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Research paper

The anti-tumour activity of rLj-RGD4, an RGD toxin protein from *Lampetra japonica*, on human laryngeal squamous carcinoma Hep-2 cells in nude mice

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ABSTRACT

Purpose: The objective of this study is to investigate the antiproliferative activity and mechanism of integrin-binding rLj-RGD4 in a Hep-2 human laryngeal carcinoma-bearing nude mouse model.
Methods: Human laryngeal squamous carcinoma cells (Hep-2) were inoculated subcutaneously into the axilla of nude mice to generate a Hep-2 human laryngeal carcinoma-bearing nude mouse model. When the Hep-2 xenograft model was successfully established, the animals were randomly separated into five groups. Three groups were treated with different dosages of rLj-RGD4. Cisplatin was administered to the positive control group, and normal saline (NaCl) was administered to the negative control group for 3 weeks. The body weights and the survival of the nude mice were evaluated, and the volumes and weights of the solid tumours were measured. The mechanism underlying rLj-RGD4 inhibition of tumour growth in transplanted Hep-2 human laryngeal carcinoma-bearing nude mice was evaluated by haematoxylin-eosin (HE) staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL), measurement of intratumoural microvessel density (MVD), Western blotting, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR).
Results: The tumour volumes and weights of the treatment groups were reduced compared with the model group, and survival times were improved by rLj-RGD4 treatment in Hep-2 human laryngeal carcinoma-bearing nude mice. The number of apoptotic Hep-2 human cells and intratumoural MVD significantly decreased after the administration of rLj-RGD4. In the xenograft tissue of animals treated with rLj-RGD4, FAK, PI3K, and Akt expression was unaltered, whereas P-FAK, P-PI3K, Bcl-2, P-Akt, and VEGF levels were down-regulated. In addition, activated caspase-3, activated caspase-9, and Bax levels were up-regulated.
Conclusion: rLj-RGD4 exhibits potent in vivo activity and inhibits the growth of transplanted Hep-2 human laryngeal carcinoma cells in a nude mouse model. Thus, these results indicate that the recombinant RGD toxin protein rLj-RGD4 may serve as a potent clinical therapy for human laryngeal squamous carcinoma.

Abbreviations: RGD, Arg-Gly-Asp; rLj-RGD4, recombinant *Lampetra japonica* (Arg-Gly-Asp) 4; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling; MVD, intratumoural microvessel density; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; AI, apoptotic index; ECM, cell-extracellular matrix; Bcl-2, B cell lymphoma/leukaemia-2; Bax, Bcl-2 associated X protein; FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IR, inhibition rate.

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1. Introduction

Head and neck cancer is the most prevalent cause of mortality in the United States [1]. Laryngeal squamous carcinoma is one of the most commonly encountered head and neck cancers. In recent decades, laryngeal squamous carcinoma has been confirmed as the 11th most common cause of cancer in men worldwide [2]. The larynx plays an important role in numerous primary functions, including swallowing, phonation, breathing, and deglutition. The loss of laryngeal function can lead to lifestyle changes and job loss, thereby negatively impacting quality of life.

Squamous cell carcinomas account for approximately 95% of all laryngeal carcinomas. Effective treatments for laryngeal squamous carcinoma represent a challenge for cancers experts. Furthermore, laryngeal squamous carcinoma is a significant cause of morbidity and mortality worldwide [3]. However, existing therapies for laryngeal squamous carcinoma, including chemotherapy, radiotherapy [4], and immunotherapy [5], are ineffective compared with surgical techniques. As such, the discovery of novel therapeutic agents for laryngeal squamous carcinoma is needed.

Integrin antagonists that inhibit tumour neovasculature and promote tumour cell apoptosis represent a promising strategy for cancer therapeutics. Integrins are a class of heterodimeric transmembrane glycoproteins on the cell surface that regulate various biological functions, including cell-cell and cellular stroma interactions [6]. Integrins, which consist of non-covalently associated α and β glycoprotein subunits, participate in the regulation of various biological events, including cell adhesion, cell signalling, and angiogenesis. Indeed, αvβ3 integrin is the most notable receptor class and has been widely studied. Changes in αvβ3 integrin expression are associated with several pathological manifestations, including tumour angiogenesis, metastasis, invasion, inflammation, osteoporosis, and rheumatoid arthritis [7–13]. Moreover, αvβ3 integrin is responsible for tumour angiogenesis and is a receptor for extracellular matrix (ECM) proteins expressing RGD (Arg-Gly-Asp) motifs [7]. In addition, αvβ3 integrin receptor expression is preferentially up-regulated on various tumour cells and is activated on tumour-associated endothelial cells during angiogenesis in various types of rapidly growing tumours, whereas it is absent on resting endothelial cells in most normal organ systems. Angiogenesis plays an important role in tumour growth and metastasis [14]. Therefore, αvβ3 integrin antagonists are considered valuable anti-tumour and anti-angiogenic agents for the diagnosis and treatment of malignant tumours [9,10,15–17].

αvβ3 integrin contains a short cytoplasmic tail that binds to adaptor proteins and a large extracellular domain that interacts with ECM ligands [18]. RGD motifs in recombinant peptides have been proposed to be important in the binding of ECM ligands to viper integrins. Assa-Munt N et al. reported that a number of peptides containing RGD motifs act as ligands of the αvβ3 integrin, promote apoptosis [19], and inhibit angiogenesis [20] and tumour progression [21]. Furthermore, a number of radiolabeled RGD peptide-based probes have been used in clinical investigations [22,23]. Therefore, the present study aimed to investigate whether rLj-RGD4 exhibits potential activity against the tumour neovasculature.

rLj-RGD4 is a novel toxin protein from the salivary gland of *Lampetra japonica* that is characterised by 4 RGD motifs. rLj-RGD4 complementary DNA (cDNA) is 174 bp long, and the 58-amino acid sequence includes 2 cysteine, 4 histidine, 7 arginine, and 6 threonine residues as well as 4 RGD motifs (Fig. 1A). To elucidate the effects of recombinant rLj-RGD4 on the inhibition of cancer growth and metastasis, we used the nude Hep-2 human laryngeal carcinoma mouse model.

2. Materials and methods

2.1. Materials

Cisplatin was purchased from Hansoh Pharmaceutical Co. (Jiangsu, China). The TUNEL apoptosis detection kit was purchased from Roche (Shanghai, China). The anti-GAPDH primary antibody was purchased from Bioworld, and the rabbit anti-CD34 was purchased from Biosynthesis Biotechnology (Beijing, China). Antifade Mounting Medium, BeyECL Plus, TEMED, a DAB Horseradish Peroxidase Colour Development Kit, anti-Bax, anti-p-PI3K, anti-PI3K, anti-Akt, anti-p-Akt, anti-cleaved caspase-3, a BCA Protein Assay kit, and a ECL Western blot substrate kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). An anti-β-actin antibody, goat anti-rabbit antibody, and anti-mouse horseradish peroxidase (HRP)-IgG were purchased from Zhongshan Golden Bridge Bio-technology Co., Ltd. (Beijing, China). Anti-FAK, anti-p-FAK, anti-Bcl-2, and anti-cleaved caspase-9 were purchased from Santa Cruz Biotechnology, Inc (California, USA); anti-VEGF was purchased from Thermo Electron Corporation (Massachusetts, USA)

2.2. Cell culture

The Hep-2 human laryngeal squamous carcinoma cell line was obtained from Yanjing Biotech (Shanghai, China). All cells were maintained in RPMI1640 (GIBCO, Carlsbad, CA) medium supplemented with 10.0% foetal bovine serum (FBS, Sigma, St. Louis, MO), 100 μg/mL streptomycin, and 100 U/mL penicillin G in a humidified incubator at 5% CO_2 and 95% air at 37 °C (Thermo Scientific, USA).

2.3. Nude mice

Eighty nude mice (40 male and 40 female) 3–4 weeks in age and 18–22 g in weight were purchased from the Animal Centre of Dalian Medical University (Dalian, China; permit number: SCXK2013-0003). All nude mice were fed a commercial diet and pure water in a room maintained at 20–22 °C at Dalian Medical University according to the nation's animal welfare requirements.

2.4. Nude mice xenograft models

Hep-2 cells in the logarithmic phase of growth were trypsinised and harvested by centrifugation. Forty nude mice (20 males and 20 females) were injected with a clonal population of Hep-2 cells (5×10^5/ml) in 0.2 ml of PBS in the right axilla. Xenograft tumour sizes were monitored by measuring two perpendicular diameters with digital callipers daily. After 7 days, the maximum diameter of the established tumours ranged from 5 to 7 mm. The 40 tumour-bearing nude mice were randomly separated into five groups: (1) normal saline (NaCl) control group, (2) 50.0 μg/kg rLj-RGD4, (3) 25.0 μg/kg rLj-RGD4, (4) 12.5 μg/kg rLj-RGD4, and (5) 3.0 mg/kg cisplatin (cis-diammineplatinum dichloride). Each mouse in the experimental group was administered two intraperitoneal injections daily for a 3-week period. Another mouse in the cisplatin group was administered one intraperitoneal injection daily for 3 weeks. Each animal's weight was measured once daily.

2.5. Tumour volume and weight

Tumour xenograft growth rates were measured every 3 days by the same researcher over the 3-week period. Tumour volumes were estimated using the following formula: $V = 1/2 \cdot a \cdot b^2$, where a and b represent the largest and shortest perpendicular axis, respectively. After 3 weeks of therapy administration, the nude mice were