

RGD-IL-24, A Novel Tumor-Targeted Fusion Cytokine: Expression, Purification and Functional Evaluation

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Abstract Targeting drugs to tumor cells is a central challenge for improving existing cancer therapies. ACDCRGDCFCG peptide (RGD-4C) binds to $\alpha v\beta 3$ integrin, which is selectively expressed in tumor blood vessels and on the surface of some tumor cells. Interleukin 24 (IL-24) is a novel cancer growth-suppressing and apoptosis-inducing cytokine. To enhance the antitumor effect, we coupled RGD-4C to the N-terminus of IL-24 and expressed RGD-IL-24 in *Escherichia coli*. Cell proliferation and adhesion experiments revealed that RGD-IL-24 specifically binds to MCF-7 cancer cells, and induces apoptosis of MCF-7 cancer cells. These studies support the use of the RGD-IL-24 protein in tumor-targeting therapy.

Keywords Apoptosis · Integrin · Interleukin 24 · RGD peptide · Tumor targeting

Introduction

Melanoma differentiation-associated gene 7 (mda-7) is a novel tumor suppressor gene first identified by subtraction hybridization from a human melanoma cell line. Based on its structure, chromosomal location, sequence homology, and cytokine-like properties, mda-7 has been renamed

interleukin 24 (IL-24) and classified as a member of the expanding interleukin 10 (IL-10) gene family [1]. Previous research has demonstrated that IL-24 can suppress growth and induce apoptosis in a broad range of cancer cells, but not in normal cells [2]. The administration of mda-7/IL-24 using adenovirus (Ad.mda-7/IL-24) has entered phase II clinical trials [3]. In addition, various reagents have been raised to address the functional activity of mda-7/IL-24 including adenovirus, plasmid expression vectors, antibodies, and various forms of purified protein [4, 5]. However, there are several problems in current virus-based gene therapies, including lack of cancer-specific targeting and insufficient tumor delivery. In addition, some carcinoma cells are resistant to adenovirus that correlated with a lack of coxsackievirus and adenovirus receptor expression [6]. So to further investigate the unique properties of IL-24 and ameliorate these problems, IL-24 protein will have to be produced by genetic engineering. Moreover, whether IL-24 protein can reach tumor tissues and cells is significant to enhance the efficacy of antitumoral effect in vivo. Therefore targeting drug to tumor cells is a central challenge for improving cancer therapies.

The arginine–glycine–aspartic acid (RGD) sequence is a conserved recognition motif between integrins and their ligands [7]. Data obtained from phage display techniques revealed that ACDCRGDCFCG (RGD-4C) can bind selectively to $\alpha v\beta 3$ integrin [8]. The expression of $\alpha v\beta 3$ integrin is significantly upregulated in angiogenic endothelial cells or some tumor cells, but is minimally expressed on quiescent endothelial cells. Because $\alpha v\beta 3$ integrin is a specific marker for neovasculature and angiogenic cells, it has emerged as a promising target for cancer therapy. Due to the interaction between RGD sequence and integrins, coupling anticancer cytokine with RGD-containing peptides can improve its antineoplastic

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activity. For instance, when linking RGD-4C peptide to the NH₂ terminus of tumor necrosis factor (TNF), it is able to target TNF to cell membrane adhesion receptors. Moreover, subnanogram doses of this conjugate are sufficient to induce antitumor effects in tumor-bearing mice [9]. In addition, linking RGD-4C peptide to mouse recombinant interleukin 12 (IL-12) can augment antitumor activity and decrease toxicity of IL-12 [10]. Therefore, targeting IL-24 to tumor cells by attaching the homing peptide RGD-4C may enhance the effectiveness of the cytokine.

In a previous study, we constructed many recombinant vectors (e.g. pET-11c and pET-28a) to express IL-24 and found that it was very difficult to isolate IL-24 in a non-fusion protein form. Here, we coupled RGD-4C to the N-terminus of IL-24 and expressed RGD-IL-24 in *Escherichia coli*. We then tested the resulting novel fusion cytokine for the ability to target tumor cells and induce apoptosis.

Materials and Methods

Cloning of the RGD-IL-24 Fusion Gene

The DNA coding RGD-IL-24 (ACDCRGDCFCG fused with the NH₂ terminus of IL-24) was amplified by PCR from a plasmid containing the IL-24 cDNA sequence, using the following primers: 5'-CCATGGCGTGCGACTGCCGTGGTGACTGCTTCTGCGGTGC CCAGGGCCAAGAAT-3' (P1); 5'-CTCGAGGAGCTTGTAGAATTTCTGCATCCAG GTCAG-3' (P2). The P1 primer contained the *Nco*I restriction site (underlined) and the encoding sequence of RGD-4C (italicized). The P2 primer contained the *Xho*I restriction site (underlined). The amplification protocol consisted of 5 min denaturation at 94°C followed by 30 cycles of PCR (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and a final 10 min at 72°C. The PCR product of RGD-IL-24 was then cloned into the pMD18-T TA cloning vector (Takara) and sequenced (Shanghai Bioengineering). Then the RGD-IL-24 fragment was inserted into the pET-22b expression vector (Novagen) resulting in plasmid pET-22b-RGD-IL-24. pET-22b expression vector contains a 6 × His tag to allow immobilized metal ion affinity purification.

Expression and Purification of RGD-IL-24

E. coli BL21 (DE3) strain (Novagen) was transformed with pET-22b-RGD-IL-24 using the calcium chloride method. The seed culture (30 ml) carrying pET-22b-RGD-IL-24 was grown overnight at 37°C and was used to inoculate 3 L fresh LB medium containing 100 µg/ml ampicillin. The culture was grown until the OD₆₀₀ reached 0.5, at which time 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG;

Promega) was added to induce protein expression. After incubation at 37°C for 4 h, the cells were harvested by centrifugation and disrupted by sonication in TE buffer (20 mM Tris, 1 mM EDTA, pH 8.5). The condition of sonication is a series of 100 × 10 s bursts of sonication with 10 s pause between each burst at 300 W power. The insoluble inclusion bodies were obtained by centrifugation at 12000g for 20 min at 4°C, and then washed with 1.0% Triton X-100 and 2 M urea. Then the inclusion bodies were dissolved in the solution buffer (20 mM Tris, 1 mM EDTA, 8 M urea, pH 8.5). The RGD-IL-24 solution was purified using a Chelating Sepharose Fast Flow (Amersham). The column was preloaded with 0.5 M Ni₂SO₄ and equilibrated with buffer A (20 mM Tris, 8 M urea, 0.5 M NaCl, 0.01 M imidazole, pH 8.5). Then the fusion protein solution was loaded at a flow rate of 0.3 ml/min and eluted with a linear gradient of imidazole from 0.01 to 0.5 M in buffer B (20 mM Tris, 8 M urea, 0.5 M NaCl, 0.5 M imidazole, pH 8.5). Renaturation of RGD-IL-24 was performed by dialysis at 4°C for two days against different refolding buffer containing 2 mM GSH (reduced glutathione hormone), 0.2 mM GSSG (oxidized form glutathione), and decreasing concentration of urea (6 M, 4 M, 2 M). Finally, the renatured RGD-IL24 protein was dissolved in phosphate-buffered saline (PBS) (0.015 M, pH 7.3) in order to perform the biological activity assay. Protein concentration was measured using the Lowry assay [11]. Protein purity and identity was checked by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The control protein, IL-24, was expressed and purified as previously described [12].

MTT Assay

Human breast cancer cell line, MCF-7, and normal human lung fibroblast cell line, NHLF, were added to 96-well flat-bottomed plates (1 × 10³ cells/well) in RPMI 1640 medium (Hyclone) containing 10% fetal bovine serum (FBS; Hyclone) and incubated at 37°C in 5% CO₂ for 24 h, followed by exposure to RGD-IL-24 protein (6 µg/ml), IL-24 protein (6 µg/ml), and PBS control for 24 h at 37°C in 5% CO₂. Surviving cells were detected by adding 25 µl (5 mg/ml) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to each well, removing the culture supernatants after 4 h, and adding 100 µl DMSO to each well. The resulting absorbance measured at 570 nm is directly proportional to the number of viable cells.

Fluorescence Staining of Apoptotic Cells

Coverslips were placed in 24-well flat-bottomed plates prior to the addition of MCF-7 cells and NHLF cells (1 × 10³ cells/well). After a 24 h incubation at 37°C in 5%

CO₂, the cells were treated with RGD-IL-24 (6 µg/ml) or PBS control for 24 h, followed by adding 15 µl Hoechst 33258 (5 mg/ml; Sigma) to each well and incubating for 30 min. The morphology of the cells was observed immediately after staining on a Nikon 90i fluorescence microscope.

Cell Adhesion Assay

Cell adhesion was assessed as described previously [9]. Briefly, polyvinyl chloride microtiter plates were coated with RGD-IL-24 (6 µg/ml) or IL-24 solutions (6 µg/ml) at 4°C overnight. After washing with 0.9% sodium chloride, each well was filled with RPMI 1640 medium containing 2% bovine serum albumin (BSA) for 2 h at 37°C and washed again. MCF-7 cells were then detached using trypsin digestion, washed three times with 0.9% sodium chloride, resuspended in incomplete RPMI 1640 medium, and added to RGD-IL-24 or IL-24-coated plates (3 × 10⁴ cells/well in 100 µl). After incubation for 1 h at 37°C, 5% CO₂, unbound cells were removed by washing with incomplete RPMI 1640 medium. Adherent cells were fixed with a solution of 3% paraformaldehyde with 2% sucrose in phosphate-buffered saline (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. Cell adhesion inhibition experiment was performed with EDTA. After coating RGD-IL-24 (6 µg/ml) on microtiter plates, MCF-7 cells were plated in RPMI 1640 medium containing 5 mM EDTA.

Immunofluorescence Assay

MCF-7 cells were treated with RGD-IL-24 (6 µg/ml) or IL-24 (6 µg/ml) for 12 h following plating on coverslip. Cells were washed thoroughly with PBS before fixation in cold acetone. The cells were exposed to mouse anti-human IL-24 monoclonal antibody (mAb) (1:500; R&D), washed,

and then exposed to fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG secondary antibody (1:20; R&D). The negative control was performed without anti-IL-24 mAb. The morphology of the cells was observed immediately after staining using a fluorescence microscope (Nikon).

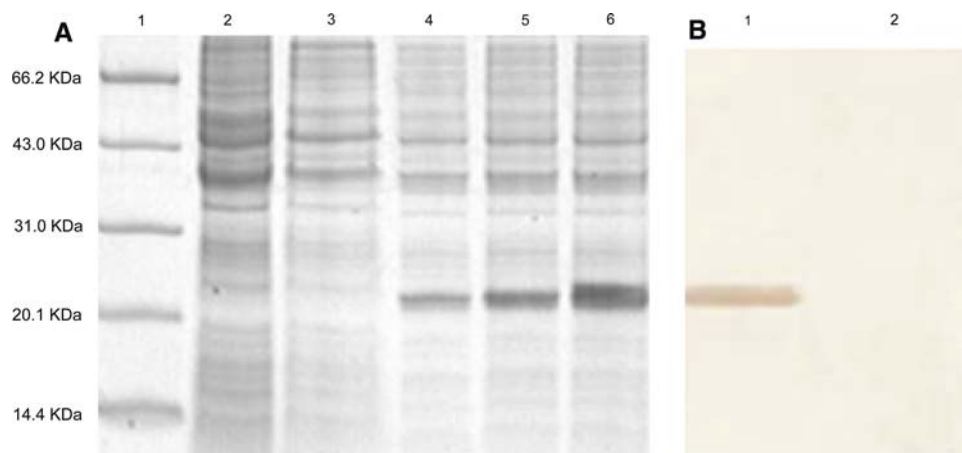
Results and Discussion

Expression and Purification of RGD-IL-24

The fusion protein RGD-IL-24, about 20 kDa in size, was expressed as inclusion bodies in *E. coli*. The expression level was about 30% of the total cellular protein (Fig. 1a). Western blot analysis confirmed that RGD-IL-24 was recognized by anti-IL-24 monoclonal antibody (Fig. 1b). Isolation of RGD-IL-24 from the inclusion bodies resulted in a preparation of approximately 52% purity. Due to a polyhistidine (6 × His) Tag in RGD-IL-24, we chose chelating affinity chromatography to purify RGD-IL-24. After the purification, the purity of RGD-IL-24 was about 90% (Fig. 2).

Despite the strong antitumor effects of mda-7/IL-24 in a broad spectrum of human cancers, several carcinoma cells are highly refractory to Ad.mda-7/IL-24 because they do not express either the coxsackievirus or adenovirus receptor. Therefore, the potential therapeutic use of IL-24 fusion protein could increase the use of this protein to cover tumors resistant to Ad.mda-7/IL-24. Direct application of the glutathione-S-transferase (GST) fusion protein GST-MDA-7 can suppress the growth of pancreatic cancer and renal cell carcinoma cell lines previously resistant to Ad.mda-7/IL-24 [4, 5]. In addition, IL-24 protein with bioactivity has been produced by transfecting 293 cells with a eukaryotic expression vector [13]. A more effective expression system will allow further investigation into the function and antitumor effect of IL-24. In previous work,

Fig. 1 Expression and identification of RGD-IL-24. **a** SDS-PAGE gel analysis of RGD-IL-24 expression. Lane 1, protein marker; Lane 2, blank plasmid pET-22b/BL21 induced for 5 h at 37°C; Lane 3, uninduced whole cell lysate of pET-22b-RGD-IL-24/BL21 at 37°C; Lanes 4–6, whole cell lysate of pET-22b-RGD-IL-24/BL21 induced at 37°C for 1, 3 and 5 h, respectively. **b** Western blot analysis of RGD-IL-24 protein. Lane 1, RGD-IL-24; Lane 2, blank control without anti-IL-24 mAb



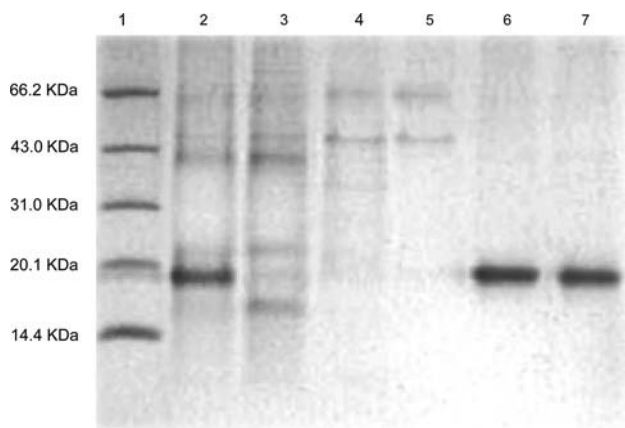


Fig. 2 SDS-PAGE gel analysis of the second purification of RGD-IL-24. Lane 1, protein marker; Lane 2, inclusion bodies of RGD-IL-24; Lane 3, flowthrough proteins; Lanes 4–5, non-target proteins of elution peak; Lanes 6–7, purified RGD-IL-24

we constructed many recombinant vectors (pET-11c, pET-28a, etc.) to express IL-24 and found that it was very difficult to obtain IL-24 in a non-fusion protein form. However, recently, we successfully expressed a fusion protein Trx-IL-24 using the pET32a vector, without losing the biological activity of IL-24 [12]. It seems that constructing a fusion protein may enhance the expression level of IL-24. However, the mechanism is not clear; it may be related to the gene character of IL-24, or the gene fusion strategy may promote the expression of the protein by overcoming many challenges including the proteolytic degradation of target proteins, protein misfolding, or poor solubility. Therefore, the strategy to construct a fusion protein of IL-24 is reasonable.

Bioactivity of RGD-IL-24

The MTT assay demonstrated that RGD-IL-24 as well as IL-24 can suppress the growth of cancer cells. In contrast, no or negligible evidence of deleterious effects was observed in control NHLF cells (Fig. 3). Hence, it appears that the RGD domain did not interfere with the antitumor effects of IL-24. The recent finding that IL-24 functions efficiently even when not secreted, due to mutation of its signal peptide, could explain why RGD-4C does not disrupt IL-24 activity [14]. In addition, another recombinant fusion protein, GST-MDA-7, induces apoptosis in diverse cancer cell lines as well as Ad.mda-7/IL-24 [4].

To confirm that the growth suppression of cancer cells treated with RGD-IL-24 resulted from the induction of apoptosis, nuclear morphology was assessed by Hoechst staining. RGD-IL-24 elicited typical apoptotic morphological changes in MCF-7 cells, including DNA fragmentation and chromatin condensation. In contrast, no significant

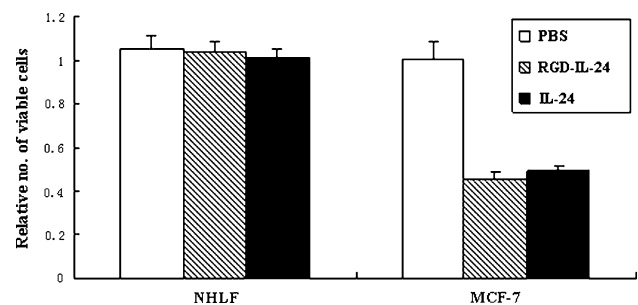


Fig. 3 Growth inhibitory effect of RGD-IL-24 on cancer cells. Cells were treated by RGD-IL-24 (6 μ g/ml) or IL-24 (6 μ g/ml) for 48 h. Cell viability was determined by the MTT proliferation assay. Triplicate experiments were performed for each cell line, and data are represented as mean \pm SD

change was found in NHLF cells or the PBS control group (Fig. 4).

There has been considerable interest in elucidating the mechanism by which IL-24 distinguishes between normal and cancer cells. The antitumor effect of IL-24 is independent of classic tumor suppressor genes, such as p53, the retinoblastoma protein (Rb), and p16, and a number of molecules mediating activity in cancer cell-specific apoptosis by IL-24 have been reported including p38, mitogen-activated protein kinase (MAPK), RNA-activated protein kinase (PKR), and the growth arrest and DNA damage inducible protein (GADD). In addition, IL-24 may kill cancer cells selectively by a mechanism potentially independent of its cytokine properties, that is, by intracellular targeting and induction of endoplasmic reticulum (ER) stress [15]. However, the precise mechanism by which IL-24 induces apoptosis is complex and requires further investigation. IL-24 has been shown to bind specific cytokine receptor complexes (IL-20R and IL-22R receptors) and consequently activate the JAK/STAT signal transduction pathway. The results of other groups have demonstrated MCF-7 cells can express IL20R1, IL20R2, and IL22R. At the same time, the expression of α v β 3 integrin is significantly upregulated in MCF-7 cells [16]. So RGD-IL-24 can simultaneously engage the α v β 3 integrin and IL24 receptor on the cells.

RGD-4C Targets IL-24 to Cancer Cells

To assess whether the RGD domain of RGD-IL-24 is functional and accessible to α v β 3 integrin, we compared cell adhesion promoted by RGD-IL-24 to that promoted by IL-24. Crystal violet staining showed adhesion and spreading of MCF-7 cells on plates coated with RGD-IL-24 (Fig. 5b) but not on plates coated with IL-24 (Fig. 5a). In addition, according to the fact that the binding between RGD-4C and α v β 3 integrin is divalent ion dependent, we performed cell adhesion inhibition experiments with

Fig. 4 The morphological features of NHLF and MCF-7 cells with Hoechst 33258 stain under fluorescence microscope (200 \times). NHLF and MCF-7 cells were treated with RGD-IL-24 (6 $\mu\text{g}/\text{ml}$) or PBS control, washed, and incubated with Hoechst 33258 for 30 min as described in section “Materials and Methods”

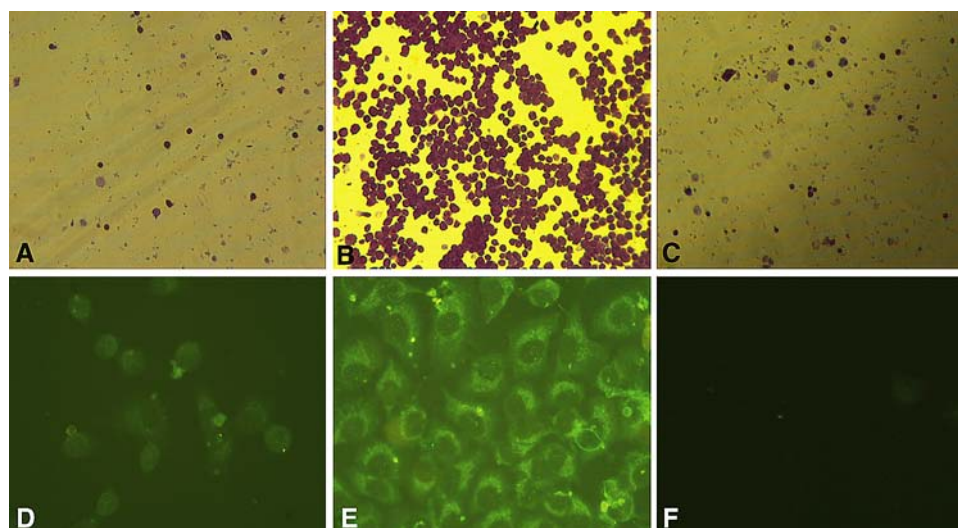
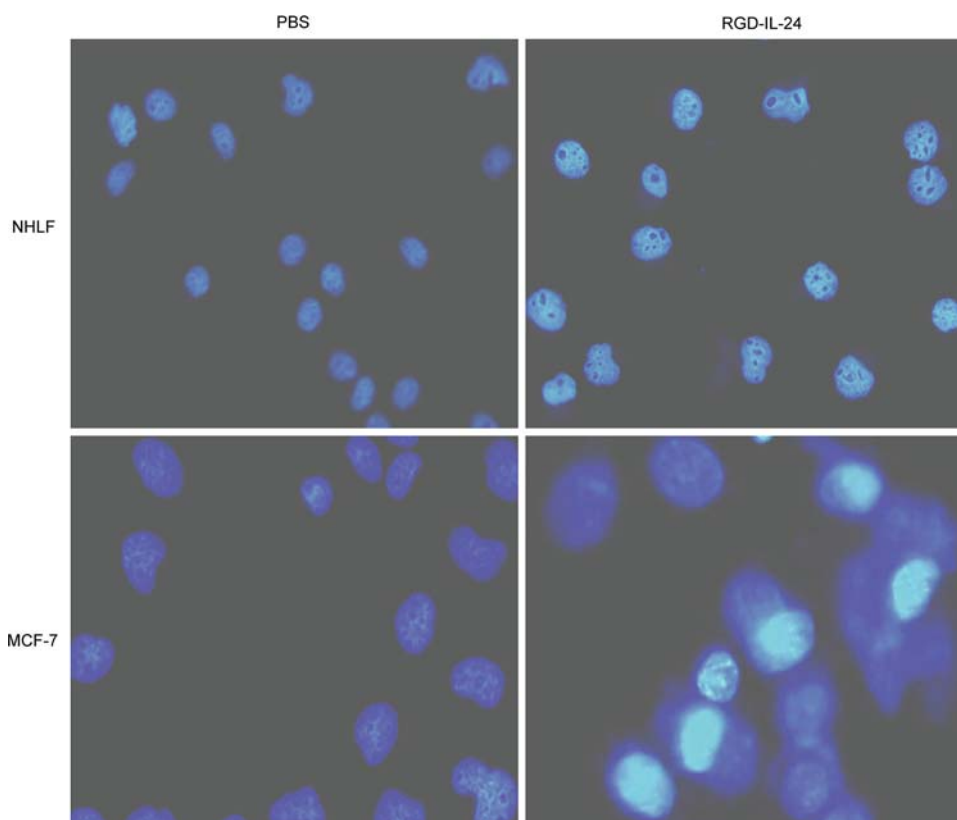


Fig. 5 The binding specificity of RGD-IL-24 to cancer cells. Microtiter wells were coated with IL-24 (6 $\mu\text{g}/\text{ml}$) (a) or RGD-IL-24 (6 $\mu\text{g}/\text{ml}$) (b), and seeded with MCF-7 cells. Cell adhesion inhibition experiment was performed with EDTA. After coating RGD-IL-24 (6 $\mu\text{g}/\text{ml}$) on microtiter plates, MCF-7 cells were plated in RPMI 1640 medium containing 5 mM EDTA (c). Images were

acquired after staining with crystal violet (200 \times). Immunofluorescence assay of MCF-7 cells treated with IL-24 (6 $\mu\text{g}/\text{ml}$) (d), RGD-IL-24 (6 $\mu\text{g}/\text{ml}$) (e) or negative control without anti-IL-24 mAb (f). Cells were washed in PBS, stained, and observed under fluorescence microscope (400 \times)

EDTA. Cell adhesion was significantly suppressed when the cells were plated in RPMI 1640 medium containing 5 mM EDTA (Fig. 5c). To further confirm the possibility of the binding specificity of RGD-IL-24, we used indirect

immunofluorescence to detect IL-24 bound to the cells. More stronger green fluorescence was observed on MCF-7 cells treated with RGD-IL-24 (Fig. 5e) than IL-24 (Fig. 5d). On the contrary, no significant fluorescence was

found on the cells without anti-IL-24 mAb (Fig. 5f). We speculate that the binding of IL-24 and its receptors is not intense, whereas the binding ability of RGD can promote the binding of RGD-IL-24 protein to MCF-7 cells. These results indicate that RGD domain of RGD-IL-24 was properly folded and able to interact with adhesion receptors, very likely $\alpha v \beta 3$ integrin, on the cell membrane. The structural basis for the heterodimeric $\alpha \beta$ integrins to their ligands is not clear. In 2002, Xiong et al. [17] reported the crystal structure of the extracellular segment of integrin $\alpha v \beta 3$ in a complex with a cyclic peptide presenting the RGD sequence. The RGD ligand binds at the major interface between the αv and $\beta 3$ subunits. The tertiary rearrangements take place in βA , the ligand-binding domain of $\beta 3$; in the complex, βA acquires two cations, one of which contacts the ligand Asp directly and the other stabilizes the ligand-binding surface.

Currently, research on RGD-4C focuses on tumor imaging using labeled RGD peptide, antiangiogenic treatment, and gene targeting therapy for cancer [18, 19]. For example, doxorubicin linked to RGD-4C has proven to be highly effective in limiting tumor growth in vivo while decreasing doxorubicin treatment-associated toxicity [20]. In addition, RGD-4C was fused to the cytokines tumor necrosis factor α (TNF- α) and interleukin 12 (IL-12) to induce antitumor effects in tumor-bearing mice, and specifically targeted αv integrin endothelial markers [9, 10].

In this report, we linked the tumor homing peptide RGD-4C to IL-24 to target IL-24 directly to cancer cells. The bioactivity of purified RGD-IL-24 was similar to that of IL-24. Moreover, the protein possibly can bind to $\alpha v \beta 3$ integrin. The targeting function of RGD-4C may enable RGD-IL-24 to exert potent antiangiogenic and antitumor effects. Taken together, the above results demonstrate that coupling IL-24 to RGD-peptide may represent a viable and promising new strategy for cancer therapy. A follow-up investigation of RGD-IL-24's antitumor activity in vivo is currently under way.

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