAIL were then analyzed by reducing SDS-PAGE and appeared at the expected molecular weight of $\sim 21,000$ and 19,000 Da, respectively (Fig. 1A). The protein spots were successfully identified to be TRAIL by the combined mass spectrometry and tandem mass spectrometry analysis (data not shown). The two mutants, RGE-L-TRAIL and RGD-L-TRAIL (C230G), were similarly prepared (data not shown).

TNF family ligands, like TRAIL, can exist in monomer, dimer, and trimer forms (32). We compared polymerization of RGD-L-TRAIL and TRAIL proteins by nonreducing SDS-PAGE and gel filtration. Under nonreducing condition, both RGD-L-TRAIL and TRAIL proteins showed bands with apparent molecular weight of 20,000, 40,000, and 60,000 Da, consistent with monomer, dimer, and trimer forms, respectively (Fig. 1A). The protein bands of reducing and nonreducing SDS-PAGE were identified as TRAIL protein by both mass spectrometry and tandem mass spectrometry analysis (data not shown). Gel filtration analysis under nondenaturing condition showed RGD-L-TRAIL

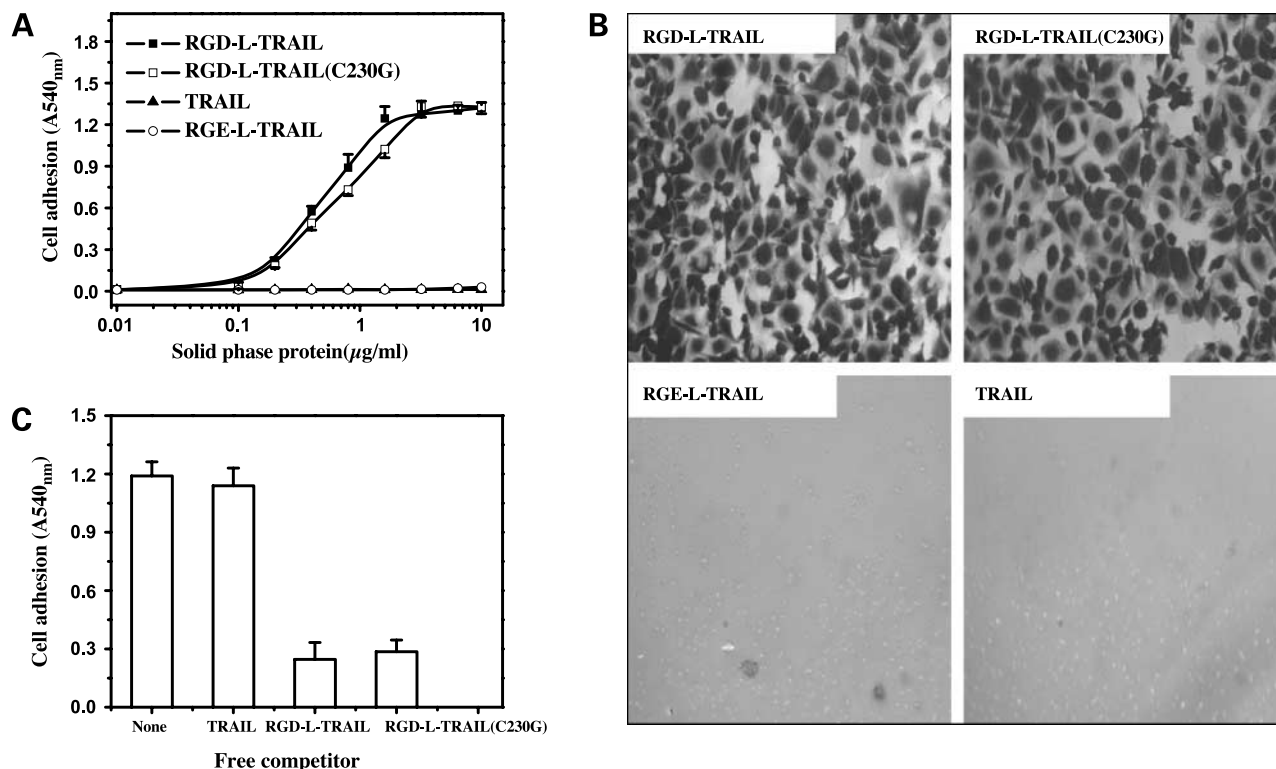


Figure 2. Effect of RGD-L-TRAIL on HDMVEC adhesion. **A**, various concentrations of RGD-L-TRAIL, TRAIL, RGD-L-TRAIL (C230G), and RGE-L-TRAIL protein were precoated on polyvinylchloride microtiter plates as solid phases. HDMVEC were then added to protein coated wells for 1.5 h at 37°C and the bound cells were quantified using crystal violet. Points, mean done in triplicate; bars, SD. **B**, adherent cells in wells coated with 6.4 $\mu\text{g}/\text{mL}$ GD-L-TRAIL, TRAIL, RGD-L-TRAIL (C230G), or RGE-L-TRAIL were visualized under the microscope ($\times 200$). **C**, HDMVEC adhesion to plates precoated with 10 $\mu\text{g}/\text{mL}$ RGD-L-TRAIL in the presence of 100 $\mu\text{g}/\text{mL}$ free TRAIL, RGD-L-TRAIL, or RGD-L-TRAIL (C230G) competitor.

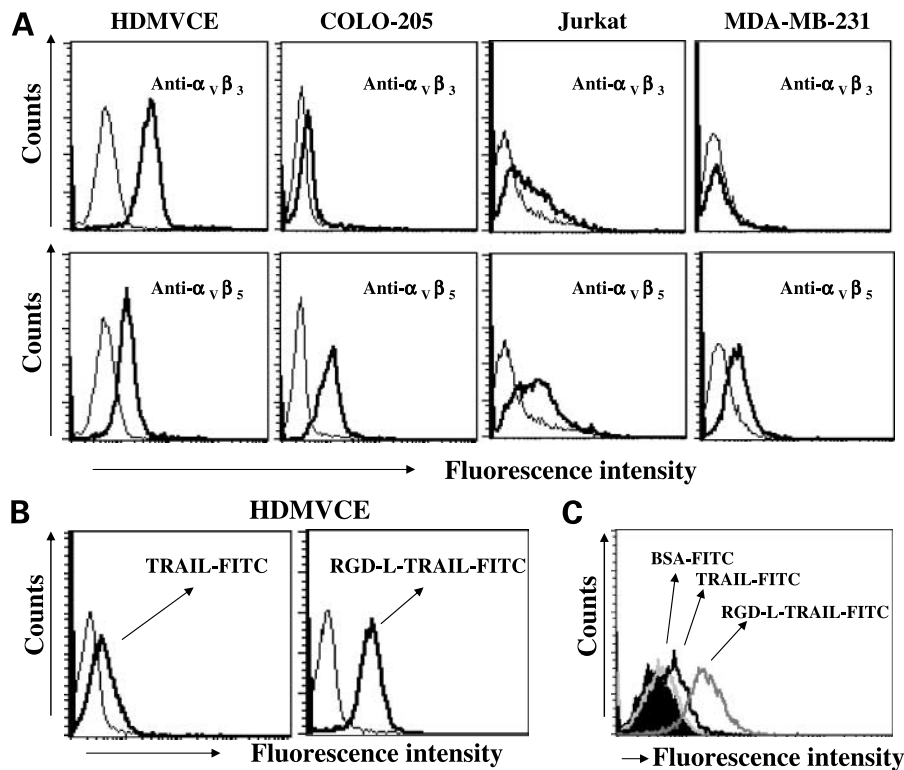


Figure 3. FACS analyses of integrin expression and RGD-L-TRAIL binding on endothelial cells and tumors. **A**, expression of integrins was analyzed using FACS in HDMVEC, COLO-205, Jurkat, and MDA-MB-231 cells. Cell suspensions (5×10^5 cells in 50 μ L PBS/FCS) were incubated for 1 h on ice with 500 ng monoclonal antibody 23C6 (anti-human $\alpha_v\beta_3$ integrin) or monoclonal antibody 1961 (anti-human $\alpha_v\beta_5$ integrin), washed twice with PBS/FCS, and incubated for an additional 30 min with goat anti-mouse IgG-FITC (diluted 1:100 in PBS/FCS; *bold dark-lined histogram*). The cells were then washed, fixed with 4% formaldehyde in PBS, and analyzed by FACS analysis. Controls cells (*shallow dark-lined histogram*) were incubated with a negative immunoglobulin G isotype primary mouse antibody and then stained with goat anti-mouse IgG-FITC. **B**, flow cytometry showing binding of FITC-labeled RGD-L-TRAIL to HDMVEC *in vitro*. HDMVEC were stained with TRAIL-FITC or RGD-L-TRAIL-FITC (*dark-lined histogram*) and then analyzed by FACS. Controls cells (*thin-lined histogram*) were incubated with BSA-FITC. **C**, RGD-L-TRAIL binding to COLO-205 tumors in BALB/c *nu/nu* mice. RGD-L-TRAIL-FITC (*dark gray-lined, open histogram*), TRAIL-FITC (*dark-lined, open histogram*), and control protein BSA-FITC (*shallow gray-lined, open histogram*) were injected into the tail vein of COLO-205 tumor-bearing mice. Thirty minutes postinjection, tumor tissues were surgically separated and tumor cells were analyzed by flow cytometry. Control cells (*black-filled histogram*) were taken from experiments without FITC-labeled protein injection.

and TRAIL proteins have nearly identical composition of monomer, dimer, and trimer forms (Fig. 1B). This excludes the possibility that the differences in antitumor activity between RGD-L-TRAIL and TRAIL are due to different amounts of polymer forms.

RGD-L-TRAIL Enhanced HDMVEC Adhesion

ACDCRGDCFC has been reported to be a ligand of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (32). The ACDCRGDCFC fusion protein was shown to bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-positive cells and promote adhesion. Therefore, we used primary HDMVEC to test whether the ACDCRGDCFC motif of RGD-L-TRAIL showed a similar phenotype. Microtiter plates with serial dilution of preimmobilized RGD-L-TRAIL, RGD-L-TRAIL (C230G), RGE-L-TRAIL, and TRAIL protein were seeded with HDMVEC and adhesion was measured 1.5 h later. As expected, HDMVEC adhered to plates immobilized with RGD-L-TRAIL and RGD-L-TRAIL (C230G) protein in a dose-dependent manner but not with TRAIL or RGE-L-TRAIL (Fig. 2A and B). These data suggest that ACDCRGDCFC motif is functional and able to trigger integrin signaling and promote endothelial cell adhesion.

To determine if the ACDCRGDCFC motif of RGD-L-TRAIL could specifically facilitate HDMVEC adhesion, we did competition experiments with free RGD-L-TRAIL, RGD-L-TRAIL (C230G), and TRAIL. Preincubation of HDMVEC with unbound RGD-L-TRAIL or RGD-L-TRAIL (C230G; 100 μ g/mL) protein greatly reduced HDMVEC adhesion to RGD-L-TRAIL-immobilized plates (Fig. 2C), whereas TRAIL itself did not affect cell adhesion. These results indicate that the ACDCRGDCFC domain of RGD-L-TRAIL is functional and interacts with integrins.

ACDCRGDCFC Motif Increased RGD-L-TRAIL Binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrin-Positive Cells

Considering that ACDCRGDCFC is a ligand for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, we analyzed expression of integrin subunits on human endothelial cells and tumor cell lines. Fluorescence-activated cell sorting (FACS) analysis showed that HDMVEC expressed the highest level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Fig. 3A) when compared with other cell lines. Moreover, our results confirmed that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are also expressed, albeit to a lesser degree, on several tumor cell lines. Jurkat cells expressed medium

level of $\alpha_V\beta_3$ and $\alpha_V\beta_5$, whereas COLO-205 cells expressed low level of $\alpha_V\beta_3$ but relatively high level of $\alpha_V\beta_5$ (Fig. 3A). MDA-231 cells did not express detectable level of $\alpha_V\beta_3$ and relatively low level of $\alpha_V\beta_5$ (Fig. 3A).

We further evaluated the direct binding ability of RGD-L-TRAIL and TRAIL to HDMVEC by using FITC-labeled proteins. FACS analysis showed that RGD-L-TRAIL bound more than TRAIL to HDMVEC (Fig. 3B). These studies support the idea that ACDCRGDCFC endowed TRAIL has enhanced binding to endothelial cells.

We also evaluated the binding capability of RGD-L-TRAIL and TRAIL to tumor cells *in vivo*. FITC-labeled RGD-L-TRAIL, TRAIL, or BSA was injected into COLO-205 tumor-bearing mice. After 30 min, tumor tissues were surgically removed and tumor cells were analyzed by flow cytometry as above. The results show that RGD-L-TRAIL exhibits higher binding ability than native TRAIL (Fig. 3C). The negative control BSA protein did not bind to tumor cells. These results further show the ability of ACDCRGDCFC endowed TRAIL to preferentially bind tumor cells and enhance its tumor-targeting ability over native TRAIL protein.

Biological Activity of RGD-L-TRAIL *In vitro*

To investigate the *in vitro* biological activity of RGD-L-TRAIL, we used COLO-205, Jurkat, and MDA-MB-231 cancer cell lines. Cells were treated with serial dilutions of RGD-L-TRAIL and TRAIL stained with Annexin V-FITC and PI followed by flow cytometry. The results are shown in Fig. 4A. The COLO-205 cells exhibited a 10-fold shift in the concentration required to induce 50% apoptosis with RGD-L-TRAIL compared with native TRAIL and Jurkat cells; only a marginal effect was seen in MDA-MB-231 cells. The EC_{50} (concentration at which 50% cell appeared apoptotic) for native TRAIL on COLO-205, Jurkat, and MDA-MB-231 cells was 3.5, 6.7, and 2.3 ng/mL, respectively. The corresponding EC_{50} improved to 0.37, 0.41, and 1.2 ng/mL when cells were treated with RGD-L-TRAIL. The increase in RGD-L-TRAIL-induced apoptosis may be due to ACDCRGDCFC receptor expression on these tumor cells, as improved apoptosis correlates with expression level of integrin receptors (see Fig. 3A).

To examine whether the enhanced bioactivity of RGD-L-TRAIL was attributed to the attachment of ACDCRGDCFC-targeting motif, we did parallel experiments on Jurkat and

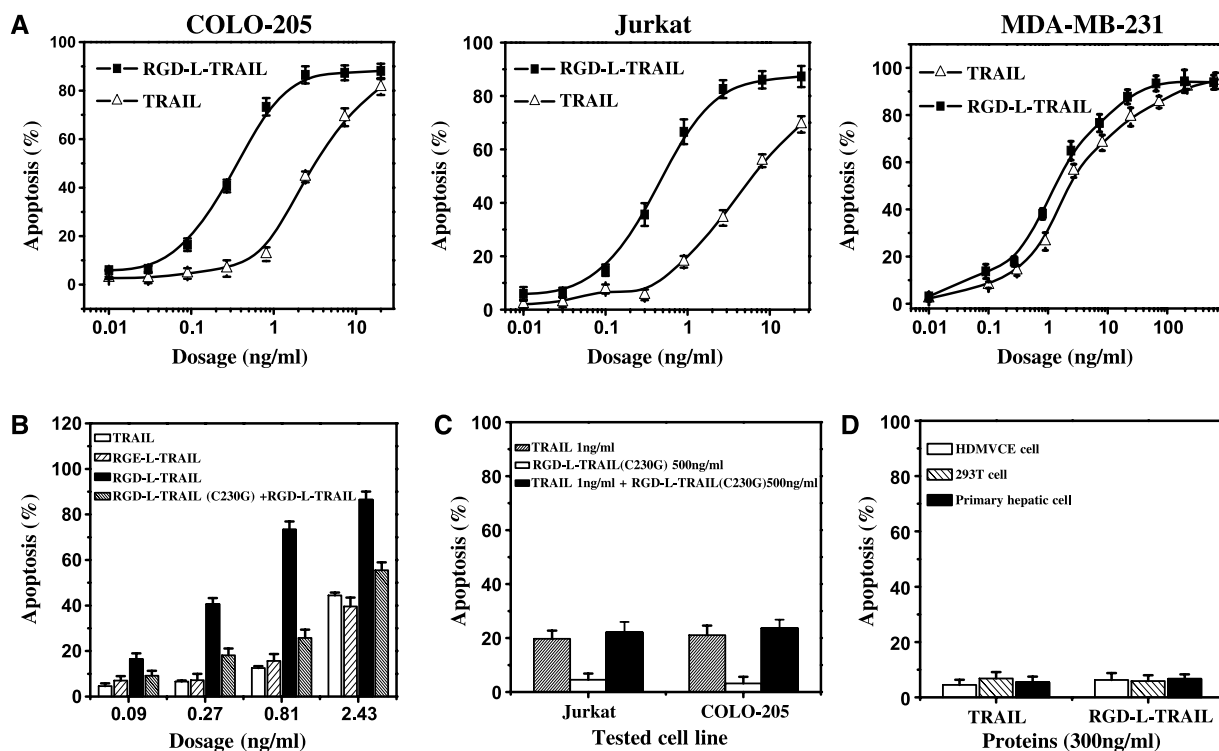


Figure 4. *In vitro* apoptosis-inducing activity of each TRAIL preparation on tumor and normal cells. After incubation with serially diluted RGD-L-TRAIL or TRAIL for appropriate times (COLO-205 incubation for 6 h; Jurkat incubation for 4 h; MDA-MB-231 incubation for 8 h; and HDMVEC, 293T, and primary hepatic cells incubation for 24 h), cells were double stained with Annexin V-FITC and PI as described in Materials and Methods and then analyzed by flow cytometry. Experiments were done independently at least three times with each cell line. Bars, SD. **A**, dose-response effect of RGD-L-TRAIL and TRAIL on COLO-205, Jurkat, and MDA-MB-231 cancer cells. ACDCRGDCFC motif in RGD-L-TRAIL fusion protein increased the apoptosis-inducing activity of TRAIL. RGD-L-TRAIL shows high apoptosis-inducing potency. **B**, COLO-205 cells were treated with serially diluted TRAIL, RGE-L-TRAIL, RGD-L-TRAIL, and RGD-L-TRAIL plus 500 ng/mL RGD-L-TRAIL (C230G). The ACDCRGDCFC motif itself does not induce apoptosis. **C**, Jurkat and COLO-205 cells were treated with TRAIL (1 ng/mL) in the absence or presence of RGD-L-TRAIL (C230G; 500 ng/mL). TRAIL and its mutants do not induce apoptosis in normal cells. **D**, normal human HDMVEC, 293T, and primary hepatic cells were treated with 300 ng/mL RGD-L-TRAIL or TRAIL for 24 h. No significant difference in apoptosis was observed between different cells.

COLO-205 cancer cell lines. Two RGD-L-TRAIL mutants were prepared: RGE-L-TRAIL with the ACDCRGDCFC motif replaced to ACDCRGECFC and RGD-L-TRAIL (C230G) with TRAIL mutation of cysteine 230 to glycine. Previous reports showed that ACDCRGECFC peptide loses the ability to bind $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins (40), whereas the cysteine 230 mutation of TRAIL completely abolished the apoptotic activity of TRAIL (37). As expected, the apoptosis-inducing ability of RGE-L-TRAIL decreased when compared with RGD-L-TRAIL and showed no significant difference when compared with TRAIL (Fig. 4B). RGD-L-TRAIL (C230G) protein exhibited no apoptotic activity in Jurkat or COLO-205 cells even at concentrations as high as 500 ng/mL (Fig. 4C); meanwhile, 10 ng/mL native TRAIL led to massive apoptosis (see Fig. 4A). The above results clearly indicate that the increased apoptotic activity of RGD-L-TRAIL is solely due to targeted TRAIL; the ACDCRGDCFC motif itself did not induce apoptosis as displayed by RGD-L-TRAIL (C230G). Our results also showed that binding of RGD-L-TRAIL to integrins was inhibited by preincubating the tumor cells with a large excess (500 ng/mL) of RGD-L-TRAIL (C230G). In this case, RGD-L-TRAIL displayed identical biological activity to TRAIL (Fig. 4B).

To further investigate the role of ACDCRGDCFC motif in TRAIL-induced cell death signaling, we treated Jurkat cells with TRAIL (1 ng/mL) in the absence or presence of RGD-L-TRAIL (C230G; 500 ng/mL) for 4 h. Apoptosis was no different in the presence or absence of RGD-L-TRAIL (C230G) as detected using FACS (Fig. 4C). Similar results were obtained for COLO-205 cells treated for 8 h (Fig. 4C). These data show the ACDCRGDCFC motif of RGD-L-TRAIL enhances apoptotic bioactivity of TRAIL by binding more RGD-L-TRAIL to target cells, but the motif itself did not cause the apoptotic signal.

In contrast to tumor cell lines, the treatment with neither RGD-L-TRAIL nor TRAIL (300 ng/mL) for 24 h displayed toxicity to human HDMVEC, 293T, or primary hepatic cells (Fig. 4D). These data indicate that RGD-L-TRAIL is nontoxic to normal cells but is able to discriminate tumor cell lines and induce apoptosis.

RGD-L-TRAIL Induced Apoptosis via FADD and Caspase-8 Pathway

TRAIL is known to induce apoptosis by recruitment of FADD and activation of the caspase-8 pathway. To confirm apoptosis induced by RGD-L-TRAIL also implemented the death receptor-FADD-caspase-8 pathway, we tested the activity of caspase-3 and caspase-8 in Jurkat cells incubated with TRAIL and RGD-L-TRAIL. After incubating cells for 2 h, RGD-L-TRAIL resulted in 2.5-fold higher caspase-3 and caspase-8 signal than native TRAIL (Fig. 5A). The activity of caspase-3 or caspase-8 induction by RGD-L-TRAIL was reduced when coincubated with RGD-L-TRAIL (C230G), a nonapoptotic TRAIL mutant (Fig. 5A). Jurkat cells deficient in FADD or caspase-8 were treated with both RGD-L-TRAIL and TRAIL and failed to show induction of apoptosis as detected by Annexin V-FITC and PI staining (Fig. 5B). These results indicate that RGD-L-TRAIL-induced

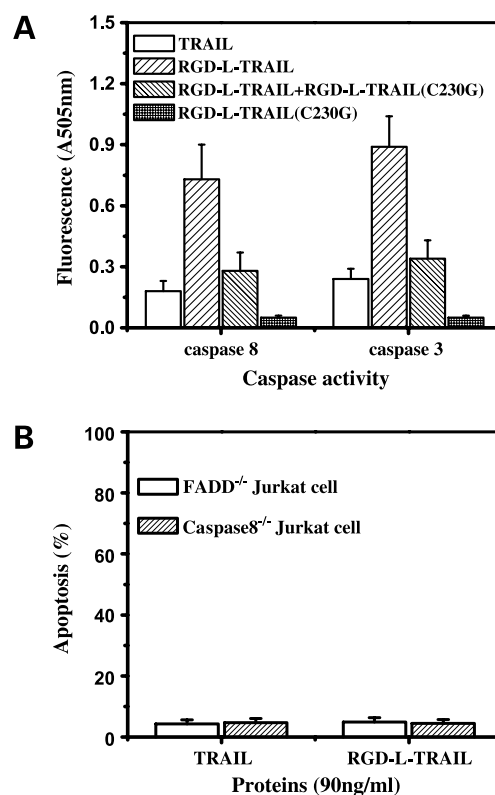


Figure 5. Role of caspase-3, caspase-8, and FADD in RGD-L-TRAIL induces apoptosis of Jurkat cells. **A**, wild-type Jurkat cells were exposed to TRAIL (1 ng/mL), RGD-L-TRAIL (1 ng/mL), RGD-L-TRAIL (C230G; 500 ng/mL), and RGD-L-TRAIL (1 ng/mL) plus 500 ng/mL RGD-L-TRAIL (C230G) for 2 h. Caspase-3 and caspase-8 activity were measured using a caspase fluorescent assay kit as described in Materials and Methods. **B**, FADD^{-/-} and caspase-8^{-/-} Jurkat cells were exposed to 90 ng/mL RGD-L-TRAIL or TRAIL for 12 h and apoptosis was analyzed by Annexin V-FITC/PI double staining as described in Materials and Methods followed by flow cytometry.

apoptosis, like TRAIL-induced apoptosis, is FADD and caspase-8 dependent.

Antitumor Activity of RGD-L-TRAIL *In vivo*

To further characterize the *in vivo* antitumor activity of RGD-L-TRAIL, COLO-205 and HT-29 xenografts were employed in athymic nude mice. Because COLO-205 is sensitive to TRAIL, we first compared the effects of single administration of RGD-L-TRAIL or TRAIL on COLO-205 tumor growth. Eight days after tumor implantation, mice were administered daily for 10 days *i.p.* with RGD-L-TRAIL (100 or 20 μ g), TRAIL (100 μ g), or control vehicle (PBS). Treatment of COLO-205 bearing mice with 100 μ g TRAIL resulted in a slight reduction in tumor size compared with PBS control (Fig. 6A). In contrast, treatment with 100 μ g RGD-L-TRAIL dramatically suppressed tumor growth compared with PBS control. Moreover, tumor inhibition by 20 μ g RGD-L-TRAIL was as effective as 100 μ g dose of TRAIL (Fig. 6A).

Previous reports have shown that combination of TRAIL and CPT-11 improves antitumor efficiency of TRAIL-sensitive COLO-205 and TRAIL-resistant HT-29 tumor

xenografts (41). To confirm these studies, we administrated RGD-L-TRAIL and TRAIL i.p. daily for 2 weeks. CPT-11 was injected i.v. once every 2 days for a total of 14 days. We used a relatively low dosage of CPT-11 (6.25 mg/kg/dose) plus RGD-L-TRAIL (100 or 30 μ g) or TRAIL (270 or 90 μ g) for COLO-205 tumors but higher dose of CPT-11 (25 mg/kg/dose) plus RGD-L-TRAIL (200 μ g) or TRAIL (400 μ g) for HT-29 tumors. Our results are shown in Fig. 6B. A combination treatment using RGD-L-TRAIL (30 or 90 μ g) and CPT-11 (6.25 mg/kg) greatly improved the tumor suppression efficacy of RGD-L-TRAIL. Moreover, combi-

nation with CPT-11 (6.25 mg/kg), administration of 100 μ g RGD-L-TRAIL achieved a higher tumor suppression effect than that of 270 μ g TRAIL, and 30 μ g RGD-L-TRAIL showed better tumor inhibition than that of 90 μ g TRAIL. Of note, 8 of the 10 animals treated with 100 μ g RGD-L-TRAIL plus CPT-11 were completely tumor free, whereas only 6 of 10 animals treated with 270 μ g TRAIL plus CPT-11 were tumor free after 36 days.

Treatment of HT-29-bearing mice with RGD-L-TRAIL alone resulted in little antitumor effect even at the highest dose (400 μ g). Nevertheless, when RGD-L-TRAIL was

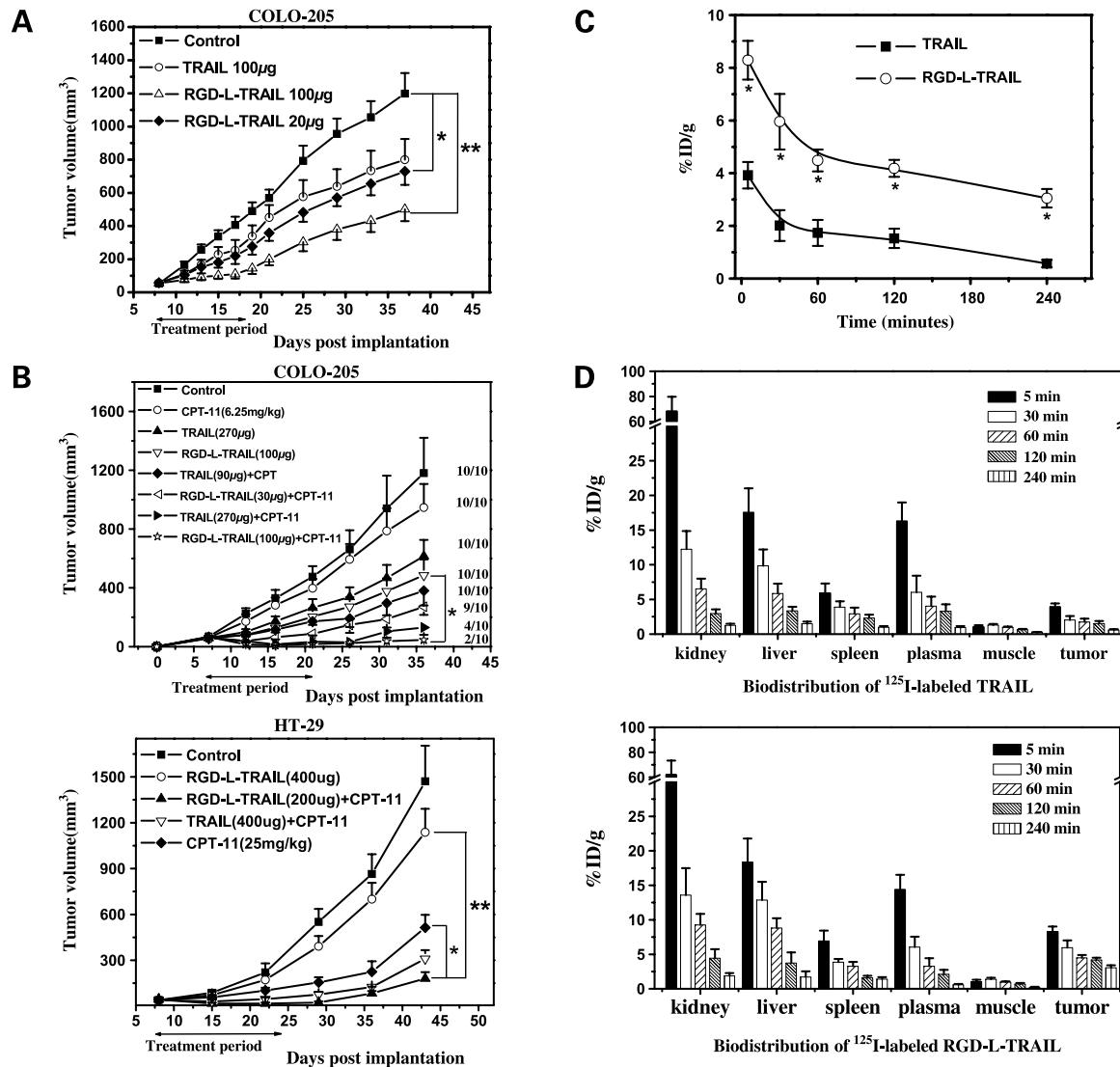


Figure 6. Antitumor effect and biodistribution of RGD-L-TRAIL in tumor-bearing mice. **A**, effect of RGD-L-TRAIL or TRAIL alone on tumor growth of COLO-205 tumor-bearing nude mice. Eight days after tumor implantation, mice were treated daily for 10 days with PBS, 100 μ g TRAIL, 100 μ g RGD-L-TRAIL, or 20 μ g RGD-L-TRAIL, respectively, by i.p. injection. RGD-L-TRAIL (20 μ g) is as effective as 100 μ g TRAIL. **B**, effects of RGD-L-TRAIL in combination with CPT-11 on COLO-205 and HT-29 tumor-bearing mice. COLO-205 tumor-bearing mice were treated with PBS, 6.25 mg/kg CPT-11 alone, 100 or 30 μ g RGD-L-TRAIL alone, 6.25 mg/kg CPT-11 plus 100 or 30 μ g RGD-L-TRAIL, or 6.25 mg/kg CPT-11 plus 270 or 90 μ g TRAIL. HT-29 tumor-bearing mice were treated with PBS, 400 μ g RGD-L-TRAIL alone, 25 mg/kg CPT-11 alone, 25 mg/kg CPT-11 plus 200 μ g RGD-L-TRAIL, or 25 mg/kg CPT-11 plus 400 μ g TRAIL. **C**, ¹²⁵I-labeled RGD-L-TRAIL and TRAIL distributed in tumor. RGD-L-TRAIL showed higher of tumor uptake than TRAIL did at each time points. **D**, biodistribution of ¹²⁵I-labeled RGD-L-TRAIL and TRAIL in COLO-205 tumor-bearing mice at different time points. Columns, mean; bars, SE. *, $P < 0.05$; **, $P < 0.01$.

combined with CPT-11, improved antitumor effects were seen in the HT-29 tumor model (Fig. 6B). In addition, less RGD-L-TRAIL was required than TRAIL to achieve tumor suppression in the HT-29 model, analogous to the COLO-205 studies. These results support the model in which attachment of TRAIL with ACDCRGDCFC peptide enhances its antitumor activity *in vivo* for both TRAIL-sensitive and TRAIL-resistant tumor models.

Targeted Accumulation of RGD-L-TRAIL in Tumor Tissue *In vivo*

To further validate the increased antitumor effect of RGD-L-TRAIL was due to increased binding capacity of tumor tissue *in vivo*, ^{125}I -labeled RGD-L-TRAIL or TRAIL was injected into COLO-205 tumor-bearing mice. As shown in Fig. 6C, RGD-L-TRAIL showed a higher accumulation (>2-fold) in tumors than TRAIL did at each time point. At 240 min postinjection, there was very low or trace amounts of native TRAIL in tumor tissue; in contrast, large amounts of RGD-L-TRAIL were maintained. However, no statistically significant difference has been found for their respective distribution in plasma, liver, kidney, spleen, and muscle (Fig. 6D).

The *in vivo* enhanced targeted delivery of RGD-L-TRAIL in tumor tissues as observed above, along with increased antitumor activity of RGD-L-TRAIL (see Fig. 6A and B), strongly support our model that ACDCRGDCFC endows TRAIL with better tumor-specific binding and improved antitumor efficacy.

Discussion

Angiogenesis, the formation of new blood vessels, plays a key role in solid tumor formation and maintenance. The number of drugs targeting the tumor vasculature with antitumor effects has dramatically increased in recent years, making angiogenesis an attractive target for cancer therapies. Targeted delivery of cytokines to the tumor vasculature increases the localized concentration to elicit significant antitumor activity. This has been shown both in primary and disseminated tumors and has greatly improved the therapeutic index. Recently, several peptides have been selected by *in vivo* phage display to selectively target the tumor vasculature and inhibit tumor growth. Herein, we report a promising strategy that elicits a significant enhancement in antitumor activity by fusing TRAIL with the peptide ACDCRGDCFC, a tumor-homing ligand. The recombinant fusion protein targeted TRAIL to tumor regions at lower concentrations than native TRAIL, thus decreasing the required therapeutic dosage. By selectively targeting tumor tissues, its net effect is increased bioavailability to tumor tissues and reduced cost for effective cancer therapy. Lastly, given that RGD-L-TRAIL is more effective in animal models at much lower concentration than native TRAIL, it may prove to cause fewer adverse side effects.

We selectively delivered TRAIL to tumors by fusing TRAIL to the ACDCRGDCFC peptide. This was based on the evidence that ACDCRGDCFC peptide could bind to

human $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (26, 42). These integrins are known to be overexpressed in human tumor blood vessels (27) and minimally expressed on resting or normal blood vessels. Several studies indicate that α_v -related integrins are good markers for tumor vascular and that integrin α_v -binding peptides can be used as modular targeting moieties (30, 32–34). In addition, several tumors have also been reported to express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (28–31). Thus, RGD-L-TRAIL could be specifically targeted to both the tumor neovasculature and tumor cells via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. In the present report, we used COLO-205 and HT-29 colon carcinomas, which express α_v integrins (43, 44), to test our model that RGD-L-TRAIL will be more effective than native TRAIL in inhibiting tumor growth. These *in vivo* transplanted animal tumor models may mimic the situation in at least a subgroup of cancer patients with increased $\alpha_v\beta_3$ and $\alpha_v\beta_5$ levels (30). Moreover, targeting $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-positive disseminated tumors would be expected to be equally inhibited using the RGD-L-TRAIL strategy.

The *in vitro* results show that RGD-L-TRAIL elicits a significant increase in the percentage of apoptotic cells when compared with native TRAIL. In the COLO-205 and Jurkat cancer cell lines, RGD-L-TRAIL exhibited an 8- to 10-fold increase in activity (Fig. 4A) when compared with TRAIL or RGE-L-TRAIL. This increase in apoptosis is attributed to RGD-L-TRAIL selectively binding integrin receptors, via the ACDCRGDCFC domain, and increasing its local concentration on the target cell surface. This allows the TRAIL domain to bind to proximal TRAIL receptors more efficiently, thereby enhancing the proapoptotic signal cascade. The enhanced level of apoptosis corresponded to the expression levels of ACDCRGDCFC receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) on three cancer cell lines tested. MDA-MB-231 cells showed only a 2-fold improvement in apoptosis with RGD-L-TRAIL possibly due to the relatively low level of integrin expression. In contrast, cells with increased $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression level (COLO-205 and Jurkat) showed a 10-fold improvement in apoptosis induction with RGD-L-TRAIL. Importantly, apoptosis could be completely inhibited when COLO-205 cells were preincubated with RGD-L-TRAIL (C230G), a TRAIL mutant capable of competing for integrin receptor binding but unable to induce apoptosis. These results convincingly show that the improved activity of RGD-L-TRAIL was due to ACDCRGDCFC targeting to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Furthermore, cell adhesion and competitive binding assays with RGD-L-TRAIL (C230G) also show that the ACDCRGDCFC domain of RGD-L-TRAIL specifically bind to integrins. This result is consistent with recent studies using the ACDCRGDCFC peptide coupled to TNF- α (32). In our study, we also observed an increase in cell staining of RGD-L-TRAIL-FITC to microendothelial cells. In contrast, the staining of TRAIL-FITC to microendothelial cells was weaker probably due to the fast-on/fast-off for ligand and receptor interactions (22). The *in vivo* tumor tissue distribution experiments using fluorescent and radioactive-labeled RGD-L-TRAIL support the tumor-specific accumulation and persistence

of RGD-L-TRAIL. The targeted delivery and accumulation on cells with expression of integrins, that is, tumor and tumor microendothelial cells, provide strong supporting evidence that RGD-L-TRAIL has potent biological activity and may be beneficial to patients with $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin-positive cancers.

The increased biological activity of RGD-L-TRAIL was further shown in a COLO-205 tumor xenograft model. At equivalent doses, RGD-L-TRAIL showed a significant improvement in tumor growth inhibition when compared with TRAIL. Moreover, only one-fifth the dose of RGD-L-TRAIL was needed to achieve a similar antitumor effect induced by TRAIL. These results suggest that fusing TRAIL with a tumor-homing peptide, such as ACDCRGDCFC, improve its bioactivity. In this study, we also carried out a detailed investigation of the antitumor effect of RGD-L-TRAIL in combination with the chemotherapeutic agent CPT-11 in TRAIL-sensitive COLO-205 and TRAIL-resistant HT-29 tumors, as numerous studies have shown that TRAIL combined with chemical agents improves antitumor effects both *in vitro* and *in vivo* (41, 45–47). We expected combined therapy with CPT-11 would enhance the antitumor effect of RGD-L-TRAIL and decrease the dose of both agents, thereby minimizing potential side effects. We show the antitumor activity of RGD-L-TRAIL was greatly enhanced using this combination strategy. Treatment with low doses of RGD-L-TRAIL (100 μ g) combined with CPT-11 (6.25 mg/kg) in TRAIL-sensitive COLO-205 tumor led to the greatest tumor suppression and even complete elimination of tumors in some mice. Importantly, when RGD-L-TRAIL was compared with TRAIL using this combined strategy, a 3- to 9-fold higher dose of TRAIL was required to achieve comparable responses in COLO-205 xenograft model. At least 2-fold more TRAIL was needed in the HT-29 tumor model for comparable tumor suppression. Overall, these data indicate the antitumor activity of RGD-L-TRAIL is superior to that of TRAIL.

In conclusion, our results suggest that RGD-L-TRAIL, containing the ACDCRGDCFC-targeting peptide, directly targets TRAIL to both the tumor vasculature and tumor cells. This unique tumor selective targeting feature may improve the therapeutic index of TRAIL by reducing the amount patients required to achieve measurable response and provide a promising avenue in cancer therapy.

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References

1. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995;3:673–82.

2. Pitti RM, Marsters SA, Ruppert S, et al. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996;271:12687–90.
3. Pan G, Ni J, Wei YF, et al. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 1997;277:815–8.
4. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. *Science* 1997;276:111–3.
5. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 1997;277:818–21.
6. Lincz LF, Yeh TX, Spencer A. TRAIL-induced eradication of primary tumour cells from multiple myeloma patient bone marrows is not related to TRAIL receptor expression or prior chemotherapy. *Leukemia* 2001;15:1650–7.
7. Clodi K, Wimmer D, Li Y, et al. Expression of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors and sensitivity to TRAIL-induced apoptosis in primary B-cell acute lymphoblastic leukaemia cells. *Br J Haematol* 2000;111:580–6.
8. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* 1999;5:157–63.
9. Locklin RM, Croucher PI, Russell RG, Edwards CM. Agonists of TRAIL death receptors induce myeloma cell apoptosis that is not prevented by cells of the bone marrow microenvironment. *Leukemia* 2007;21:805–12.
10. Gazitt Y. TRAIL is a potent inducer of apoptosis in myeloma cells derived from multiple myeloma patients and is not cytotoxic to hematopoietic stem cells. *Leukemia* 1999;13:1817–24.
11. Roth W, Isenmann S, Naumann U, et al. Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. *Biochem Biophys Res Commun* 1999;265:479–83.
12. Sanlioglu AD, Koksali IT, Karacay B, et al. Adenovirus-mediated IKK β KA expression sensitizes prostate carcinoma cells to TRAIL-induced apoptosis. *Cancer Gene Ther* 2006;13:21–31.
13. Lawrence D, Shahrokh Z, Marsters S, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* 2001;7:383–5.
14. Hilliard B, Wilmen A, Seidel C, et al. Roles of TNF-related apoptosis-inducing ligand in experimental autoimmune encephalomyelitis. *J Immunol* 2001;166:1314–9.
15. Cretney E, McQualter JL, Kayagaki N, et al. TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L suppresses experimental autoimmune encephalomyelitis in mice. *Immunol Cell Biol* 2005;83:511–9.
16. Janssen EM, Droin NM, Lemmens EE, et al. CD4⁺ T-cell help controls CD8⁺ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 2005;434:88–93.
17. Hamilton SE, Wolkers MC, Schoenberger SP, Jameson SC. The generation of protective memory-like CD8⁺ T cells during homeostatic proliferation requires CD4⁺ T cells. *Nat Immunol* 2006;7:475–81.
18. Lamhamedi-Cherradi SE, Zheng SJ, Maguschak KA, Peschon J, Chen YH. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL^{-/-} mice. *Nat Immunol* 2003;4:255–60.
19. Lamhamedi-Cherradi SE, Zheng S, Tisch RM, Chen YH. Critical roles of tumor necrosis factor-related apoptosis-inducing ligand in type 1 diabetes. *Diabetes* 2003;52:2274–8.
20. Zheng SJ, Wang P, Tsabary G, Chen YH. Critical roles of TRAIL in hepatic cell death and hepatic inflammation. *J Clin Invest* 2004;113:58–64.
21. Kelley SK, Harris LA, Xie D, et al. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of *in vivo* efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* 2001;299:31–8.
22. Bremer E, Samplonius DF, Peipp M, et al. Target cell-restricted apoptosis induction of acute leukemic T cells by a recombinant tumor necrosis factor-related apoptosis-inducing ligand fusion protein with specificity for human CD7. *Cancer Res* 2005;65:3380–8.
23. Bremer E, Samplonius DF, van Genne L, et al. Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR. *J Biol Chem* 2005;280:10025–33.

24. Bremer E, Samplonius D, Kroesen BJ, et al. Exceptionally potent anti-tumor bystander activity of an scFv:sTRAIL fusion protein with specificity for EGP2 toward target antigen-negative tumor cells. *Neoplasia* 2004;6:636–45.
25. Bremer E, Kuijlen J, Samplonius D, et al. Target cell-restricted and -enhanced apoptosis induction by a scFv:sTRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2. *Int J Cancer* 2004;109:281–90.
26. Pasqualini R, Koivunen E, Ruoslahti E. α_v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol* 1997;15:542–6.
27. Max R, Gerritsen RR, Nooijen PT, et al. Immunohistochemical analysis of integrin $\alpha_v\beta_3$ expression on tumor-associated vessels of human carcinomas. *Int J Cancer* 1997;71:320–4.
28. Natali PG, Hamby CV, Felding-Habermann B, et al. Clinical significance of $\alpha(v)\beta_3$ integrin and intercellular adhesion molecule-1 expression in cutaneous malignant melanoma lesions. *Cancer Res* 1997;57:1554–60.
29. Kemperman H, Wijnands YM, Roos E. α_v Integrins on HT-29 colon carcinoma cells: adhesion to fibronectin is mediated solely by small amounts of $\alpha_v\beta_6$, and $\alpha_v\beta_5$ is codistributed with actin fibers. *Exp Cell Res* 1997;234:156–64.
30. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* 1998;279:377–80.
31. Carreiras F, Denoux Y, Staedel C, et al. Expression and localization of α_v integrins and their ligand vitronectin in normal ovarian epithelium and in ovarian carcinoma. *Gynecol Oncol* 1996;62:260–7.
32. Curnis F, Gasparri A, Sacchi A, Longhi R, Corti A. Coupling tumor necrosis factor- α with α_v integrin ligands improves its antineoplastic activity. *Cancer Res* 2004;64:565–71.
33. Yokoyama Y, Ramakrishnan S. Addition of integrin binding sequence to a mutant human endostatin improves inhibition of tumor growth. *Int J Cancer* 2004;111:839–48.
34. Dickerson EB, Akhtar N, Steinberg H, et al. Enhancement of the antiangiogenic activity of interleukin-12 by peptide targeted delivery of the cytokine to $\alpha_v\beta_3$ integrin. *Mol Cancer Res* 2004;2:663–73.
35. Harper N, Hughes M, MacFarlane M, Cohen GM. Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis. *J Biol Chem* 2003;278:25534–41.
36. Ganten TM, Koschny R, Sykora J, et al. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. *Clin Cancer Res* 2006;12:2640–6.
37. Seol DW, Billiar TR. Cysteine 230 modulates tumor necrosis factor-related apoptosis-inducing ligand activity. *Cancer Res* 2000;60:3152–4.
38. Kim SH, Kim K, Kwagh JG, et al. Death induction by recombinant native TRAIL and its prevention by a caspase 9 inhibitor in primary human esophageal epithelial cells. *J Biol Chem* 2004;279:40044–52.
39. Zitzmann S, Ehemann V, Schwab M. Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells *in vivo*. *Cancer Res* 2002;62:5139–43.
40. Su ZF, Liu G, Gupta S, et al. *In vitro* and *in vivo* evaluation of a Technetium-99m-labeled cyclic RGD peptide as a specific marker of $\alpha(V)\beta(3)$ integrin for tumor imaging. *Bioconjug Chem* 2002;13:561–70.
41. Gliniak B, Le T. Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity *in vivo* is enhanced by the chemotherapeutic agent CPT-11. *Cancer Res* 1999;59:6153–8.
42. Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. *Biotechnology (NY)* 1995;13:265–70.
43. Koretz K, Bruderlein S, Henne C, et al. Comparative evaluation of integrin α - and β -chain expression in colorectal carcinoma cell lines and in their tumours of origin. *Virchows Arch* 1994;425:229–36.
44. Enns A, Korb T, Schluter K, et al. $\alpha_v\beta_5$ -integrins mediate early steps of metastasis formation. *Eur J Cancer* 2005;41:1065–72.
45. Ray S, Shyam S, Fraizer GC, Almasan A. S-phase checkpoints regulate Apo2 ligand/TRAIL and CPT-11-induced apoptosis of prostate cancer cells. *Mol Cancer Ther* 2007;6:1368–78.
46. Voortman J, Resende TP, Abou El Hassan MA, Giaccone G, Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. *Mol Cancer Ther* 2007;6:2103–12.
47. Wang S, El Deiry WS. Inducible silencing of KILLER/DR5 *in vivo* promotes bioluminescent colon tumor xenograft growth and confers resistance to chemotherapeutic agent 5-fluorouracil. *Cancer Res* 2004;64:6666–72.