Original Article

rLj-RGD4, an RGD toxin protein from Lampetra japonica, can inhibit proliferation and migration of human laryngeal squamous carcinoma Hep-2 cells

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Abstract: rLj-RGD4, the recombinant Lampetra japonica (Arg-Gly-Asp) 4, is a recombinant soluble version of the wild-type rLj-RGD3 protein found in the salivary gland of Lampetra japonica, which is characterized by 4 RGD motifs. Our previous report demonstrated that rLj-RGD4 has anti-tumor activity against human laryngeal squamous carcinoma Hep-2 cells in nude mice. However, the mechanism by which rLj-RGD4 inhibits the growth of transplanted Hep-2 cells in the nude mouse model remains unclear. In this study, we investigated the anti-proliferative activity and the effects of rLj-RGD4 on the migration and invasion of Hep-2 cells. Our results indicated that either rLj-RGD3 or rLj-RGD4 can inhibit cell proliferation via inducing apoptosis in Hep-2 cells. In addition, a Transwell assay showed that rLj-RGD4 can suppress Hep-2 cell adhesion, invasion, and migration in a dose-dependent manner. Further examination revealed that rLj-RGD4 has a destructive effect on the cytoskeleton of Hep-2 cells, which might be responsible for its effects on tumor invasion and migration. Moreover, we also found that rLj-RGD4 treatment can regulate the expression levels of p-FAK, p-PI3K, p-Akt, VEGF, Bcl-2, Bax, caspase-3, and caspase-9. Overall, these findings suggested that rLj-RGD4 might be a potential clinical anti-tumor therapeutic for the treatment of human laryngeal squamous carcinoma.

Keywords: rLj-RGD4, RGD toxin protein, cisplatin, Hep-2 cells

Introduction

Head and neck cancer is one of the most prevalent causes of mortality in the United States [1], and laryngeal squamous cell carcinoma is one of the most common malignant tumors in the head and neck, accounting for 2-3% newly diagnosed cases of head and neck cancer worldwide each year [2-4]. To date, although several anti-tumor therapeutic strategies, including resection operation, radiotherapy, chemotherapy, and immunotherapy, have proven to be effective therapies for the treatment of laryngeal squamous cell carcinoma [5, 6], the 5-year survival rate is still only 60% for patients with laryngeal squamous cell carcinoma after treatment [7]. In addition, impaired laryngeal function can negatively impact patients’ quality of life, given that a functional larynx is required for breathing, swallowing, and phonation [8]. Therefore, there are major unmet needs in laryngeal squamous cell carcinoma management that might be addressed through the discovery of a new therapy for the treatment of head and neck cancer.

Cell-cell and cell-extracellular matrix (ECM) interactions are regulated by integrins expressed on the cell surface [9]. One well-studied example, αvβ3 integrin, is closely associated with several biological processes, including cell adhesion, signal transduction, and angiogenesis [10-16], a key process in tumor growth and metastasis [17]. Upregulation of αvβ3 integrin receptor has been found in multiple tumor cell types, and notably, αvβ3 integrin is activated on tumor-associated endothelial cells (ECs) during angiogenesis in many rapidly growing
tumors but not on resting ECs in most healthy organ systems [12, 15, 16]. Given that αvβ3 integrin is a receptor for ECM proteins expressing the RGD (Arg-Gly-Asp) motif [10], the RGD motif is likely associated with the downstream effects of αvβ3 integrin activation, including tumor angiogenesis.

The RGD motif, which is widely present in the ECM, has been found in the salivary gland secretions of many blood-sucking animals, including snake, leeches, ticks, and lampreys, and thus, proteins containing the RGD motif were also called RGD toxin proteins [18-23]. Several studies indicated that RGD toxin proteins can competitively inhibit cell proliferation, tumor invasion, and tumor cell migration by binding to the RGD domains of the highly expressed integrins on tumor cells or vascular ECs [24-26]. Moreover, recent studies reported that a number of synthetic peptides containing RGD motifs also act as ligands of integrins to promote apoptosis and inhibit tumor progression [27, 28]. Notably, rLj-RGD3, a novel toxin protein from the salivary gland of *Lampetra japonica* that is characterized by 3 RGD motifs, was shown to inhibit cell proliferation in various tumors [28]. To obtain a more effective RGD toxin protein with lower immunogenicity, we obtained a recombinant rLj-RGD4 protein (rLj-RGD4), which contains 58 amino acids, including 2 cysteine, 4 histidine, 7 arginine, and 6 threonine residues as well as 4 RGD motifs. Our previous study indicated that rLj-RGD4 exhibits potent in vivo activity and inhibits the growth of transplanted Hep-2 cells in a nude mouse model [29]. However, the mechanisms by which rLj-RGD4 inhibits tumor growth, invasion, and migration remain unclear.

In this study, we analyzed the effects of different doses of rLj-RGD4 on the viability, adhesion, invasion, and migration of human laryngeal carcinoma Hep-2 cells as well as cytoskeletal transformation. Our results indicate that the expression levels of cytoplasmic aspartate specific cysteine protease caspase-3 and caspase-9, B lymphocyte tumor 2 gene (Bcl-2), Bcl-2 associated X protein (Bax), focal adhesion kinase (FAK), phosphatidylinositol kinase (PI3K), protein kinase B (Akt), and vascular endothelial growth factor (VEGF) in Hep-2 cells were altered in response to treatment with different doses of rLj-RGD4, suggesting that rLj-RGD4 has potential value for the treatment of human laryngeal squamous carcinoma.

**Materials and methods**

**Cell culture and treatment**

The human laryngeal squamous carcinoma cell line Hep-2 was obtained from Yanjing Biotechnology Company (Shanghai, China). The cells were maintained in RPMI 1640 medium (Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco) and 1:100 penicillin/streptomycin (Solarbio, Beijing, China) in a humidified incubator (Thermo Scientific, USA) with 5% CO₂ at 37°C. rLj-RGD3 and rLj-RGD4 were obtained from the College of Life Sciences, Liaoning Normal University (Dalian, Liaoning Province, China). Cisplatin was purchased from Jiangsu Haosen Pharmaceutical Ltd (Jiangsu, China). Different doses of drugs or PBS were added in the cell culture medium to evaluate their potential effects in Hep-2 cells.

**WST-1 cell proliferation and cytotoxicity assay**

To determine the effects of rLj-RGD4 on Hep-2 cell proliferation, WST-1 cell proliferation was examined, and a cytotoxicity assay was performed. Briefly, Hep-2 cells were inoculated in a 96-well plate (1×10⁵ cells/well) and incubated in RPMI 1640 medium with or without different doses of rLj-RGD4 treatments, as well as with 10 μM cisplatin as a positive control (6 wells/group). After 24 h of incubation, 10 μl WST solution (Beyotime, Beijing, China) was added in each well and incubated for 4 hr at 37°C. The plate was then read at a reference wavelength of 450 nm. The experiments were repeated independently three times.

**Cell adhesion assay**

The procedure was described in our previous report [29]. Briefly, 0.1 mg/mL fibronectin (FN; BD Biosciences, San Jose, CA, USA) was added in 96-well plate (40 μl per well) at 4°C overnight. After blocking with 1% bovine serum albumin at 37°C for 2 hr, the plate was washed with PBS three times. Hep-2 cells (1×10⁵ cells/well) were incubated in RPMI 1640 medium with or without different concentrations of rLj-RGD4 (1.5875, 3.175, and 6.25 μM) were inoculated and incubated at 37°C for 2 hr. The adhered cells were then fixed with 4% formaldehyde (PFA) for 10 min before washing with PBS. After treatment with 1% sodium dodecyl sulfate (SDS), the plate was read at a wavelength of 570 nm. The experiments were repeated independently three times.
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Transwell assay

According to our previous reported procedure [29], the migration assay was performed using a 24-well chamber plate with 8-μm pore size polycarbonate membrane filters (Corning Costar, Cambridge, MA, USA). For the invasion assay, the polycarbonate membrane filters were treated with Matrigel (4 mg/mL; 60 μL each well; BD Bioscience). Aliquots of 1×10^5 Hep-2 cells were added in the upper chamber, which contained different concentrations of rLj-RGD4 (1.5875, 3.175, and 6.25 μM), PBS (negative control), or 10 μM cisplatin a positive control. Complete medium containing with basic fibroblast growth factor (bFGF; 3 ng/mL; Roche, Basel, Switzerland) was added into the lower chamber. After incubation at 37°C in a 5% CO₂ incubator for 6 hr, the upper surface of the membranes was scraped with a cotton swab to remove non-migratory cells. The filters were then washed with PBS, fixed, and stained with 0.1% crystal violet for 30 min. After washing with PBS, the migratory cells were evaluated by counting the cells that migrated to the lower side of the filter by bright field microscopy and analyzed using Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA). The experiments were repeated independently three times.

Phalloidin treatment

Phalloidin treatment was performed to detect the effects of rLj-RGD4 on cytoskeleton transformation of Hep-2 cells. Briefly, 2×10^6 Hep-2 cells were seeded onto sterilized coverslips in the 6-well plate. After 24 hr of incubation, different concentrations of rLj-RGD4 (5, 10, or 20 μM), PBS, or 10 μM cisplatin, were added to induce apoptosis, respectively. Twenty-four hours later, the cells were washed twice and fixed with 4% PFA for 30 min before phalloidin (Sigma, St. Louis, MO, USA) staining. The cytoskeletal transformation was observed with immunofluorescence microscopy.

Apoptosis detection

Overall, 2×10^6 cells Hep-2 cells were seeded on the sterilized coverslips in the 6-well plate. After 24 hr of incubation, different concentrations of rLj-RGD4 (5, 10, or 20 μM), PBS, or 10 μM cisplatin, were added to induce apoptosis. Twenty-four hours later, the cells were fixed with 4% PFA for 30 min before washing with PBS three times. The Hoechst 33342 solution (Beyotime) was then added to detect the apoptosis of Hep-2 cells according to the standard protocol. The apoptotic cells were observed and photographed under an immunofluorescent microscope. The experiments were repeated independently three times.

Protein extraction and western blot analysis

Total cellular protein of the rLj-RGD4-treated Hep-2 cells was extracted in lysis buffer for 30 min. After centrifugation, the protein concentration in the supernatant was determined. The sample was separated for SDS-PAGE electrophoresis and detected with the desired antibodies including: p-PI3K antibody (1:1000), PI3K antibody (1:1000), Bcl-2 antibody (1:1000), Akt antibody (1:1000), p-Akt antibody (1:1000; all from Beyotime), and β-actin antibody (1:1000; Thermo Electron Corporation, MA, USA), respectively. On the next day, the membranes were washed with PBS-Twenty 20 (PBS-T) three times, after incubation with the corresponding secondary antibodies (all from Beyotime). The signals were detected using an enhanced chemiluminescence (ECL) solution from Beyotime, and the fluorescence intensity of each target band was quantified using the Gel-Pro Analyser 4.0 software (Media Cybernetics, Inc.).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5’→3’)</th>
<th>Anti-sense primer (5’→3’)</th>
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<tr>
<td>VEGF</td>
<td>AGGAGTACCCCTGATGAGATCGAGTA</td>
<td>TGGTGAGGTTTGTATCCGCATA</td>
</tr>
<tr>
<td>BCL2</td>
<td>ACCGTGTTGAGGAGCTCTTT</td>
<td>GCCGCTCAGGACCTCACT</td>
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<tr>
<td>Caspase 9</td>
<td>GCCAGGCAGCTGATCATGACT</td>
<td>TCTAGGCTGCTGCTGACA</td>
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<tr>
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<td>GCAATCTGCTAAGGCGCTGAG</td>
</tr>
<tr>
<td>β actin</td>
<td>GGGAAATCGTGCGTGACAATT</td>
<td>GGAACCCGTCTATTGCAAT</td>
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Table 1. Primers used in real-time PCR analysis
Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. qRT-PCR was performed using the SYBR PrimeScript RT-PCR kit (Takara Biotechnology [Dalian] Co. Ltd, China) on a ABI Prism 7500 Real-time PCR System (Applied Biosystems, USA) according to the manufacturer’s instructions. The primer sequences used for VEGF, BCL2, BAX, AKT, caspase 3, caspase 9, PI3K, FAK, and β-actin are shown in Table 1. The PCR protocol included a denaturation program (95°C for 2 min), followed by 40 cycles of the amplification and quantification program (95°C for 5 sec, 60°C for 25 sec, 72°C for 25 sec). Each sample was replicated three times.

Statistical analysis

All results were analyzed by SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) and presented as the arithmetic mean ± standard error (SE). Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by multiple mean comparison by the Student’s t-test. $P<0.05$ was considered indicative of a statistically significant difference.

Results

Inhibitory effects of rLj-RGD3 and rLj-RGD4 on viability of Hep-2 cells

To investigate the effects of rLj-RGD3 and rLj-RGD4 on the viability of Hep-2 cells, RPMI 1640 medium containing different concentrations of rLj-RGD3 (2.5, 5, 10, 20, or 40 µM) and rLj-RGD4 (12.5, 25, 50, 100, and 200 µM) was added in Hep-2 cells. RPMI 1640 medium with or without Hep-2 cells was regarded as a negative control. After 24 hr of incubation, WST-1 cell proliferation and cytotoxicity assay were performed to determine the effects of rLj-RGD3 or rLj-RGD4 on the viability of Hep-2 cells. As shown in Figure 1A, either rLj-RGD3 or rLj-RGD4 can inhibit the viability of Hep-2 cells, and their 50% inhibiting concentrations ($IC_{50}$) were 10.497 µM and 56.096 µM, respectively, indicating that rLj-RGD4 might have a lower tox-
Figure 2. The expression levels of Bcl-2, Bax, caspase-3, and caspase-9 in rLj-RGD4- or cisplatin-treated Hep-2 cells. (A) The expression levels of Bcl-2, Bax, caspase-3, caspase-9, and β-actin were examined by western blotting; (B) Quantitative analysis of relative expression levels of Bcl-2/Bax and caspase-3/caspase-9 in cells treated with rLj-RGD4 or cisplatin; (C) The mRNA levels of Bcl-2, Bax, caspase-3, and caspase-9 were examined by qRT-PCR assay; quantitative analysis of the ratio of Bcl-2/Bax expression at the protein level (D) or mRNA level (E). The results are representative of three independent experiments and are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 compared with the control. *P<0.05, **P<0.01, ***P<0.001 compared with the control.
icity for the inhibition of Hep-2 cell viability. Moreover, our further results revealed that rLj-RGD4 inhibited cell viability in a dose-dependent manner in Hep-2 cells, and displayed a stronger inhibitory effect than 10 µM cisplatin treatment in Hep-2 cells at 100 µM concentration (Figure 1B). Together, these findings suggest that rLj-RGD4 might have potential value for the treatment of human laryngeal squamous cell carcinoma.

Notably, we noticed that treatments with difference concentrations of either rLj-RGD4 (5, 10, and 20 µM) or cisplatin (10 µM) could induce cell apoptosis. We expected that the effects of rLj-RGD4 might be associated with the induction of cell apoptosis. Hoechst 33324 staining was performed to detect cell apoptosis with or without treatments with differing concentrations of rLj-RGD4 (25, 50, and 100 µM) in a dose-dependent manner in Hep-2 cells (Figure 1C). Together, these findings indicate that rLj-RGD4 may inhibit cell viability by inducing cell apoptosis in Hep-2 cells.

Because cytoskeletal transformation plays an important role during cell apoptosis and the cytoskeleton formation in apoptotic cells may be clustered or degraded. By contrast, normal Hep-2 cells remained intact, and a further phalloidin assay was performed to examine the formation of the cytoskeleton of Hep-2 cells with or without rLj-RGD4 treatments. As shown in Figure 1C, the treatments with different concentrations of rLj-RGD4 (25, 50, and 100 µM) could destroy the cytoskeleton of Hep-2 cells in a dose-dependent manner, suggesting that the effects of rLj-RGD4 might also be related to cytoskeleton transformation in Hep-2 cells.

rLj-RGD4 can induce apoptosis through regulating the mitochondrion-related apoptotic pathway

To address the mechanism of rLj-RGD4-induced apoptosis in Hep-2 cells, western blot analysis and qRT-PCR assay were performed to determine the expression levels of apoptosis-related protein, including caspase-3, caspase-9, Bax, and Bcl-2, and after rLj-RGD4 or cisplatin treatment in Hep-2 cells (Figure 2). We found that the expression level of Bcl-2, an anti-apoptotic protein, was down-regulated, whereas apoptotic protein, Bax expression, was up-regulated in a dose-dependent manner in rLj-RGD4-treated Hep-2 cells, suggesting the...
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Bcl-2/Bax signaling pathway may be associated with rLj-RGD4-induced apoptosis in Hep-2 cells. In addition, another apoptosis-related protein family, the caspase protein family, was also determined by using western blot analysis and qRT-PCR assay. Our results indicated that rLj-RGD4 treatment can significantly promote the expression levels of caspase-3 and caspase-9 proteins in Hep-2 cells. As we know, both Bcl-2/Bax pathways and the caspase family were involved in mitochondrion-related apoptosis and one of the important mechanisms for apoptosis [30]. These results suggest that rLj-RGD4 might induce apoptosis by regulating the expression levels of certain endogenous mitochondrion-related factors, including Bcl-2, Bax, caspase-3, and caspase-9, in Hep-2 cells.

Inhibitory effects of rLj-RGD4 on cell adhesion, invasion, and migration in Hep-2 cells

Because cell-cell adhesion between tumor cells and the ECM play an important role during tumor invasion and migration [31], further investigations were performed to clarify whether the interaction of rLj-RGD4 and fibronectin was involved in Hep-2 cell adhesion, invasion and migrations. Our results indicated that rLj-RGD4 treatment can significantly inhibit cell adhesion between Hep-2 cells and fibronectin in a dose-dependent manner (Figure 3A). In addition, we noticed that the inhibitory effect of 6.25 µM rLj-RGD4 treatment was similar to that of 10 µM cisplatin treatment in Hep-2 cells (Figure 3A), suggesting that rLj-RGD4 treatment might be an effective potential clinical
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therapeutic for patients with laryngeal squamous cell carcinoma. Furthermore, as shown in Figure 3B-D, a Transwell assay demonstrated that cell invasion and migration were remarkably inhibited in rLj-RGD4-treated Hep-2 cells, compared with those in non-treated Hep-2 cells, and this effect followed a dose-dependent manner (1.5625, 3.125, and 6.25 µM). Thus, as expected, these results suggested that rLj-RGD4 can significantly inhibit cell adhesion, invasion, and migration in Hep-2 cells.

rLj-RGD4-inhibited cell adhesion, invasion and migration by inactivating the PI3K/Akt pathway

To clarify the mechanism of rLj-RGD4-inhibited cell adhesion, invasion and migration, we then examined the expression levels of the FAK/PI3K/Akt pathway-related proteins, which may be closely related to cell adhesion, invasion and migration, in rLj-RGD4- or cisplatin-treated Hep-2 cells. Western blot and qRT-PCR assays indicated that rLj-RGD4 treatment can significantly inhibit the phosphorylation of FAK, PI3K, and Akt in a dose-dependent manner (Figure 4). However, the regulation of endogenous FAK, PI3K, and Akt expression was undetectable in either rLj-RGD4- or cisplatin-treated Hep-2 cells, suggesting that rLj-RGD4 can only inhibit activation of the PI3K/Akt signaling pathway and that the inactivation mediated by the PI3K/Akt pathway might be involved in the inhibition of cell adhesion, invasion and migration in Hep-2 cells.

rLj-RGD4 treatment can down-regulate the level of VEGF expression

As is known, angiogenesis plays important roles in tumor growth and proliferation [32], we expected that rLj-RGD4 also may inhibit cell proliferation by regulating angiogenesis. To clarify the relationship between rLj-RGD4 and angiogenesis, we determined the expression levels of VEGF, a classical angiogenic biomarker [17], in rLj-RGD4- or cisplatin-treated Hep-2 cells. Our results demonstrated that VEGF expression in Hep-2 cells at either the protein or mRNA level was significantly inhibited upon treatment with rLj-RGD4 or cisplatin (Figure 5), suggesting that the inhibitory effect of rLj-RGD4 on cell proliferation was also associated with the regulation of VEGF expression in Hep-2 cells.

Discussion

Our previous study demonstrated that rLj-RGD4 exhibits potent in vivo activity and inhibits the growth of transplanted Hep-2 cells in a nude mouse model. In the present study, we found that rLj-RGD4 treatment significantly inhibit cell viability (Figure 1), and its inhibitory effect might be associated with the induction of apoptosis through regulation of the Bcl-2/
Bax and caspase pathways (Figure 2), as well as interruption of cytoskeleton transformation in Hep-2 cells (Figure 1C). Further experiments revealed that rLj-RGD4 can down-regulate the expression level of VEGF to inhibit the angiogenic process, which might be involved in the inhibition of Hep-2 cell proliferation (Figure 5). Moreover, our results demonstrated that rLj-RGD4 treatment remarkably inhibited Hep-2 cell adhesion, invasion, and migration (Figure 3) through inactivating the FAK/PI3K/Akt pathway (Figure 4). Our findings clarified the molecular mechanism of the effects of rLj-RGD4 on Hep-2 cell viability, cell adhesion, invasion, and migration, suggesting that rLj-RGD4 may serve as a potent clinical therapeutic for human laryngeal squamous carcinoma.

The WST-1 proliferation assay revealed that either rLj-RGD3 or rLj-RGD4 can inhibit cell proliferation in a dose-dependent manner. Interestingly, the effective drug concentrations of rLj-RGD3 and rLj-RGD4 for the inhibition of cell proliferation differed (25-40 μM vs. 12.5-200 μM; Figure 1A and 1B), suggesting that the toxicity of rLj-RGD4 was lower than that of rLj-RGD3. In addition, further examination indicated that rLj-RGD4 treatment can induce cell apoptosis and destroy cytoskeletal transformation (Figure 1C) in Hep-2 cells, which is similar with the results in cisplatin-treated Hep-2 cells.

Apoptosis is mainly induced by two possible pathways, the exogenous apoptotic pathway and the mitochondrion-regulated endogenous apoptotic pathway [33, 34]. Previous studies revealed that caspase-3 and caspase-9 play important roles in the mitochondrion-regulated endogenous apoptotic pathway [35, 36]. In addition, several reports indicated that certain proteins containing the RGD motif can induce apoptosis by regulating the expression levels of caspase-3 and caspase-9 proteins [37-41]. We therefore expected that rLj-RGD4, which contains a four-repeat-RGD-motif, might bind to integrin ligands to activate the caspase-3 and caspase-9 pathways on the cell membrane and subsequently induce apoptosis [38, 40, 41]. In addition, similar with the functions of the caspase family, the Bcl-2/Bax pathway, including an anti-apoptotic factor (Bcl-2) and an apoptotic factor (Bax), also was found to be closely associated with the mitochondrion-regulated endogenous apoptotic pathway [42, 43]. Our results indicated that rLj-RGD4 treatment can up-regulate Bax expression and down-regulate Bcl-2 expression, which increases the release of cytochrome C and the permeability of the mitochondrial membrane and results in the activation of caspase-3 and caspase-9 [44, 45]. All these results indicate that rLj-RGD4 treatment can induce apoptosis of Hep-2 cells through the mitochondrion-regulated endogenous apoptotic pathway.

As is widely known, tumor invasion and migration are key steps during tumor metastasis [46]. Tumor invasion and migration are basic biological features of laryngeal carcinoma, which is associated with the highest mortality among head and neck cancers. A previous report indicated that the cell adhesion effect of tumor cells and ECM may be involved in this process [31]. Our results demonstrated that rLj-RGD4 can bind to fibronectin in a dose-dependent manner, which might represent a possible mechanism for the inhibition of invasion and migration in rLj-RGD4-treated Hep-2 cells (Figure 3A).

Integrin ligands have been found to regulate various biological functions, including cell-cell and cellular stroma interactions, cell proliferation, EC migration, angiogenesis, and metabolism. FAK, a key factor in the downstream of integrin pathway, plays important roles in cellular metabolism and migration in ECs through binding to ECM ligands [47, 48]. In addition, FAK-regulated Akt expression is involved in EC proliferation and migration [49, 50]. Our results showed that the phosphorylation of FAK was inhibited, whereas FAK expression was not regulated in rLj-RGD4-treated Hep-2 cells (Figure 4), suggesting that rLj-RGD4 can only regulate the activity of FAK in a dose-dependent manner. Therefore, we expected that rLj-RGD4 might block the phosphorylation of FAK to inhibit FAK/ECM ligand interaction, which would result in the inhibition of proliferation and migration in rLj-RGD4-treated Hep-2 cells. Furthermore, we also found that rLj-RGD4 treatment can only inhibit the expression levels of p-PI3K and p-Akt, but not PI3K and Akt (Figure 4). Phosphorylated PI3K can mediate the activation of many cell signaling pathways, which might result in cell apoptosis, angiogenesis, tumor invasion, and migration. Ge et al. found that proteins containing the RGD motif can regulate angiogenesis through inhibiting the PI3K/Akt pathway [51]. Therefore,
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we expect that inactivation of the FAK/PI3K/Akt pathway might be associated with the rLj-RGD4-induced inhibition of Hep-2 cell adhesion, invasion, and migration.

Angiogenesis plays an important role in tumorigenesis and tumor progression [17, 52]. Several reports have indicated that the effects of αvβ3 integrin are involved in angiogenesis and tumor invasion [53-55]. A number of RGD-containing peptides, which exhibit competitive anti-angiogenic activity, have been used in clinical investigations. Cilengitide, a cyclic RGD peptide, has displayed an anti-angiogenic effect in vitro and in vivo in a phase III clinical trial stage [56, 57]. Moreover, previous studies demonstrated that αvβ3 integrin can activate VEGF, the most important angiogenic factor [58], as well as its downstream signaling molecules [59-62]. Thus, the possible mechanism of rLj-RGD4-inhibited VEGF expression might be associated with the blockade of αvβ3 integrin signaling through competitive interaction with the αvβ3 integrin receptor on the cell membrane.

In summary, our results indicate that rLj-RGD4 can regulate cell adhesion, invasion, migration, and angiogenesis with less toxicity than the classic chemotherapy agent. Also, we found that rLj-RGD4-induced apoptosis was mediated through the Bcl-2/Bax and caspase pathways. Further investigations showed that the FAK/PI3K/Akt pathway might be involved in the changes in cell adhesion, invasion, and migration in rLj-RGD4-treated Hep-2 cells. Moreover, we found that the expression level of VEGF in Hep-2 cells can be regulated by rLj-RGD4 treatment. Therefore, our findings on the effects of rLj-RGD4 might contribute to the development of a new therapeutic strategy for patients with laryngeal squamous carcinoma.

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Disclosure of conflict of interest

None.

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