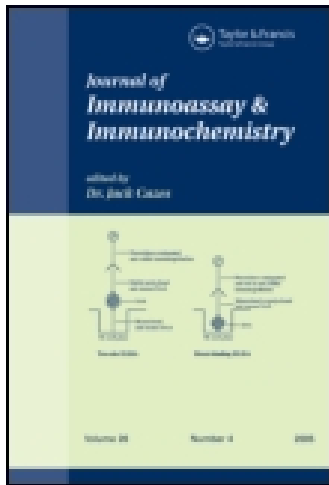


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EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RGD-mda-7, A HIS-TAGGED mda-7/IL-24 MUTANT PROTEIN

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EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RGD-mda-7, A HIS-TAGGED mda-7/IL-24 MUTANT PROTEIN

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□ RGD peptide (Arg-Gly-Asp tripeptide) binds to integrin $\alpha V\beta_3$ and $\alpha V\beta_5$, which is selectively expressed in tumor neovasculature and on the surface of some tumor cells. Some studies showed that coupling the RGD peptides to anticancer drugs yielded compounds with increased efficiency against tumors and lowered toxicity to normal tissues. The melanoma differentiation-associated gene-7/interleukin-24 gene (*mda-7/IL-24*) is a novel tumor-suppressor/cytokine gene that exhibits potent tumor-suppressive activity without damaging normal cells. To enhance the antitumor effect, we inserted a glycine residue into the wild type (*mda-7/IL-24*) between ¹⁶⁴Arg and ¹⁶⁵Asp to form a RGD peptide, named RGD-*mda-7*, then expressed RGD-*mda-7* in *Escherichia coli*. Herein, we describe the expression and purification of RGD-*mda-7*. We detected the characterizations of immunostimulatory activity, tumor targeting, potent cytopathic effect, and apoptosis inducing exploited by RGD-*mda-7* in tumor cells, and also compared these characterizations with *wmda-7/IL-24*. The data showed that RGD-*mda-7* had more potent tumor targeting and apoptosis-inducing effects than *wmda-7/IL-24*.

Keywords antitumor, integrin $\alpha V\beta_3/\alpha V\beta_5$, *mda-7/il-24*, mutant, RGD

INTRODUCTION

The *mda-7/IL-24* gene was isolated by means of subtraction hybridization from human melanoma cells induced to undergo terminal differentiation by treatment with fibroblast interferon and mezerein.^[1] Based on its structure,

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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.^[2-4] The results of in vitro studies, in vivo animal studies, and a phase I clinical trial all indicate that MDA-7/IL-24 has the ability to selectively induce apoptosis in numerous human cancer cell lines.^[5-9] In contrast, no significant growth-inhibitory effect occurred when this gene was transduced into normal human breast or prostate epithelial, endothelial, melanocyte, astrocyte, or fibroblast cells.^[4,8,10] Intriguingly, in addition to its direct apoptosis-inducing properties, mda-7/IL-24 also shows antiangiogenic, radiosensitizing, immunostimulatory, and potent “bystander” antitumor activity.^[3,11-13]

Integrin $\alpha V\beta_3$, $\alpha V\beta_5$ is overexpressed both on multiple tumor cells (osteosarcomas, neuroblastomas, glioblastomas, melanomas, lung carcinomas, breast cancer, and so on) and the activated endothelial cells of tumors' neovasculature.^[14-17] Integrin $\alpha V\beta_3$, $\alpha V\beta_5$ serves as a receptor for a variety of extracellular matrix proteins with the exposed RGD peptide sequence.^[18] When coupled to the anticancer drug, the RGD peptide could develop targeting and enhance the antitumor effect of the drug.^[19] Moreover, RGD-containing peptides are able to directly induce apoptosis by triggering conformational changes that promote pro-caspase-3 autoprocessing and activation.^[20] Thus, to further augment cancer-specific targeting and enhance the antitumor effect, mda-7/IL-24 mutant RGD-mda-7 was constructed and its bioactivity investigated.

Here, we describe the cloning, expression, purification, renaturation, and characterization of mda-7/IL-24 mutant RGD-mda-7. The final purity of RGD-mda-7 was 90%. Our results indicate that RGD-mda-7 could bind to integrin $\alpha V\beta_3$, $\alpha V\beta_5$ and enhance wtmda-7/IL-24 antitumor effect in tumor cell lines MCF-7 and Ket-3, but not affect normal cell line Normal Human Lung Fibroblasts (NHLF). Furthermore, we document that RGD-mda-7 has stronger antitumor effect compared with wtmda-7/IL-24. These results showed that the process for purification of RGD-mda-7 was effective.

MATERIALS AND METHODS

Reagents and Bacterial Strains

The pET-28a(+) expression vector and the *E. coli* strain BL21 (DE3) were purchased from Novagen (Philadelphia, PA, USA). The BL21 (DE3) cells harbor chromosomal kanamycin-resistant gene and grow in overnight express instant TB medium (Novagen) with 15 $\mu\text{g}/\text{mL}$ kanamycin. The *E. coli* strain DH5 α was maintained in our laboratory. All restriction endonucleases, DNA polymerase, and T4 DNA ligase were purchased from Promega (San Luis Obispo, CA, USA). Plasmid extraction kit and PCR production

purification kit were purchased from Promega. BugBuster Ni-NTA His. Bind Purification Kit and Protein Refolding Kit were purchased from Novagen.

Construction of mda-7/IL-24 Mutant RGD-mda-7

The whole cDNA coding region of mda-7/IL-24 gene was kindly provided by Professor Liu (Xin-Yuan Liu, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) and used as a template for overlapping polymerase chain reaction (PCR). Four primers for PCR were designed as follows based on the mda-7/IL-24 sequence and the pET-28a(+) vector:

P1: 5'-TGTGCATATG *GACGACGACGACAAGGCCAGGGCCAAG*-3'

P2: 5'-*ACTGTCACCTCTGATGGAAAACATC*-3'

P3: 5'-*ATCAGAGGTGACAGTGCACACAGGC*-3'

P4: 5'-TGTGGGATCCTCAGAGCTTGTAG-3'

The P1 had enterokinase restriction sites are shown in italics. The P2 and P3 had the overlapping complementary 15-base sequence shown in italics, which resulted in the extra codon *GGT* encoding the glycine between Arg¹⁶⁴ and Asp¹⁶⁵ to form a RGD motif, while P1 and P4 had Nde and BamH recognition sites (underlined), respectively, for directed cloning into vector. After first round of PCR using P1/P2 and P3/P4, respectively, agarose gel purification was performed for the two products. A second round of PCR followed using the two purified products as templates and the P1, P4 primers to obtain the fragment encoding mutant RGD-mda-7. The fragment was digested with Nde and BamH, and then was inserted in-frame into the pET-28a(+) expression vector to construct the pET28-RGD-mda-7 plasmid. The mutations were confirmed by nucleotide sequencing (Invitrogen, Carlsbad, CA, USA). Similarly, the wtmda-7/IL-24 was inserted pET-28a(+) vector to construct the pET28-mda-7/IL-24 plasmid. pET-28a(+) expression vector contains a 6 × His-tag to allow immobilized metal ion affinity purification.

Expression of wtmda-7/IL-24 and RGD-mda-7

The expression plasmids pET28-RGD-mda-7 and pET28-mda-7/IL-24 were screened in DH5 α and confirmed by DNA sequencing. The successfully constructed plasmids were used to transform *E. coli* strain BL21(DE3) (resistant to 15 μ g/mL kanamycin) competent cells. The positive clone cells were inoculated in 5 mL overnight in express instant TB medium with 15 μ g/mL kanamycin at 37°C by shaking at 300 rpm for 8 h, 10 h, 12 h,

14 h, 16 h, 18 h, and 20 h, respectively. Every experimental group was accompanied by a control of non-induced cultures. At indicated times, the OD₆₀₀ of the cultured medium was tested, and then the harvested cells were taken and analyzed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15% separating gel, 4% stacking gel).

Renaturation of wtmda-7/IL-24 and RGD-mda-7

Two 1 L flasks containing 50 mL overnight express instant TB medium were inoculated to a starting OD₆₀₀ of about 0.6 from the cultured supplement with 15 µg/mL kanamycin. Cells were grown at 37°C by shaking at 300 rpm for 16 h. The cells were harvested from liquid culture by centrifugation at 10,000 × g for 10 min. Decanted the supernatant and allowed the pellet to drain, removing as much liquid as possible. The cell pellet (0.407 g) completely resuspended in room temperature using 5 mL BugBuster Protein Extraction Reagent by pipetting, and added 10 µL (250 units) benzonase nuclease and 10 kU rLysozyme solution. The cell suspension was incubated on a shaking platform at a slow setting for 20 min at room temperature. The inclusion bodies were collected by centrifugation for the suspension at 5000 × g for 15 min at 4°C, and the supernatant was removed with a pipette.

The pellet was thoroughly resuspended in 10 mL 1 × IB wash buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100) with adding 2 M urea. The suspension was centrifuged at 5000 × g for 15 min and the pellet saved. The pellet was washed three times, the cleared inclusion bodies were collected by centrifugation at 10,000 × g for 10 min, the supernatant was decanted, and the last traces of liquid were removed by tapping the inverted tube on a paper towel. 10 ml 1 × solubilization buffer (500 mM CAPS, pH 11.0)/N-lauroylsarcosine was added to the inclusion bodies and gently mixed by repeated pipetting, and incubated at room temperature for 15 min. The solution was clarified by centrifugation at 10,000 × g for 10 min at room temperature. The supernatant containing the solubilized protein was transferred carefully into a clean tube, avoiding pelleted debris.

The solubilized protein was renatured by dialysis. The solubilized protein (10 mL) was slowly added to 1000 mL dialysis buffer (20 mM Tris-HCl, 0.1 mM DTT, pH 8.5) for 8 h at 4°C. The buffer was changed and dialyzed for an additional 8 h. The dialysis was continued through two additional changes (8 h each) with the dialysis buffer II lacking DTT. After dialysis, the protein solution was analyzed by SDS-PAGE.

Purification of wtmda-7/IL-24 and RGD-mda-7

2 mL of the 50% Ni-NTA His · Bind slurry was added to 8 mL of 1X Ni-NTA bind buffer (300 mM NaCl, 50 mM sodium phosphate buffer,

10 mM imidazole, pH 8.0) and mix gently. When the resin settled by gravity, 8 mL of the supernatant with a pipette. The dialyzed protein solution was added to the Ni-NTA His·Bind slurry and mixed gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min. The solution-Ni-NTA His·Bind mixture was load into a column with the bottom outlet capped. The bottom cap was removed and the column flow-through that was saved for SDS-PAGE analysis was collected. The column was washed with 2 × 8 mL 1X Ni-NTA wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0), and wash fractions were collected for SDS-PAGE analysis. The protein was eluted with 4 × 1 mL 1X Ni-NTA elution buffer (300 mM NaCl; 250 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0) and collected in four tubes, and the fractions were analyzed by SDS-PAGE. Protein purity and identity was checked by SDS-PAGE and Western blotting.

Immunostimulatory Activity of RGD-mda-7

Human peripheral blood cells (PBMC) were obtained from healthy volunteer donors by centrifugation over Ficoll–Hypaque density gradient separation. Monocytes were cultured in RPMI 1640 medium containing 100 u/mL penicillin, 100 mg/L streptomycin, and 10% fetal bovine serum at 1×10^6 cells/mL, with the wtmda-7/IL-24 and RGD-mda-7 recombinant proteins added at the designated concentrations. The cells were incubated at 37°C in a 95% humidified 5% CO₂ environment. At indicated times, the culture supernatants were harvested, and the amounts of IL-6, TNF- α , and INF- γ were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (ZhongShan GoldenBridge Company, China).

Cell Adhesion Assay and Adhesion Inhibiting Assay

Polyvinyl chloride microtiter plates (96 well) were coated with RGD-mda-7 (8 μ g/mL) and wtmda-7/IL-24 solutions (8 μ g/mL) at 4°C overnight. After being washed with 0.9% sodium chloride, each well was filled with RPMI 1640 medium containing 1% bovine serum albumin (BSA) for 2 h at 37°C and washed again. For adhesion assay, MCF-7 cells and Ket-3 cells were detached using trypsin digestion, washed three times with 0.9% sodium chloride, and resuspended in incomplete RPMI 1640 medium. Then the cells were added to RGD-mda-7 or wtmda-7/IL-24 coated plates with 2×10^4 cells/well in 100 μ L. After incubation for 1 h at 37°C in 5% CO₂, unbound cells were removed by washing with incomplete RPMI 1640 medium. Adherent cells were fixed with a solution of 4% paraformaldehyde

in phosphate-buffered saline (PBS) (pH 7.2) and stained with 0.5% crystal violet, then observed using a microscope. Absorbance from the plates was read on an ELX-800 spectrometer reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 490 nm. The experiment was repeated more than 4 times. For the adhesion-inhibiting assay, MCF-7 cells and Ket-3 cells were stained with monoclonal antibodies directed against specific integrins. The antibodies that were used include LM609 (anti-avb3) and 15F11 (anti-avb5). Antibodies were incubated with cells in suspension for 30 min at an antibody concentration of 15 $\mu\text{g}/\text{mL}$. Then do in the same way as the adhesion assay.

Cell Viability Assay

Cells were plated in 96-well plates and treated with the RGD-mda-7 and wtmda-7/IL-24 proteins at the designated concentrations. To further evaluate the function of RGD-mda-7 in growth inhibition, we added increasing concentrations of an anti-MDA7 neutralizing antibody to RGD-mda-7 treated cells. At the indicated times, the medium was removed and fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL, 100 μL) was added to each well. Cells were incubated at 37°C for 4 h. Then the medium was removed, and 150 μL solubilization solution (DMSO) was added and mixed thoroughly. Absorbance from the plates was read on an ELX-800 spectrometer reader at 490 nm.

Apoptosis Assays

Cells were analyzed for apoptosis, using the DAPI staining (KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, cells were plated in 24-well plates and treated with RGD-mda-7 and wtmda-7/IL-24 proteins, and incubated at 37°C in humidified 5% CO_2 for 72 h. Cells were washed twice with cold PBS (pH 7.2) and stained with 1 $\mu\text{g}/\text{mL}$ DAPI (KeyGen Biotech Co., Ltd., China) at 37°C for 30 min. Nuclear morphology was observed using a fluorescence microscope (Nikon, ECLIPSE-Ti, Tokyo, Japan).

Western Blotting Analysis

Cell lines were grown on 10-cm plates, and protein extracts were prepared with RIPA buffer containing a cocktail of protease inhibitors. A measure of 50 mg of protein was applied to 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with polyclonal (Santa Cruz, CA, USA) or monoclonal (Ambion, Austin, TX, USA) antibodies to mda-7/IL-24 and polyclonal antibodies to bax, bcl-2, caspase-3.

Statistical Analysis

Values were expressed as mean \pm S.D., and statistical analysis of the results was carried out by one-way analysis of the variance (ANOVA) followed by Duncan's new multiple range method or Newman-Keuls test. P-values <0.05 were considered significant.

RESULTS AND DISCUSSION

Construction Mutant RGD-mda-7

By means of overlapping PCR, we inserted a Gly between Arg164 and Asp165 into wtmda-7/IL-24 and created mutant RGD-mda-7. The RGD-mda-7 was cloned into pET-28a(+) and transformed into DH5 α competent cells. The expected mutation was confirmed by DNA sequencing. As shown in Figure 1, we successfully inserted extra codon **GGT** (Gly) between codon **AGA** (Arg¹⁶⁴) and **GAC** (Asp¹⁶⁵) to form a RGD motif in wtmda-7/IL-24. Glycosylation is not mandatory for inducing cell death or bystander activities in different cancer cells.

Expression and Purification of wtmda-7/IL-24 and RGD-mda-7

The transformed BL21 (DE3) cells were inoculated to overnight express instant TB medium at 37°C by shaking at 300 rpm. After being cultured for 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, and 20 h, respectively, the OD₆₀₀ of the cultured medium was tested. As shown in Figure 2A, the OD₆₀₀ of the cultured medium was rising faster in less than 16 h, but was stalemating after more than 16 h.

Following, the cells were grown in two 1 L flask containing 50 mL overnight express instant TB medium at 37°C by shaking at 300 rpm for 16 h. The cells were dissolved in SDS-PAGE sample buffer and subjected to

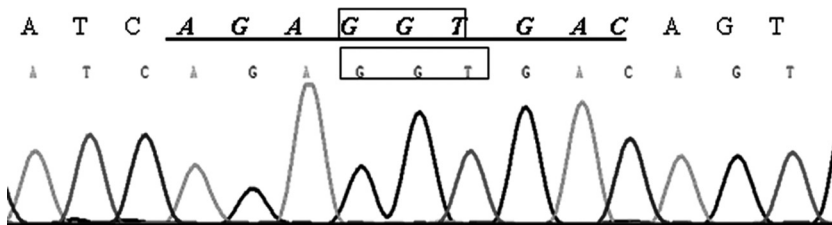


FIGURE 1 DNA sequence alignment of (A) wtmda-7/IL-24 and (B) RGD-mda-7. The sequencing shows that the codon GGT(Gly) was inserted between codon AGA(Arg) and GAC(Asp), and successfully formed RGD domain of RGD-mda-7.

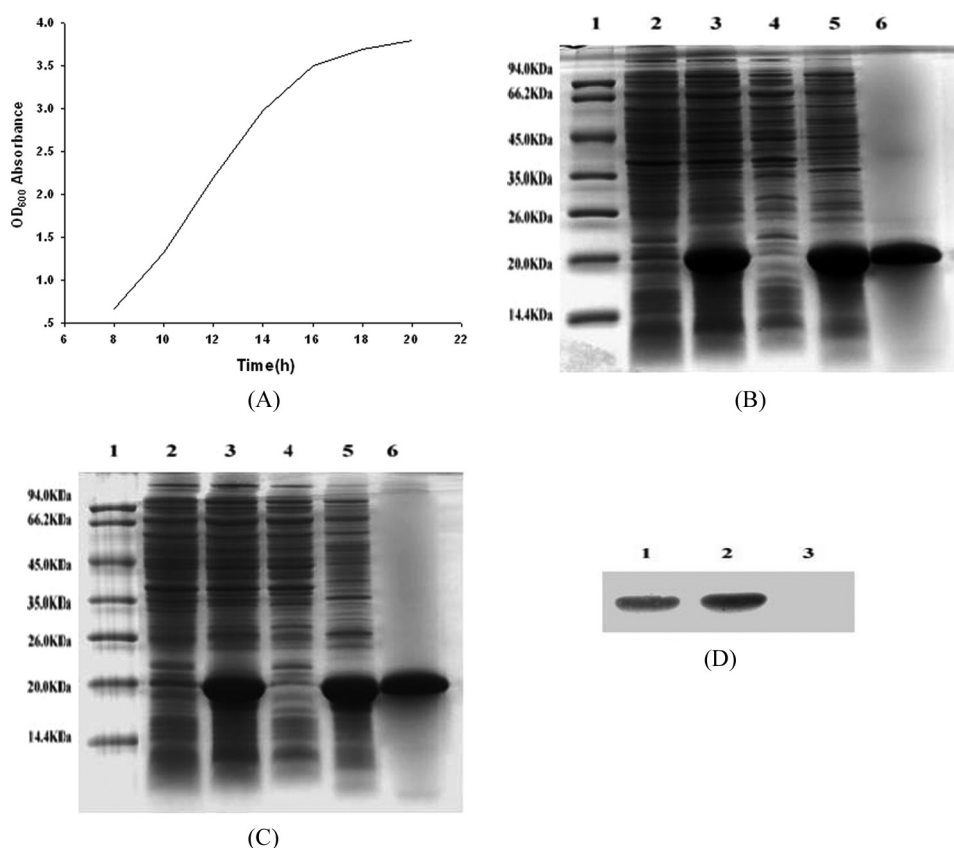


FIGURE 2 Expression, purification, and identification of RGD-mda-7 and wtmda-7/IL-24. (A) The OD₆₀₀ absorbance of the cultured medium was rising faster within 16 hours, and then was stalemating after more than 16 hours. (B) SDS-PAGE gel analysis of RGD-mda-7. Lane 1, protein marker; Lane 2, pET-28a-RGD-mda-7/BL21 uninduced whole cell lysate; Lane 3, pET-28a-RGD-mda-7/BL21 induced whole cell lysate; Lane 4, pET-28a-RGD-mda-7/BL21 induced whole cell lysate supernatant; Lane 5, inclusion bodies of RGD-mda-7; Lane 6, the purification of RGD-mda-7. (C) SDS-PAGE gel analysis of wtmda-7/IL-24. Lane 1, protein marker; Lane 2, pET-28a-wtmda-7/IL-24/BL21 uninduced whole cell lysate; Lane 3, pET-28a-wtmda-7/IL-24/BL21 induced whole cell lysate; Lane 4, pET-28a-wtmda-7/IL-24/BL21 induced whole cell lysate supernatant; Lane 5, inclusion bodies of wtmda-7/IL-24; Lane 6, the purification of wtmda-7/IL-24. (D) Western blot analysis of RGD-mda-7 protein. Lane 1, wtmda-7/IL-24; Lane 2, RGD-mda-7; Lane 3, blank control without anti-IL-24 mAb.

SDS-PAGE. The result showed that the recombinant wtmda-7/IL-24 and RGD-mda-7 proteins were expressed efficiently with the yield accounting for 30% of total bacterial proteins. SDS-PAGE analysis also showed that the recombinant proteins were mainly in the precipitation of cell lysate, indicating that the recombinant proteins existed predominantly in the inclusion bodies. After washing with $1 \times$ IB wash buffer and 2 mol/L urea, the recombinant proteins were renatured by dialysis according to the steps of the manual.

The dialyzed proteins were purified with nickel metal-affinity resin columns for single-step purifications of His-tagged in wtmda-7/IL-24 and RGD-mda-7. The purity of purified proteins was about 90% (Figures 2B and 2C). Western blot analysis confirmed that RGD-mda-7 was recognized by anti-mda-7/IL-24 monoclonal antibody (Ambion) (Figure 2D).

Immunostimulatory Activity of RGD-mda-7

Data indicate that mda-7/IL-24 protein functions as a pro-Th1 cytokine in PBMC and induces secretion of IL-6, TNF- α , and INF- γ .^[2,3] To address the biological function of RGD-mda-7, its induction of IL-6, TNF- α , and INF- γ secretion by PBMC was examined. Optimum levels of wtmda-7/IL-24 and RGD-mda-7 to induce maximal IL-6, TNF- α , and INF- γ secretion were determined in a dose-response curve. Moreover, as shown in Figure 3, the abilities of inducing IL-6, TNF- α , and INF- γ productions by RGD-mda-7 were higher than wtmda-7/IL-24, suggesting that the immunostimulatory activity of purified RGD-mda-7 was as effective as that of wtmda-7/IL-24. It is well documented that inserted Gly has no effect on the immunostimulatory activity of the wtmda-7/IL-24 protein.

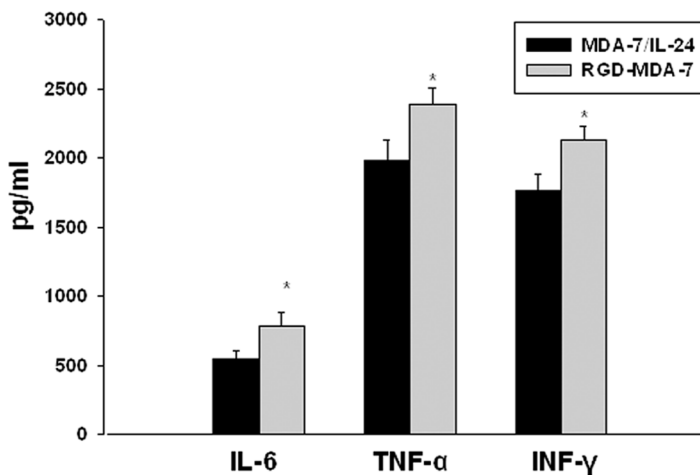


FIGURE 3 Effect of RGD-mda-7 on IL-6, INF- γ , and TNF- α inflammatory cytokine secretion from PBMC. Two milliliters per well (1×10^6 cells/mL) PBMC were plated in a 24-well plate and cultured with 8 μ g/mL wtmda-7/IL-24 and RGD-mda-7. Supernatants were harvested at 48 h and analyzed for cytokine content by ELISA according to the manufacturer's instructions. Data were expressed as mean \pm S.D. from six independent groups (n = 6). *P < 0.05 vs. wild type mda-7/IL-24.

RGD Motif Targets mda-7/IL-24 to Cancer Cells

Integrin $\alpha V\beta_3$, $\alpha V\beta_5$ plays a significant role in tumor progression, invasion, and metastasis, and is a receptor for RGD tripeptide sequence.^[18] To investigate whether the RGD domain of RGD-mda-7 is functional and accessible to integrin $\alpha V\beta_3$, $\alpha V\beta_5$, we observed cell adhesion enhanced by RGD-mda-7 and compared to wtmda-7/IL-24. As shown in Figures 4A and 4B, adhesion and spreading of MCF-7 cells and Ket-3 cells occurred on plates coated with RGD-mda-7 but not on plates coated with wtmda-7/IL-24. Moreover, adhesion of MCF-7 cells and Ket-3 cells, which were incubated with antibodies LM609 (anti- $\alpha V\beta_3$) and 15F11 (anti- $\alpha V\beta_5$), was reduced on plates coated with RGD-mda-7. These results indicate that RGD domain of RGD-mda-7 was properly folded and able to interact with integrin $\alpha V\beta_3$, $\alpha V\beta_5$, and targets mda-7/IL-24 to cancer cells.

The mda-7/IL-24 binds to its cognate receptor complexes consisting of two sets of heterodimeric chains, IL-20R1 and IL-20R2, or IL-22R1 and IL-20R2, and activates signal transducer and activator of transcription (STAT) 3 signaling. However, mda-7/IL-24 can induce cancer-selective apoptosis even in the absence of JAK/STAT signaling. As shown in Figure 4, nonspecific adhesion occurred on plates coated with wtmda-7/IL-24; therefore, we speculate that the levels of wtmda-7/IL-24 receptors are very low on MCF-7 cells and Ket-3 cells, or binding of wtmda-7/IL-24 and its receptors is not intense. In the present study, the results showed that the RGD can promote the binding ability of RGD-mda-7 protein to MCF-7 cells and Ket-3 cells. Similarly to our findings, Zarovni's study found RGD-TNF induced antitumor effects without shedding of sTNF-R2.^[21]

Potent Cytopathic Effect Induced by RGD-mda-7

To assess the cytopathic effect of RGD-mda-7, MTT assay was also performed to examine cell viability. Cells (MCF-7 and Ket-3) were plated in 96-well plates and treated with RGD-mda-7 compared with wtmda-7/IL-24 at different concentrations as indicated. At the same time, we added increasing concentrations of an anti-MDA7 neutralizing antibody to RGD-mda-7 treated cells. As shown in Figure 5, treatment of MCF-7 and Ket-3 cells with RGD-mda-7 resulted in a time- and dose-dependent cytopathic effect (CPE), and as was expected, RGD-mda-7 could kill tumor cells more powerfully than wtmda-7/IL-24. Apparently, RGD-mda-7 achieved antitumor efficacy that was much more superior to wtmda-7/IL-24 by RGD sequence to interact with Integrin $\alpha V\beta_3$, $\alpha V\beta_5$ in the tumor cell membrane. Moreover, in MCF-7 cells and Ket-3 cells, neutralization of RGD-mda-7 significantly decreased cell killing ($P < 0.01$) compared to the addition of a nonspecific IgG antibody (Figure 5C). These results suggest that the

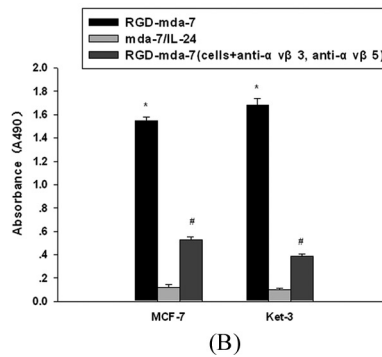
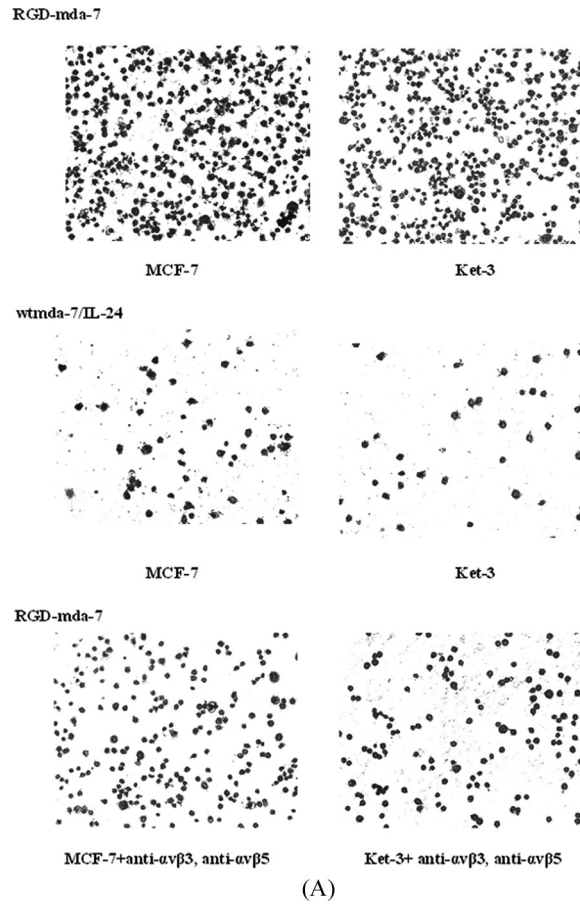


FIGURE 4 The binding specificity of RGD-mda-7 to cancer cells. Microtiter wells were coated with RGD-mda-7 (8 $\mu\text{g}/\text{mL}$) or wtmda-7/IL-24, and seeded with MCF-7; Ket-3 cells which were incubated with antibodies anti- $\alpha\text{V}\beta_3$ and anti- $\alpha\text{V}\beta_5$. (A) Images were acquired after staining with crystal violet (original magnification $\times 100$). (B) Absorbance was read at 490 nm. Data were expressed as mean \pm S.D. from six independent groups ($n = 6$). * $P < 0.05$ vs. wild type mda-7/IL-24, # $P < 0.05$ vs. MCF-7, Ket-3 cells.

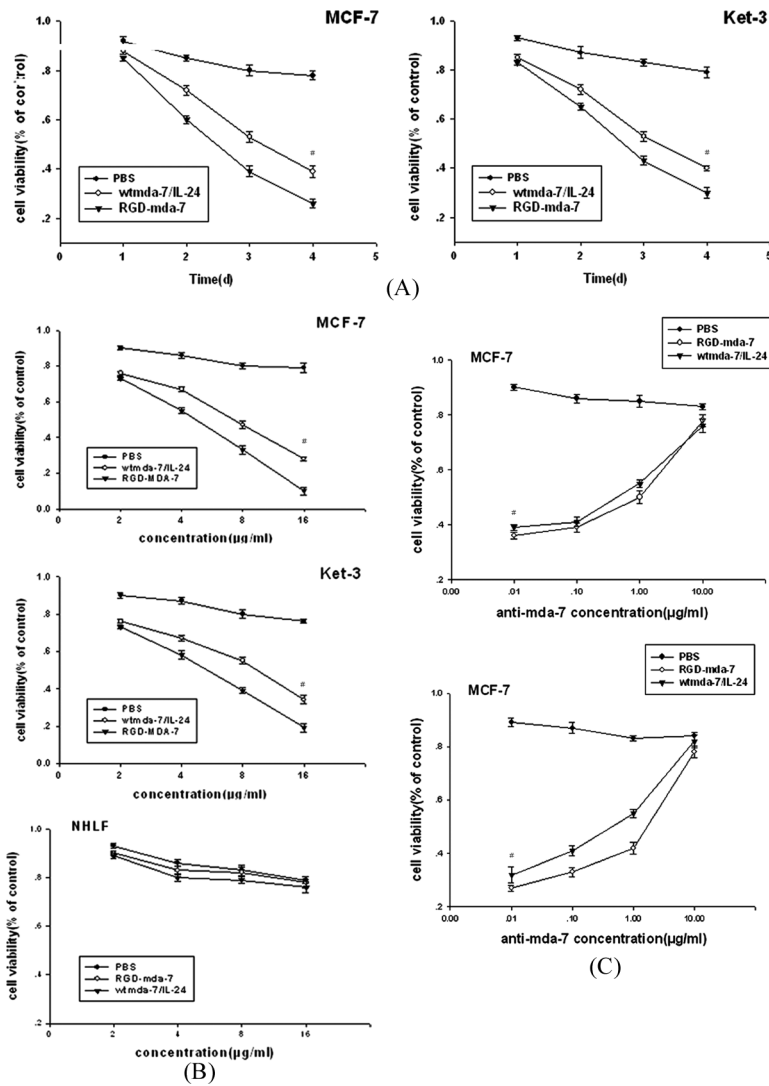


FIGURE 5 The effect of RGD-mda-7 on the growth of tumor cells. (A) Tumor cells were treated with RGD-mda-7, wtmda-7/IL-24, and PBS, respectively, at an concentration of 8 µg/mL. On days 1, 2, 3, and 4 post-treatment, cell viability was determined by the MTT proliferation assay. (B) Tumor cells were treated with RGD-mda-7, wtmda-7/IL-24, and PBS for 48 h, respectively, at different concentrations of 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL. Cell viability was determined by the MTT proliferation assay. (C) The anti-MDA-7 neutralizing antibody to inhibit the antitumor effect of RGD-MDA7. Data were expressed as mean ± S.D. from six independent groups (n = 6). P < 0.05 (RGD-mda-7 vs. wild type IL-24).

inserted Gly into the wtmda-7/IL-24 does not disrupt its biological function and selective killing effects in tumor cells, and that RGD-mda-7 inhibits the growth of tumor cells more effectively than wtmda-7/IL-24.

RGD-mda-7 Induces Apoptosis in Tumor Cells but Not in Normal Fibroblasts

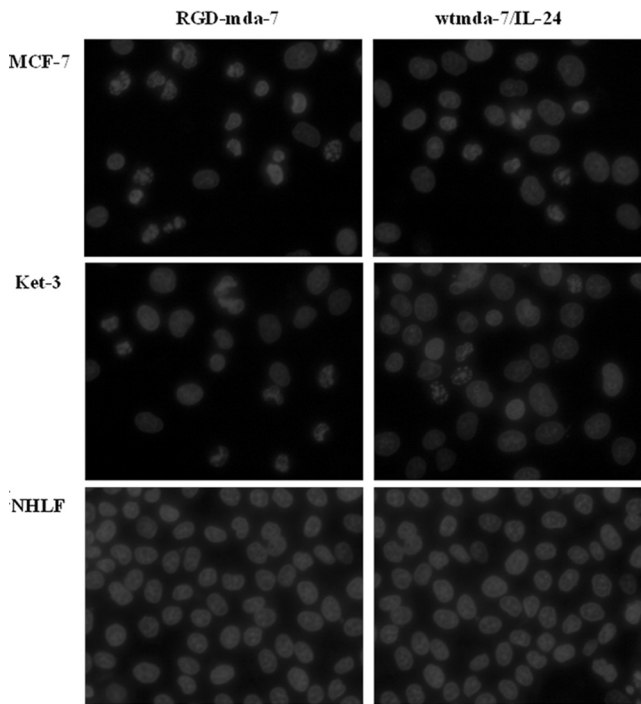
Following treatment of RGD-mda-7 or wtmda-7/IL-24, tumor cells (MCF-7 and Ket-3) and normal cells (NHLF) were analyzed for apoptotic change by DAPI staining. After 72 h, nuclear morphology was observed by DAPI staining. RGD-mda-7 elicited typical apoptotic morphological changes in tumor cells, including chromatin condensation and apoptotic body (Figure 6A). In contrast, no significant change was found in NHLF cells control group. Moreover, as shown in Figure 6B, statistical analysis showed RGD-mda-7 had much stronger apoptosis-induced activity in tumor cells than that induced by wtmda-7/IL-24. A possible explanation for the difference is that the RGD domain of RGD-mda-7 further enhanced the antitumor potency of wtmda-7/IL-24.

A recent phase I clinical trial indicates that mda-7/IL-24 is safe and induces significant clinical activity in patients with solid tumors.^[22] These observations confirm the cancer therapeutic properties of this intriguing cytokine. The combination of direct cancer specific apoptosis induction and indirect antitumor properties make mda-7/IL-24 to an ideal cancer therapeutic. The most important fail-safe for mda-7/IL-24 is its non-toxicity towards normal cells that might be exploited to target expression of mda-7/IL-24 to both cancer and normal cells, the latter serving as a reservoir for continuous secretion of mda-7/IL-24 protein, thereby exerting profound bystander antitumor effects. Though the secretion of mda-7/IL-24 is associated with glycosylation, Sauane et al. has verified that glycosylation is not mandatory for inducing cell death or bystander activities in different cancer cells.^[23] It is imperative that mda-7/IL-24 be delivered by a vector system that employs a universal entry system in the cells and is also shielded from immunologic clearance from the circulation.

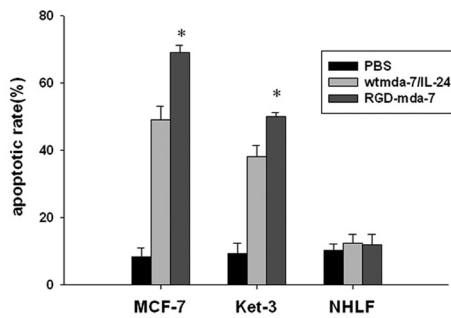
Activation of Caspase-3 and Up-Regulated Ratio of BAX to BCL-2 Following Treatment with RGD-mda-7

Since mda-7 induces apoptosis in tumor cells, the activation of caspases-3, pro-apoptotic protein bax, and anti-apoptotic protein BCL-2 were studied by Western blot analysis at 48 h after RGD-mda-7 or wtmda-7/IL-24 treatment in MCF-7 and Ket-3 cells. These results showed that RGD-mda-7 group showed a higher up-regulated ratio of BAX to BCL-2, and a higher cleaved level of caspase-3, but not in NHLF cells (Figures 7A and 7B).

A number of studies have sought to identify molecules involved in apoptotic signaling by Ad-mda7 treatment of tumor cells. Apoptotic mediators that are up-regulated or activated by mda7/IL-24 include caspases, p53,



(A)



(B)

FIGURE 6 RGD-mda-7 induced apoptosis in tumor cells but not normal cells. Tumor cells (MCF-7 and Ket-3) and NHLF cells were treated with RGD-mda-7, wtmda-7/IL-24, and PBS, respectively, at a concentration of 8 $\mu\text{g}/\text{mL}$. (A) 72 h after treatment, cells were analyzed by DAPI staining. In the two tumor cell lines, RGD-mda-7 induced apparent increase of apoptotic cell death as compared with wtmda-7/IL-24, while no increase in apoptotic cell was observed in NHLF cells. (B) Quantitative representation of the proportion of MCF-7 and Ket-3 and NHLF cells manifesting apoptotic changes. Data were expressed as mean \pm S.D. from six independent groups ($n = 6$). * $P < 0.05$ vs. wild type IL-24. (Original magnification $\times 400$.)

BAX, BAK, TRAIL, Fas, and DR4.^[24] Signaling molecules implicated in mda7/IL-24 mediated apoptosis include PKR, p38 MAPK, PI3 K, JNK, and GSK-3.^[24,25] It is evident, based on existing data, that the wide spectrum

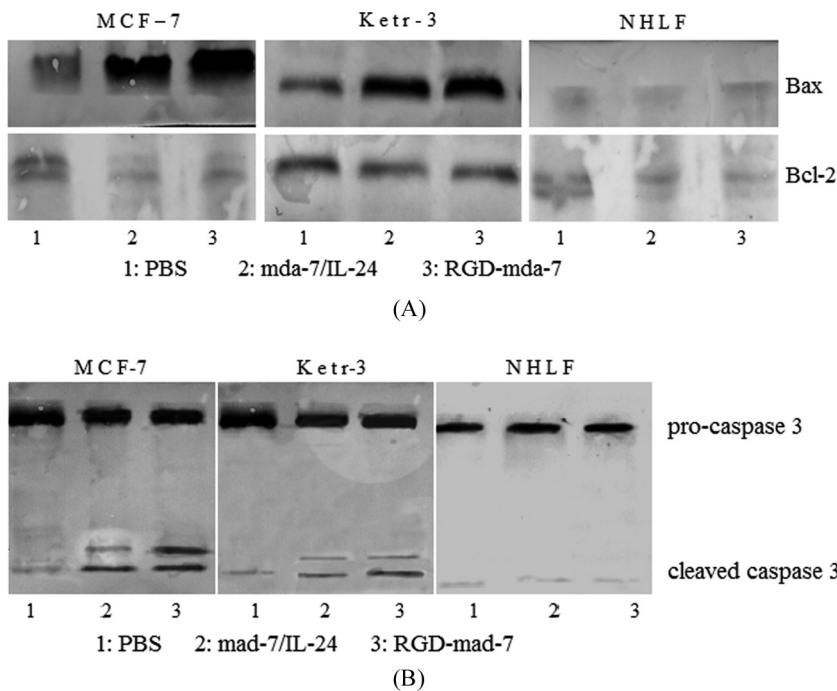


FIGURE 7 Mediation of apoptosis by RGD-mda-7 via up-regulated ratio of BAX to BCL-2 and caspase3 activation. MCF-7, Ket-3, and NHLF cells were treated with RGD-mda-7 or wtmda-7/IL-24. After 48 h, proteins were then extracted from cells and separated by SDS-PAGE. Bax, Bcl-2, and caspase-3 were observed after treatment with RGD-mda-7 or wtmda-7/IL-24 in MCF-7 and Ket-3 cells. No cleavage of caspase-3 was detectable in any PBS group. In NHLF cells activation of caspase-3 was not seen.

of tumor-specific apoptotic activity of mda-7/IL-24 involves multiple pathways. These molecules appear to be regulated only in tumor cells; however, it is not clear which are critical for tumor cell apoptosis. Our research showed a higher up-regulated ratio of BAX to BCL-2, and a higher cleaved level of caspase-3 was observed in tumor cells treated with RGD-mda-7 (Figure 7). These results demonstrate that RGD-mda-7 induced apoptosis and activated a common caspase cascade including cleavage of caspase-3.

In conclusion, we inserted Gly into mda-7/IL-24 amino acid sequence to construct an a RGD domain, and named RGD-mda-7, then expressed RGD-mda-7 in *Escherichia coli*. We describe the expression, purification, and characterization of RGD-mda-7. The mutant RGD-mda-7 not only disrupts mda-7/IL-24 biological function, but also enhances growth suppression and apoptosis in human tumor cell lines (MCF-7 and Ket-3), but not in normal cell line (NHLF). Although the antitumor mechanism RGD-mda-7 should be further elucidated for future use, our preliminary data indicate that RGD-mda-7 is an effective approach to enhancing the antitumor potency of wtmda-7/IL-24.

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