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Short Communication

Effects of the recombinant toxin protein rLj-RGD3 in multidrug-resistant human breast carcinoma cells

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We have previously reported that Lj-RGD3, a novel RGD-toxin protein, was isolated from the buccal gland of *Lampetra japonica*. The recombinant protein rLj-RGD3 has anti-invasive and anti-adhesive activity in tumor cells (HeLa cells) and endothelial cells (ECV304 cells) *in vitro*, and inhibits αvβ3, αvβ5, and β1 integrin-mediated adhesion. In this study, we investigated the bioactivity of rLj-RGD3 in the drug-resistant MCF-7/Adr breast carcinoma cell line and drug-sensitive parental line MCF-7, and found that rLj-RGD3 inhibited the growth of both cell lines. Biological function studies revealed that rLj-RGD3 could induce the apoptosis in MCF-7/Adr, which was more prevalent than that in the drug-sensitive parental line MCF-7. In addition, rLj-RGD3 inhibited the adhesion of MCF-7/Adr cells to fibronectin. Furthermore, rLj-RGD3 prevented invasion of MCF-7/Adr cells through an artificial matrigel basement membrane. In summary, rLj-RGD3 may be used as a potential drug in multidrug-resistant breast cancer therapy.

Keywords *Lampetra japonica*; RGD-toxin protein; integrin; breast cancer

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Introduction

Integrins are important α/β heterodimeric cell surface glycoproteins and receptors that are involved in both cell–cell and cell–extracellular matrix (ECM) interactions [1]. Interactions between cells and the ECM control a number of biological functions, including cell proliferation, survival, migration, and differentiation. Adhesion of cells to the ECM is mediated by the binding of cell surface integrins to the ECM, and this binding activates intracellular signaling cascades and tension-dependent changes in cell morphology and cytoskeletal structure [2,3]. Many studies have indicated that peptides containing the RGD sequence could bind competitively to integrins on the surface of tumor cells and inhibit binding of the cancer cells to the ECM [4,5]. Therefore, disrupting these integrins interactions with the ECM should have significant anti-tumor activities.

In our previous studies, we isolated and characterized three novel RGD-toxin proteins from the buccal gland secretion of *Lampetra japonica*: Lj-RGD1, Lj-RGD2, and Lj-RGD3. Then we cloned the Lj-RGD3 gene and obtained the recombinant protein rLj-RGD3. In 2010, Wang *et al.* [6] reported that rLj-RGD3 inhibited basic fibroblast growth factor (bFGF)-induced cell proliferation, fibrinogen-dependent platelet aggregation, integrins (αvβ3, αvβ5, and β1)-mediated adhesion, bFGF-induced angiogenesis, and experimental tumor metastasis. Furthermore, they suggested that the RGD motifs in Lj-RGD3 appeared to be in a linear array and were not cyclic like the snake venom disintegrins, which might account for the difference in the integrin binding affinity of rLj-RGD3. There are some studies about the effect of rLj-RGD3 on ECV304 cells and HeLa cells; however, the biological effects of rLj-RGD3 on multidrug-resistant human breast carcinoma cells have not yet been investigated.

In this study, we showed for the first time the effects of rLj-RGD3 in MCF-7/Adr cells and MCF-7 cells. Results indicated that rLj-RGD3 could be valuable in the development of anti-multidrug-resistant tumor agents.

Materials and Methods

Cell line and cell culture

The human breast carcinoma cell line MCF-7, which is sensitive to adriamycin *in vitro*, and its adriamycin-resistant variant MCF-7/Adr were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 1% antibiotics (1 × penicillin/100 U/ml streptomycin) (Gibco, Grand Island, USA). In addition, MCF-7/Adr cells were maintained in culture containing 1 μg/ml of adriamycin. Cells were cultured in an incubator with a controlled humidified

atmosphere containing 5% CO_2 at 37°C. Cell culture media and reagents were purchased from Gibco.

Purification of recombinant Lj-RGD3

Escherichia coli BL21 with pET28a-(Lj-RGD3) was kept in our lab. Recombinant Lj-RGD3 was purified as described previously [6]. Briefly, *E. coli* BL21 with pET28a-(Lj-RGD3) was grown at 30°C for 12 h in Luria–Bertani medium, and then 1 mM (final concentration) of isopropyl β-D-thiogalactoside was added to induce recombinant protein (rLj-RGD-His) expression in soluble form. The fusion protein, rLj-RGD3-His, was purified with His·Bind columns according to the manufacturer's protocol (Novagen, Gibbstown, USA). Cells were harvested and resuspended in ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and sonicated on ice. After centrifuging at 14,000 *g* for 20 min, the supernatant was filtered through a 0.45-μm membrane and incubated with Ni^{2+}-nitrilotriacetate resin. Bound fusion protein was eluted with increasing amounts of imidazole and then dialyzed against phosphate-buffered saline (PBS). The protein concentration was determined by a bicinchoninic acid assay (Beyotime Biotechnology, Haimen, China), and the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Cell proliferation assay

The effects of rLj-RGD3 on cellular proliferation were measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2×10^3) were plated onto 96-well plates and maintained in complete medium with 3 ng/ml of bFGF (PeproTech EC, London, UK) for 24 h. Then, the cells were treated with rLj-RGD3 at various concentrations for 24 h. The cells were incubated with MTT (5 mg/ml; Invitrogen, Carlsbad, USA) for 4 h at 37°C, and 150 μl of dimethyl sulfoxide was added for 10 min at room temperature to solubilize the crystals. The absorbance values were measured by a microplate reader (Bio-Rad, Hercules, USA) at a wavelength of 490 nm.

Apoptosis assays

To confirm that rLj-RGD3 causes apoptosis in human breast carcinoma cells, a Hoechst apoptosis kit (Beyotime Biotechnology) and a DNA ladder kit (Beyotime Biotechnology) were used. For the Hoechst staining assay, MCF-7 cells (1×10^6) were plated on coverslips in six-well plates and left overnight. The next day, cells were treated with PBS or rLj-RGD3 for 24 h. The cells were then fixed in methanol/acetic acid (3 : 1) for 10 min. After fixation, the cells were washed with PBS and stained with Hoechst 33258 for 15 min, and nuclear morphology was examined by fluorescence microscopy. The cells with apoptotic morphology, characterized by condensed or fragmented nuclei, were counted.

For the DNA ladder detection assay, MCF-7 cells (2×10^6) were treated with rLj-RGD3 for 24 h and collected. The DNA ladder extraction was performed as described in the instruction manual of the DNA ladder kit. The eluents containing the DNA pellets were electrophoresed on a 1.5% agarose gel, which was then stained with ethidium bromide (EtBr). The gel was examined and photographed by an ultraviolet gel documentation system.

Staining of cells for F-actin

The effects of rLj-RGD3 on the cell F-actin structure were visualized by staining F-actin with fluorescein isothiocyanate (FITC)–phalloidin (Enzo Life Sciences, Farmingdale, USA) according to the manufacturer's instructions. Briefly, cells were treated with 10 μM of rLj-RGD3 for 4 h. The treated monolayers were washed once with PBS (pH 7.4) at 37°C. Then, cells were fixed for 20 min in 3% formaldehyde at room temperature. After being washed three times with PBS, cells were incubated with 0.1% Triton X-100 in PBS for 5 min. The cells were washed three times with PBS. They were then treated with 5 mg/ml of FITC-conjugated phalloidin in PBS for 30 min. After two washes in PBS to remove any trace of non-specific fluorescence, the cells were examined for cytoskeletal actin under a fluorescence microscope.

Cell adhesion assay

A cell adhesion assay [7] was used to investigate the ability of rLj-RGD3 to inhibit MCF-7/Adr cell adhesion to immobilized fibronectin (FN). FN fragments were non-covalently immobilized through passive adsorption to 96-well microtiter plates. Briefly, FN (1 μg/well) was reconstituted in 0.5 M NaCl–0.05 M Tris (pH 7.5) and incubated from 4 to 24 h at room temperature. The polystyrene surface was subsequently blocked with 5% bovine serum albumin (BSA; Sigma, St Louis, USA) overnight at 37°C. Prior to use, the plates were washed four times with PBS, and excess liquid was removed from the wells. Cells (10^5 cells/ml) were treated with or without rLj-RGD3 protein for 30 min before application to microtiter wells (100 μl/well) that were pre-coated with FN. Pretreated cells were allowed to adhere for 1 h at 37°C. After non-adherent cells were washed away, the number of adherent cells for each condition was estimated with a MTT assay.

In vitro cell migration and invasion assay

The inhibitory effect of rLj-RGD3 on MCF-7/ADR cell invasion was demonstrated *in vitro* using 24-well transwell units (Corning, New York, USA). These units had 8-μm pore size polycarbonate filter coated with ECMatrix gel (Chemicon, Temecula, USA) to form a continuous thin

layer. Cells (3×10^5) were harvested in serum-free medium containing 0.1% BSA and were added to the upper chamber of the unit. The lower chamber contained 500 μl of RPMI 1640. Cells were incubated for 24 h at 37°C with 5% CO_2. At the end of incubation, the cells on the upper surface of the filter were completely removed by wiping the filter with a cotton swab. Then, the filters were fixed in methanol and stained with Wright–Giemsa. Cells that had invaded the matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of ×400. Triplicate samples were acquired, and the data were expressed as the average cell number of five fields.

Statistical analysis

SPSS12.0 software was used. The data were expressed as the mean ± SD, and Student's *t*-test was used to determine the significance of differences in multiple comparisons. $P < 0.05$ was considered to be statistically significant. Each assay was performed at least three times.

Results

rLj-RGD3 protein inhibits proliferation and induces apoptosis in human breast carcinoma cells

MTT assays were carried out to examine the effect of rLj-RGD3 on the growth of human breast carcinoma cells. As shown in **Fig. 1**, rLj-RGD3 could inhibit the proliferation of MCF-7 cells and MCF-7/Adr cells in a dose-dependent manner, with IC_{50} values of 35 and 7.9 μM, respectively. However, the IC_{50} of rLj-RGD3 was much lower in MCF-7/Adr cells [**Fig. 1(B)**] than that in MCF-7 cells [**Fig. 1(A)**], indicating that rLj-RGD3 inhibits cell growth more robustly in MCF-7/Adr than in MCF-7.

To investigate the effect of rLj-RGD3 on the survival of human breast carcinoma cells, a Hoechst apoptosis kit and a DNA ladder kit were used. Human breast carcinoma cells, incubated with rLj-RGD3 protein or PBS, were stained with Hoechst 33258. The cells treated with rLj-RGD3 protein show chromatin condensation in their nuclei [**Fig. 2(A)**]. In both MCF-7 and MCF-7/Adr cells, cells undergo apoptosis in a concentration-dependent manner after treatment with rLj-RGD3. However, almost no apoptotic nuclei were detected in the cells that were treated with PBS [**Fig. 2(A)**]. In addition, a genomic DNA ladder formation was clearly observed when human breast carcinoma cells were treated with 15 μM of rLj-RGD3 protein for 24 h [**Fig. 2(B)**].

rLj-RGD3 induces the aggregation of F-actin

Apoptosis is closely associated with changes in the organization of F-actin, which is related to the formation of apoptotic bodies. To examine whether rLj-RGD3 is associated with the redistribution of actin, F-actin was stained with FITC-phalloidin and visualized by fluorescent microscope. The staining pattern of the actin in untreated MCF-7 and MCF-7/Adr cells showed a continuous lined distribution at the cell cytoskeleton and borders (**Fig. 3**). In contrast, the actin architecture in cells that were pretreated with rLj-RGD3 is disorganized and aggregated. The incubation of MCF-7/Adr with 10 μM rLj-RGD3 resulted in a centripetal retraction of the perijunctional actin filaments with separation of actin from the apical cellular membrane (**Fig. 3**). In contrast, treatment with 10 μM rLj-RGD3 only slightly changed the appearance of MCF-7. This result was consistent with those found by the cell proliferation assay.

rLj-RGD3 inhibits the adhesion of human breast carcinoma cells to FN

Our previous studies indicated that rLj-RGD3 blocks integrin-mediated cell adhesion to vitronectin (VN) through binding to the αvβ3, αvβ5, and β1 integrins [6]. We therefore sought to determine whether the rLj-RGD3 could inhibit α5β1–FN interactions. The ability of rLj-RGD3 to

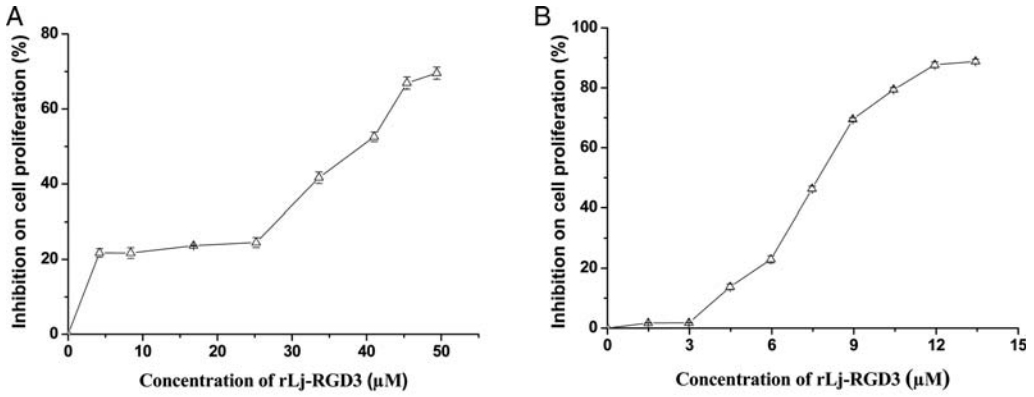

Figure 1 Cytotoxicity of rLj-RGD3 protein in human breast carcinoma cells MCF-7 (A) or MCF-7/Adr (B) cells were treated with indicated amount of rLj-RGD3 protein for 24 h, and a MTT assay was used to examine proliferation. These data were obtained from three independent experiments.

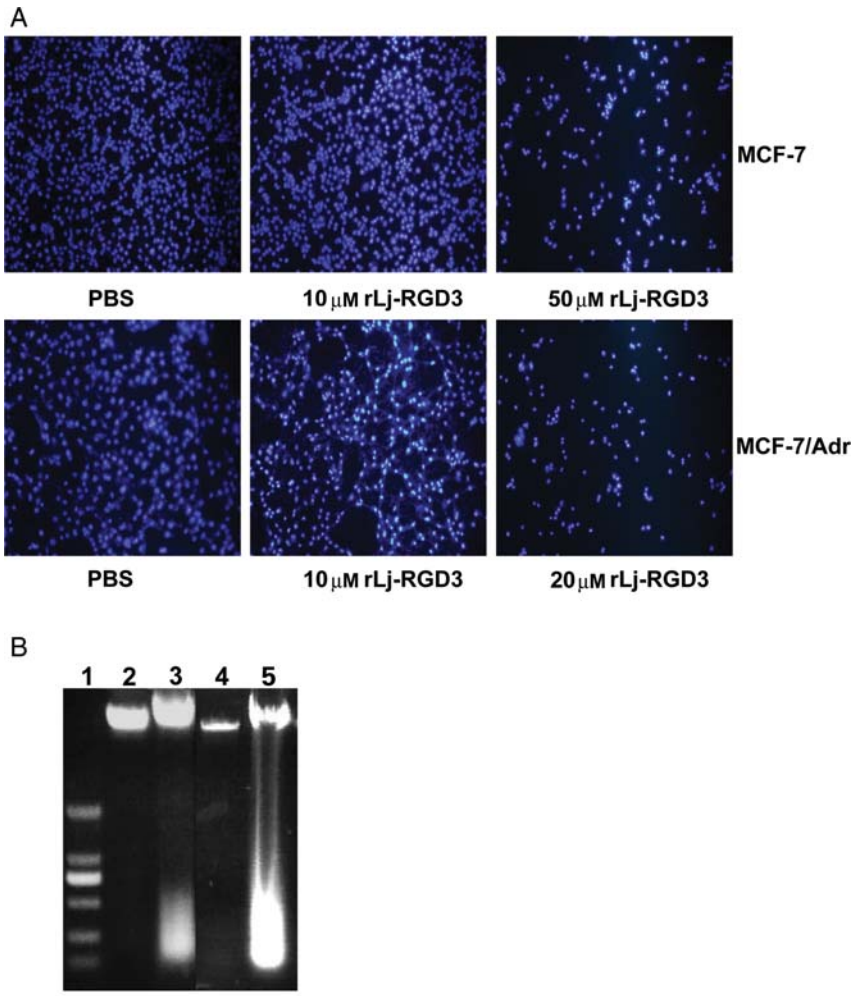

Figure 2 rLj-RGD3 induces apoptosis in MCF-7/Adr cells (A) rLj-RGD3 protein induced chromatin condensation in human breast carcinoma cells. The cells were stained with a fluorescent DNA-binding dye, Hoechst 33258, to identify the apoptotic nuclei. Pictures (×100) were taken under an Olympus microscope. (B) MCF-7 cells were incubated with PBS (lane 2) or rLj-RGD3 (lane 3), and MCF-7/Adr cells were incubated with PBS (lane 4) or rLj-RGD3 (lane 5). All cells were incubated for 24 h and lysed. Lane 1 is the DNA marker. The total cellular DNA was isolated and subjected to electrophoresis.

inhibit the adhesion of MCF-7/Adr cells on immobilized FN was investigated. The adhesion of human breast carcinoma cells to FN was abrogated by rLj-RGD3 (**Fig. 4**), indicating that rLj-RGD3 strongly inhibited the adhesion of MCF-7/Adr cells to FN. In other words, rLj-RGD3 can competitively inhibit the adhesion of human breast carcinoma cell to FN.

rLj-RGD3 inhibits migration and invasion in MCF-7/Adr cells

Previous studies have shown that rLj-RGD3 is capable of inhibiting migration of ECV304 [6]; thus, we also examined whether the rLj-RGD3 could influence the motility of human breast carcinoma cells. The ability of cells to migrate through uncoated porous filters was examined in a Transwell migration assay [**Fig. 5(A)**]. The results showed that rLj-RGD3 significantly inhibits the migration of human breast carcinoma cells. To examine whether the rLj-RGD3 in human breast carcinoma cells affected their invasive ability, we performed an *in vitro* ECMatrix gel analysis. The MCF-7/Adr cell line differs from the parent MCF-7 line by demonstrating much higher invasion activity *in vitro* [8]. The results showed that the number of rLj-RGD3-treated MCF-7/Adr cells passing through the ECMatrix gel was markedly lower than the number of untreated MCF-7/Adr cells [**Fig. 5(B)**].

Discussion

RGD motifs and other integrin-binding sites have been found in snake venom and the buccal gland of *L. japonica*, enabling them to affect processes such as blood coagulation [4,6,9–14]. Disintegrins, which are RGD-containing peptides, can serve as antagonists targeting a broad range

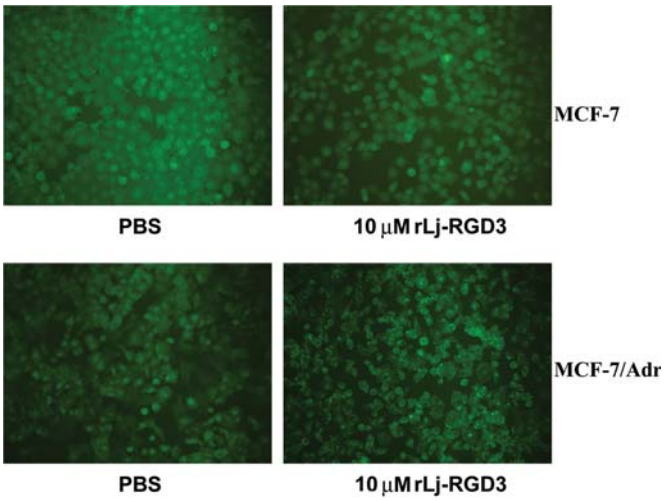

Figure 3 Immunocytochemical staining of F-actin The cells were treated with PBS or 10 μM rLj-RGD3 for 4 h. The cells were fixed and actin filaments were visualized by FITC-phalloidin. The aggregation of actin filaments after treatment with 10 μM rLj-RGD3 was observed in MCF-7/Adr cells.

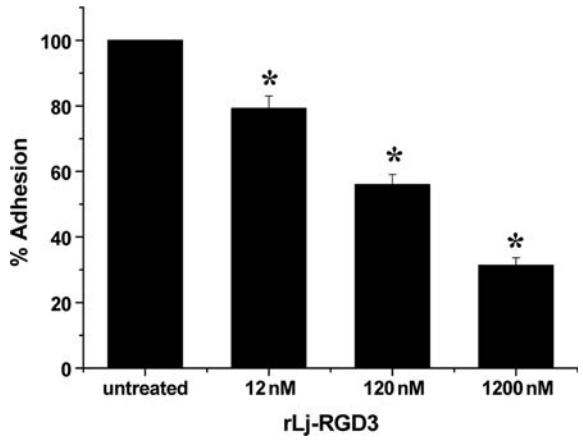

Figure 4 rLj-RGD3 inhibits adhesion of MCF-7/Adr cells to FN Pretreatment of MCF-7/Adr cells with various concentrations of rLj-RGD3 for 30 min inhibited the adhesion of MCF-7/Adr cells to immobilized FN. Pretreated cells were allowed to adhere for 1 h at 37°C. The number of adherent cells for each condition was estimated using the MTT assay after non-adherent cells were washed away. The adhesion in cells treated with rLj-RGD3 was significantly less ($*P < 0.05$) than the control group (untreated). These data were obtained from three independent experiments.

of integrins, can disrupt many of these tumor cell–tissue interactions. For this reason, integrins present a very attractive target for anticancer drug development. Because the disintegrin class of integrin antagonists consists of small peptides, which neither activate the immune system nor appear to be toxic, their potential side effects are minimized. Therefore, disintegrins represent an attractive alternative to conventional anti-angiogenic agents. In our previous study, we have cloned the Lj-RGD3 gene from the buccal gland of the lampreys and obtained the recombinant protein rLj-RGD3 [6]. We found that rLj-RGD3 is a novel RGD-toxin protein which has the typical characteristics of inhibiting platelet aggregation and angiogenesis.

In 2010, Karp [15] reported that the RGD motifs of integrin-binding ligands (such as fibrinogen, FN and VN) were shown to bind to integrins, then activate the signaling pathway involved in cell growth and proliferation. In this study, rLj-RGD3 incubated in MCF-7/Adr for 24 h causes significant anti-proliferative effects. In contrast, incubation with the same concentration of rLj-RGD3 in MCF-7 cells does not change. The difference of rLj-RGD3 inhibition in MCF-7 versus MCF-7/Adr cells may be determined by different cell surface expression of integrins. Our previous research showed that rLj-RGD3 protein inhibited β1 integrin-mediated adhesion of ECV304 cells [6]. Therefore, we strongly suggest that rLj-RGD3 can serve as strong antagonist of β1 integrin. Previous studies from our lab (data not shown) and others [8] showed that MCF-7 and MCF-7/Adr cells were strongly different in the integrins expressed. However, the detailed mechanism need to be further investigated. Apoptosis was detected by analyzing morphological changes of the nucleus, e.g. heterochromatin condensation and nuclear fragmentation. Our data indicated that same concentration of rLj-RGD3 induced apoptosis between the two cell types with significant differences. This is consistent with our cell proliferation assay results. These results demonstrate that rLj-RGD3 induces apoptosis in MCF-7/ADR cells more robustly than in MCF-7 cells.

To assess the ability of rLj-RGD3 to block processes critical to tumor survival and progression (adhesion, migration, and invasion), we measured the inhibitory effect of rLj-RGD3 in MCF-7/Adr cells. Major cytoskeletal filaments, including microtubules, cytokeratin, and actin, are degraded during the execution phase of apoptosis [16]. When cell apoptosis is underway, actin depolymerization and changes in cell structure occur. Vicrostatin, designed based on the sequence of contortrostatin (CN) and engineered as a novel recombinant disintegrin, induced disruption of the actin cytoskeleton in human umbilical vein endothelial cells and mediated the dissociation of talin from the cytoplasmic domain of the β1 integrin. Therefore, we examined the morphological changes in human breast carcinoma cells treated with rLj-RGD3 by FITC–phalloidin staining. The data indicated that the aggregation of actin filaments induced by rLj-RGD3, may initiate apoptosis in MCF-7/Adr cells.

Invading cancer cells express various adhesion molecules to facilitate their interaction with the matrix. These interactions enable the tumor cells to migrate, to escape from their primary site, and ultimately to attach to the matrix of a vessel wall, leading to extravasation [17]. In a

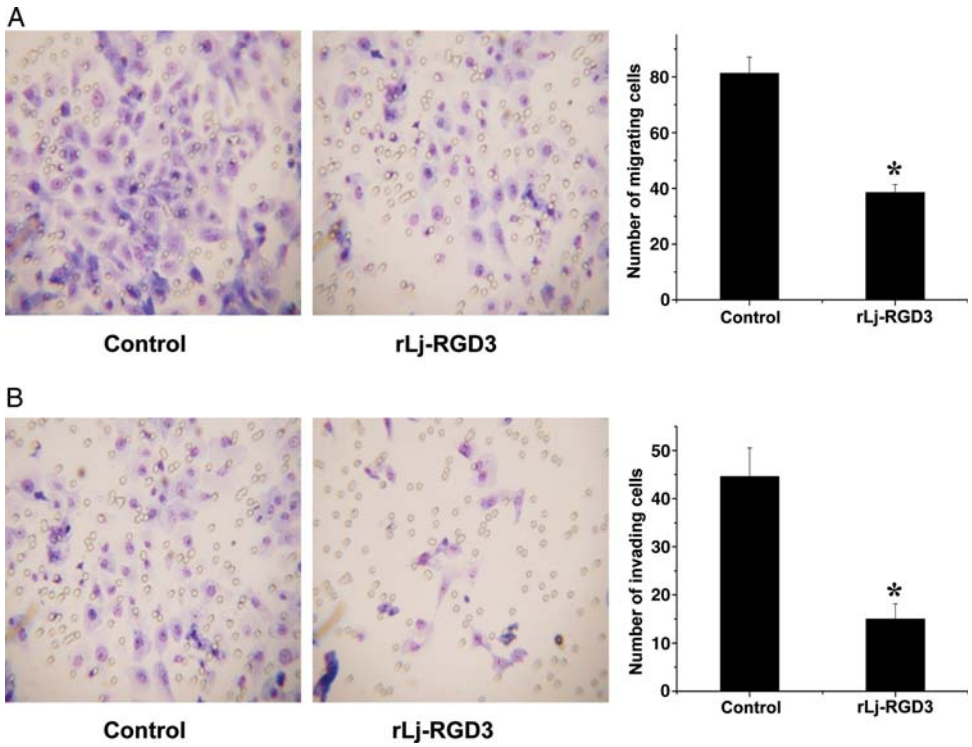

Figure 5 rLj-RGD3 inhibits migration and invasion in MCF-7/Adr cells *In vitro* transwell (A) or ECMatrix gel (B) analyses were performed. Wright–Giemsa staining results for the lower surface filters showed that the cells passed through the filter and attached to the lower side of the filter (×400). The average number of cells that invaded through the filter was determined. The migration and invasion in cells treated with rLj-RGD3 was significantly less (*$P < 0.05$) than the control cells. These data were obtained from three independent experiments.

previous study, we have shown that rLj-RGD3 can abrogate adhesion of cells to VN through binding αvβ3 integrin. Morozevich *et al*. [8] reported that α5β1, the FN-specific receptor, was a major integrin expressed in MCF-7/Adr cells. Similarly, rLj-RGD3 strongly inhibits the adhesion of human breast cancer cells to immobilized FN probably by binding to α5β1 integrin. Cell migration is an essential process in organ homeostasis. Errors during this process have serious consequences, including vascular disease, tumor formation, and metastasis. An understanding of the mechanism by which cells migrate may lead to the development of novel therapeutic strategies for controlling, for example, invasive tumor cells. In our results, rLj-RGD3 protein can effectively inhibit migration and invasion in MCF-7/Adr cells.

Calderwood [18] reported that the antitumor activity of disintegrin is based not only on blocking integrin adhesion, but also on disrupting the actin cytoskeleton and dissociating the complex between talin and the β1 integrin cytoplasmic tail. Our results were consistent with the findings from these studies. Thus, we believe that recombinant toxin protein rLj-RGD3 would be beneficial for chemotherapy in multidrug-resistant human breast carcinoma cells. Although we only evaluated the bioactivity of rLj-RGD3 in MCF-7 and MCF-7/Adr cells in this study, we would select various human tumor cell lines to further evaluate the role of rLj-RGD3 for cancer therapy in following studies. The precise mechanisms of how rLj-RGD3 affect cancer cells remain to be further clarified.

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