Low concentrations of the recombinant toxin protein rLj-RGD3 suppress TNF-α-induced human renal carcinoma cell invasion

Minli Jin1, Rong Xiao1, Jihong Wang1, Xin Liu1, Yu Liu1, Zhuang Xue1, Li Lv2, Yuanyuan Zheng1, and Qingwei Li1*

1College of Life Sciences, Liaoning Normal University, Dalian 116021, China
2College of Pharmacy, Dalian Medical University, Dalian 116044, China
*Correspondence address. Tel/Fax: +86-411-85827799; E-mail: liqw@263.net

A hallmark of renal cell carcinoma (RCC) invasion is the degradation of the extracellular matrix (ECM) by the local production of gelatinase enzymes. Matrix metalloproteinase-9 (MMP-9)-induced cancer cell invasion is one of the pivotal steps in cancer metastasis. It has been reported that tumor necrosis factor-α (TNF-α), a regulator of MMP-9, can induce invasion in human renal carcinoma cells. Previous work in our laboratory has shown that rLj-RGD3, a recombinant RGD (Arg–Gly–Asp)-toxin protein from the buccal gland secretion of Lampetra japonica, possesses anti-tumor activity. In this study, we demonstrated that rLj-RGD3 suppressed TNF-α-induced MMP-9 secretion in 786-0 cells (human renal carcinoma cells). To investigate the regulatory effect of rLj-RGD3 on TNF-α-induced MMP-9 secretion, we pre-treated cells with rLj-RGD3. Interestingly, rLj-RGD3 had no significant effect on the constitutive secretion of MMPs. However, low concentrations of rLj-RGD3 decreased TNF-α-induced MMP-9 secretion. Functional studies revealed that rLj-RGD3 induced apoptosis and significantly inhibited the proliferation, migration, and invasion of 786-0 cells. Furthermore, the actin architecture in cells pre-treated with rLj-RGD3 was aggregated and disorganized. Our findings suggest that rLj-RGD3 may be used as a potential drug in renal cancer therapy.

Keywords renal cancer; MMP-9; RGD-toxin protein; Lampetra japonica

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Introduction

Renal cell carcinoma (RCC), which accounts for 3% of adult malignancies, is an aggressive vascular cancer that is characterized by lack of early warning signs, resistance to standard radiation and chemotherapy treatments, and poor outcome due to metastasis [1]. Once metastases have occurred, the average survival is only 4 months, and only 10% of patients will survive for 1 year. One of the general properties of the cancer cells is their ability to invade surrounding tissues and metastasize to other organs [2]. Tumor invasion requires the degradation of the extracellular matrix (ECM) proteins and the migration of cells [3]. Lysis of the ECM by specific proteinases, including matrix metalloproteinase-2 (MMP-2) and MMP-9, is involved in cell invasion [1]. Therefore, MMPs are potential promising therapeutic targets in cancer treatment [4]. MMPs are a family of structurally related zinc-dependent endopeptidases capable of degrading the ECM and basement membrane, both in physiological conditions and in pathological events [5]. Elevated expression of MMPs contributes to various pathological processes, including angiogenesis, osteoarthritis, rheumatoid arthritis, invasion, and metastasis in carcinomas [6–8]. Among the members of the MMP family, MMP-2 (gelatinase A or 72 kDa type IV collagenase) and MMP-9 (gelatinase B or 92 kDa type IV collagenase) have been implicated in tumor invasion and metastasis. Zhang et al. [9] reported that there is a positive correlation between the expression of MMP-9 and the pathological and histological grade in RCC cases.

Tumor necrosis factor-α (TNF-α) is one of the inflammatory cytokines that are associated with malignant tumor progression. Specifically, studies on the effects of TNF-α using experimental models of invasion and metastasis have shown that it can act as a tumor-promoting factor in melanoma [10]. TNF-α has been reported to upregulate the expression of integrin subunits and increase the interaction of human melanoma cells with ECM substrates [11,12]. In addition, TNF-α has been shown to induce MMP-9 expression, which, in turn, degrades the ECM during inflammatory responses.

In our previous studies, we isolated and characterized the following novel RGD-toxin proteins from the buccal gland secretion of Lampetra japonica: Lj-RGD1, Lj-RGD2, and Lj-RGD3. We cloned the Lj-RGD3 gene, generated the recombinant protein rLj-RGD3, and results showed that
rLj-RGD3 inhibited HeLa and MCF-7/Adr cell invasion and chicken chorioallantoic membrane angiogenesis. Furthermore, rLj-RGD3 decreased the adhesion of tumor cells (HeLa and MCF-7/Adr cells) and endothelial cells (ECV304 cells) in vitro. The mechanisms of rLj-RGD3 action included the inhibition of vitronectin or fibronectin-dependent tumor cell adhesion, destruction of the cytoskeleton, and blockade of tumor cell invasion through a reconstituted basement membrane (Matrigel) [13,14]. These findings led to further investigations about the functional effect of rLj-RGD3 on human carcinoma cell invasion in vitro.

In the present study, we sought to determine whether rLj-RGD3 had an anti-tumor effect on 786-O cells. We postulated that the anti-angiogenic and anti-invasive activity of rLj-RGD3 might be due to the modulation of MMP-9 secretion associated with the migration and invasion of tumor cells.

**Materials and Methods**

**Purification of recombinant Lj-RGD3**

*Escherichia coli* BL21 with pET28a-(Lj-RGD3) was provided by Dr Jihong Wang at Liaoning Normal University. Recombinant Lj-RGD3 was purified as described previously [14]. Briefly, *E. coli* BL21 with pET28a-(Lj-RGD3) was grown at 30°C for 12 h in Luria–Bertani medium, and then isopropyl-β-D-thiogalactoside (IPTG, 1 mM, final concentration) was added to induce recombinant protein (rLj-RGD3-His) expression in soluble form. The fusion protein, rLj-RGD3-His, was purified with His Bind Columns according to the manufacturer’s protocol (Novagen, Darmstadt, Germany). Cells were harvested and resuspended in ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) and sonicated on ice. After centrifuging the lysate at 14,000 g for 20 min, the supernatant was filtered through a 0.45-μm membrane and incubated with Ni-NTA resin. Bound fusion protein was eluted with increasing concentrations of imidazole and then dialyzed against phosphate-buffered saline (PBS). The protein concentration was determined by a bicinchoninic acid assay (Enzo Life Sciences, Lausen, Switzerland) according to the manufacturer’s instructions. Briefly, cells were treated with 2 μM of rLj-RGD3 for 4 h and 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, and then treated with 5 mg/ml of fluorescein isothiocyanate (FITC)-conjugated phalloidin in PBS for 30 min. After two washes with PBS to remove any traces of non-specific fluorescence, the cells were examined for cytoskeletal actin under a fluorescence microscope.

**Cell proliferation assay**

The effects of rLj-RGD3 on cellular proliferation were measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2 × 10^4) were plated onto 96-well plates and maintained in complete medium with 3 ng/ml of basic fibroblast growth factor (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 ml of dimethyl sulfoxide was added for 10 min at room temperature to solubilize the crystals. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, USA).

**Apoptosis assays**

To confirm that rLj-RGD3 causes apoptosis in human renal carcinoma cells, a Hoechst apoptosis kit (Beyotime Biotechnology) and a DNA ladder kit (Beyotime Biotechnology) were used. For the Hoechst staining assay, 786-0 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, 786-0 cells (2 × 10^5) were treated with rLj-RGD3 for 24 h and collected. DNA ladder extraction was performed as described in the instruction manual of the DNA ladder kit. The eluents containing DNA were electrophoresed on a 1.5% agarose gel, which was then stained with ethidium bromide. The gel was examined and photographed by an ultraviolet light gel documentation system.

**Staining of cells for F-actin**

The effects of rLj-RGD3 on the F-actin structure of the cell were visualized by staining F-actin with FITC-phalloidin (Enzo Life Sciences, Lausen, Switzerland) according to the manufacturer’s instructions. Briefly, cells were treated with 2 μM of rLj-RGD3 for 4 h and 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, and then treated with 5 mg/ml of fluorescein isothiocyanate (FITC)-conjugated phalloidin in PBS for 30 min. After two washes with PBS to remove any traces of non-specific fluorescence, the cells were examined for cytoskeletal actin under a fluorescence microscope.
In vitro cell migration and invasion assay
The inhibitory effect of rLj-RGD3 on 786-0 cell migration was investigated in vitro using 24-well transwell units (Corning, New York, USA). These units had an 8 μm pore size polycarbonate filter that formed a continuous thin layer. Cells (3 × 10^5) were harvested in serum-free medium containing 0.1% bovine serum albumin (BSA) and transferred to the upper chamber of the unit. The lower chamber contained 500 μl of RPMI-1640 medium. Cells were incubated for 8 h at 37°C under 5% CO₂. The invasion assay was carried out as previously described with some modifications [14]. Briefly, polycarbonate filters were coated with 10 mg of Matrigel and placed in a transwell chamber. Cells (3 × 10^5) were harvested in serum-free medium containing 0.1% BSA and added to the upper chamber of the unit. The lower chamber contained 500 μl of RPMI-1640 medium. Cell suspensions, containing 3 × 10^5 cells in RPMI-1640 medium without fetal bovine serum (FBS), were loaded into the upper compartment of the chamber in the presence of rLj-RGD3 (100 nM) or TNF-α (10 ng/ml). After incubation for 24 h at 37°C, the filters were fixed with methanol, and the cells that had invaded through the basement membrane were counted.

Gelatin zymographic assay
MMP secretion was determined as previously described [15]. 786-0 cells were incubated with or without TNF-α (10 ng/ml) for 24 h under serum-free conditions in the presence or absence of rLj-RGD3 (100 nM) or other agents (doxycycline). The cell culture medium was analyzed for proteins with gelatinolytic activity by the observation of substrate lysis in 10% SDS-polyacrylamide gels containing 1 mg/ml gelatine. Gels were washed with 2.5% Triton X-100 for 1 h and incubated for 16 h at 37°C in 50 mM Tris-HCl (pH 7.5) containing 10 mM CaCl₂. Gels were stained with Coomassie Brilliant Blue R-250 (0.2%) in 40% methanol and 10% acetic acid.

Statistical analysis
The SPSS 12.0 software (IBM, New York, USA) was used for statistical analyses. The data are expressed as the mean ± SD, and Student’s t-test was used to determine the significance of differences in multiple comparisons. A P value of <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 of human renal carcinoma cells
MTT assays were carried out to examine the effect of rLj-RGD3 on the growth of the human renal carcinoma cell line 786-0. As shown in Fig. 1, rLj-RGD3 inhibited the proliferation of 786-0 cells in a dose-dependent manner, with an IC₅₀ value of 2.15 μM. To investigate the effect of rLj-RGD3 on the survival of 786-0 cells, a Hoechst apoptosis kit and a DNA ladder kit were used. Following incubation with rLj-RGD3 protein or PBS, the cells were stained with Hoechst 33258. The cells treated with rLj-RGD3 protein showed nuclear chromatin condensation [Fig. 2(A)]. The cells underwent apoptosis in a concentration-dependent manner after treatment with rLj-RGD3. However, virtually no apoptotic nuclei were detected in the cells that were treated with PBS [Fig. 2(A)]. In addition, the formation of a genomic DNA ladder was clearly observed when 786-0 cells were treated with 10 μM rLj-RGD3 protein for 24 h [Fig. 2(B)].

rLj-RGD3 induces the aggregation of F-actin
F-actin was stained with FITC-phalloidin and visualized using a fluorescent microscope. In untreated 786-0 cells, actin showed a typical, continuous distribution along the cell cytoskeleton and borders (Fig. 3). In contrast, pre-treatment with 2 μM rLj-RGD3 caused aggregation and disorganization of the cellular actin architecture (Fig. 3).

rLj-RGD3 inhibits the migration and invasion of 786-0 cells
The ability of cells to migrate through uncoated porous filters was examined in a Transwell migration assay. The results showed that rLj-RGD3 significantly inhibited the migration of human renal carcinoma cells [Fig. 4(A,C)]. To examine whether rLj-RGD3 affected the invasive ability of human renal carcinoma cells, we performed an in vitro ECMMatrix gel analysis. The results showed that the number of rLj-RGD3-treated 786-0 cells passing through the
ECMatrix gel was markedly lower than the number of untreated cells [Fig. 4(B,C)].

rLj-RGD3 suppresses TNF-α-induced 786-0 cell invasion

A matrigel invasion assay was employed to investigate the role of rLj-RGD3 in the invasiveness of 786-0 cells. rLj-RGD3 and TNF-α were present in the upper chamber, and FBS was used as chemoattractant in the lower chamber. TNF-α, a regulator of many angiogenic genes, including MMP-9 [16], induced cell invasion, as shown in Fig. 4(D). This cell invasion was reduced by >60% in the presence of 100 nM rLj-RGD3 [Fig. 4(E,F)].

discussion

The survival rate of patients with malignant carcinomas is extremely poor because of recurrent tumor cell invasion and angiogenesis. Therefore, the application of anti-invasion or anti-angiogenic agents may hold tremendous promise. Adhesion molecules, including integrins, have been found to be critically involved in tumor cell invasion [17,18]. A number of integrin antagonists have been proposed as anti-invasion or anti-angiogenic agents. Studies have demonstrated that antagonism of certain integrin receptors, such as αvβ3, αvβ5, and β1, can block angiogenesis, tumor cell migration, and metastasis [19–22]. During metastasis, cancer cells undergo several processes including proliferation, matrix remodelling, invasion, migration, and differentiation, and the adhesion molecules involved in these processes are potential drug targets. Our results demonstrated that rLj-RGD3 was able to inhibit the proliferation of 786-0 cells in a dose-dependent manner. However, the IC_{50} of rLj-RGD3 was much lower in human renal carcinoma cells than in human breast carcinoma cells [14], indicating that rLj-RGD3 inhibited cell growth more robustly in 786-0 than in breast carcinoma cells. Hoechst 33258 and DNA ladder assays revealed that rLj-RGD3 induced apoptosis in renal carcinoma cells. During apoptosis, actin depolymerization and changes in cell structure occur, and apoptosis is closely associated with these changes in the organization of F-actin, which is related to the formation of apoptotic bodies. Therefore, we examined the morphological changes in 786-0 cells treated with rLj-RGD3 by FITC-phalloidin staining. Our data indicated that rLj-RGD3 might initiate apoptosis in 786-0 cells by inducing actin filament aggregation. Our previous studies have shown that rLj-RGD3 can reduce the invasiveness of tumor cells (HeLa and MCF-7/Adr cells) and endothelial cells (ECV304 cells) [13,14]. However, the mechanisms by which rLj-RGD3 reduces cell invasion in vitro are largely uninvestigated. Thus, we examined whether the rLj-RGD3 could alter the invasive ability of
786-0 human renal carcinoma cells, and data showed that rLj-RGD3 protein effectively inhibited the migration and invasion of 786-0 cells.

Integrins are known to play a critical role in these physiological events by regulating interactions between cells, as well as between cells and the ECM, both of which determine the invasiveness of cancer cells [23]. The biological components of cell invasion include cell adhesion to the ECM, degradation of the ECM with the aid of proteolytic enzymes, and mechanisms to generate cell locomotion [24]. There are several reports suggesting that integrin signalling is associated with MMP expression and cell migration. The expression and secretion of MMPs involved in ECM degradation can be regulated de novo. Because degradation of the matrix by proteolytic enzymes is a crucial step for the invasion of tumor cells, we examined whether rLj-RGD3 decreased the invasiveness of renal carcinoma cells by altering the cellular proteolytic capacity. We measured and compared the levels of MMP-2 and MMP-9 in conditioned medium from untreated and rLj-RGD3-treated cells. Our results demonstrated that rLj-RGD3 did not significantly affect the level of secretion of MMPs, strongly indicating that the proteolytic system is not implicated in rLj-RGD3-mediated inhibition of renal carcinoma cell invasion in vitro.

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TNF-α is a key pro-inflammatory cytokine that is secreted by activated macrophages and monocytes [25]. TNF-α expression is elevated in various human cancers and correlates positively with tumor grade and poor prognosis [26,27]. In 2006, Roomi et al. [28] reported that exposure of the renal carcinoma cell line 786-0 to TNF-α increased secretion of MMP-9 and that MMP-9 activity was decreased by exposure to inhibitors, such as doxycycline. Therefore, we investigated the role of rLj-RGD as a therapeutic drug for the inhibition of TNF-α-induced MMP-9 expression, which is a hallmark of cancer
metastasis and invasion in renal cancer cells. We showed that exposure of the highly metastatic RCC cell line 786-0 to TNF-α increased the secretion of MMP-9, but not MMP-2. MMP-2 and MMP-9 activity were decreased by exposure to doxycycline. The rLj-RGD3 protein was shown to have a strong effect on TNF-α-induced MMP-9 secretion in human renal carcinoma cells and significantly decreased the invasiveness of these cells. As MMPs are important in diverse pathologies, including tumor progression, discovering the mechanisms by which MMPs are inhibited by rLj-RGD3 could be helpful in treating various diseases.

In conclusion, we demonstrated that rLj-RGD3 cell invasion by a cellular process unrelated to proteolytic degradation of the ECM in vitro. TNF-α-induced MMP-9 secretion and cell invasion are decreased by a low concentration of rLj-RGD3 in 786-0 cells. These data suggest that rLj-RGD3 inhibits tumor progression in a novel manner, but the molecular mechanism remains to be elucidated.

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