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Research Article

Antitumor Effects of RHAMM-Target Peptides on Prostate Tumor Xenografts in Nude Mice

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ABSTRACT

Prostate cancer is a high incidence disease in men and a major cause of cancer deaths. RHAMM-target peptides used for treatments of prostate tumors. Peptides were added to the PC3mLN4 cells (GEGEEGEE, DFGEEAEE and RYQLHPYR, final concentration 40 $\mu\text{g} / \text{ml}$, dose for inoculating the mouse 2.5 mg / kg). 45 nude mice were injected with 0.1 ml of PC3mLN4 cell suspension of 2×10^7 cells/ml subcutaneously. The nude mice models were randomly divided into five groups of 8 in each group. The standard models in mice were judged by 100% tumor grafting. The results showed that GEGEEGEE peptide inhibited tumor growth by 58%, DFGEEAEE peptide inhibited tumor growth by 63.5%, but RYQLHPYR peptide significantly inhibited of tumor growth by 94.6%. These results have demonstrated that RHAMM-target peptides have a therapeutic potential for the treatment of cancer.

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Introduction

Oncological diseases occupy the second place in mortality in the world after cardiovascular diseases. This complex disease is still a serious problem for the scientific and medical community. Cancer is not a separate disease, but a group of diseases characterized by unregulated growth of abnormal cells. The driving force behind this uncontrolled growth is a series of mutations that cause aberrant expression of the gene products necessary for the regulation of cell proliferation, survival and growth. Therefore, cancer arises from defects in the most basic biological functions of cells: the ability to respond to growth signals, use cell death programs to eliminate unnecessary, redundant, or damaged cells, as well as the formation of new blood vessels and the ability to penetrate tissues. The challenge facing clinicians and researchers looking for effective therapeutic approaches for the treatment of cancer is to eliminate cancer cells while maintaining normal, healthy tissue.

Currently, prostate cancer is one of the most common malignant neoplasms in men. In the world, the incidence of prostate cancer occupies the 7th-8th place (accounting for about 6%). A feature of prostate cancer is late diagnosis, when the tumor is diagnosed in stage III-IV [1]. Although significant progress has been made in the treatment of cancer in recent years, most modern cancer treatments include surgery

or chemo, radiation and hormone therapy, which have changed little over the past decade. The effectiveness of chemotherapy is often low, and patients suffer from the non-specificity and toxicity of existing anticancer drugs. For example, conventional chemotherapeutic drugs (that is, DNA alkylating agents) that target proliferating cancer cells also damage healthy growing cells but may not eliminate eliminating or non-proliferating cancer cells [2, 3].

In addition, the development of drug resistance of the tumor is often observed during chemotherapy, which may be associated with impaired drug delivery or detoxifying enzymes that affect the interaction between the drug and its target. Defects in DNA repair mechanisms and apoptosis or cell death pathways can also lead to the development of resistance to anticancer drugs. Even if the initial treatments were successful, the risk of cancer recurrence remains a problem for patients. The quest to find new cures for cancer has spurred research on the evolution of small molecules as cures, but problems with unforeseen inappropriate effects have created the need for alternative approaches. In this regard, a new class of antitumor reagents, which is based on peptides, has appeared. These low molecular weight molecules can be modeled from endogenous proteins, peptide synthesis is cheap and cost-effective, and they can be easily modified. Peptides are used both in the diagnosis and in the treatment of cancer, providing specificity for tumor tissues, reduce the likelihood of drug resistance and have low toxicity.

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Each tumor has its own specific characteristics (sometimes specific for the patient, the so-called molecular signature-signature), which are expressed in large quantities in the tumor and / or are located in a different place compared to normal tissue. This tumor-specific "signature," makes it possible to develop targeted agents for the early detection, diagnosis and treatment of cancer. Synthetic polypeptides are excellent candidates for cancer therapy. In particular, peptide ligands have advantages due to their small size, easy and affordable production, high specificity and remarkable flexibility with respect to their sequence and conjugation potential. In combination with chemotherapeutic drugs / or nanocarriers, they are well established for targeted delivery, ensuring high chemotherapy efficacy, due to the reduction of side effects.

In a new era of personalized / precise medicine, the goal of therapeutic treatment is to use tumor-specific and patient-specific genetic and molecular features to select specific targeted therapy for each patient [4-6]. It is known that the microenvironment of the tumor cell, namely, the extracellular matrix, is of great importance in the progression of cancer [7-9]. The polysaccharides and extracellular matrix proteins play an important role in the transformation of a normal cell into a cancerous cell and in the further progression of cancerous disease [10, 11]. In many tumors, hyaluronic acid (HA) makes up the bulk of the extracellular matrix and is involved in cell migration, division, angiogenesis, inflammation, wound healing, and tumor growth [12-14]. HA function depends on the molecular weight of this polysaccharide. High molecular weight HA is responsible for structural functions, while low molecular weight HA, binding to hyaluronic receptors (RHAMM, CD44), transmits signals through cell pathways that control proliferation, differentiation, adhesion, motility and invasiveness of tumor cells [15-18]. HA is a physiological ligand of the RHAMM receptor (also known as CD168 or HMMR, a mediated mobility hyaluronan receptor). On model tumor systems, it was shown that the RHAMM receptor contains the binding site of HA, tubulin, and special areas necessary for the manifestation of its oncogenic properties. HA plays an important role in the progression and prognosis of prostate cancer through RHAMM-mediated signaling pathways.

A number of studies have shown that in the tissues and metastases of prostate tumors, elevated levels of HA and RHAMM receptor are observed, which together with HYAL-1 hyaluronidase contribute to tumor growth and are associated with a poor prognosis [19-21]. Therefore, HA and the RHAMM receptor are promising targets for the development of drugs for the treatment of cancer, and in particular, prostate cancer. Several studies have shown that RHAMM is immunogenic antigen in acute and chronic leukemia, breast cancer and is involved in a joint signaling pathway with the androgen receptor in prostate cancer [22-24]. RHAMM receptor is used for immunotherapy; for example, vaccination with the RHAMM-R3 peptide has been used in acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma [25]. In addition, blocking the HA binding center at the RHAMM receptor and modulating its function may inhibit tumor development.

Thus, it was shown that 4-methylumbelliferone (a dietary supplement and a HA inhibitor) is a powerful apoptotic reagent with strong anti-invasive and anti-angiogenic properties directed against prostate cancer cells [26]. Therefore, one of the promising methods for treating prostate

cancer is the development of new effective HA inhibitors that selectively block the RHAMM receptor and its signaling pathways. Previously, RHAMM target peptides that specifically interact with the RHAMM receptor have been identified and characterized [27]. It has been established that RHAMM target peptides compete for a binding site with HA, selectively bind to the recombinant RHAMM protein, easily penetrate into tumor cells and are stable in blood serum [27]. However, the antitumor activity of RHAMM target peptides in mouse xenograft models has not been investigated. In this work, we studied the antitumor effect of RHAMM-targeted peptides on the development of prostate cancer.

Methods

I Peptide Synthesis

The synthesis of peptides (GEGEEGEE, DFGEEAEE and RYQLHPYR) was performed as described previously [28].

II Analysis of the Effect of Peptides on Tumor Growth in Xenografts (Mouse Tumor Growth Model)

Tumor Material for Transplantation: Human prostate cancer cells (PC3mLN4 cell line) were obtained from the American Type Culture Collection (Manassas, Virginia, Merck, USA). To obtain standard grafting material, cells were cultured in DMEM supplemented with 10% (v / v) fetal bovine serum and 100 u / ml penicillin-streptomycin and 10 mM HEPES, pH 7.2. Cells were maintained in a logarithmic growth phase at 37°, 5% CO₂ and 90% humidity.

III Animals

The experiment used 45 immunodeficient mice (NOD CRISPR Prkdc Il2r Gamma (NCG) Triple-Immunodeficient Mouse Model, Nomenclature: NOD-Prkdcem26Cd52Il2rgem26Cd22 / NjuCrI) with no transplant immunity. Male mice (4 weeks old, 16-18 g) were purchased from Charles River (San Francisco, CA, USA). The mice were kept in the SPF zone of a specialized air-conditioned vivarium compartment under normalized temperature and humidity conditions using sterile extruded feed, sterile water and sterile paper litter in compliance with the requirements to conventional animals. For introduction into the experiment, mice were ranked by body weight with a spread of no more than 10% in different individuals (n = 8). Mice were kept individually at 24 ± 1°C in a room with controlled illumination (light: 7: 00-19: 00, darkness: 19: 00-7: 00 h) and relative humidity 55% ± 5%.

IV Agent

The peptide substance (GEGEEGEE, DFGEEAEE and RYQLHPYR) is a white crystalline powder in a vial. The substance was dissolved in a sterile 0.9% sodium chloride solution to the desired concentrations.

V Xenograft Transplantation

PC3mLN4 cells were grown to a logarithmic phase, then a cell suspension was prepared by trypsinization (0.25% trypsin-EDTA) to a cell concentration of 2 × 10⁷ / ml. Peptides were added to the cells

(GEGEEGEE, DFGEEAEE and RYQLHPYR, final concentration 40 μg / ml, dose for inoculating the mouse 2.5 mg / kg). An equal amount of growth medium (sterile) was added to the control cells (without peptides). In the experiment, 5 groups of mice were used: 1 group was used to control tumor growth (CTG), they were injected with cells without treatment with peptides ($n = 8$), 4 groups ($n = 8$) received cells treated with peptides.

A cell suspension (100 μl , cell concentration 2×10^7 / ml) was injected into the left subcutaneous anterior part, into the armpit of 4-week-old mice under aseptic conditions. The standard models in mice were judged by 100% tumor grafting.

VI Evaluation of the Antitumor Effect

To identify tumor nodes under the skin in mice, visual observation and palpation of the transplantation site were performed. Tumor growth was observed within 8 days after inoculation, with a diameter of 2 mm, which indicated that the tumor model was successfully created. In mice, as soon as tumors began to develop, continuous measurements of tumor size were carried out using a caliper. The kinetics of tumor-left growth was observed within a month after transplantation according to the size of the tumor nodes. Palpable nodes, tumor length (a) and width (b) were measured every 2 days to construct a tumor growth curve. Tumor volume was calculated in dynamics using the following formula ($V = a \times b \times c$, mm^3), which allowed us to calculate the average volumes (V_n) and the relative growth rate of tumor nodes as the volume ratio (V_n / V_{n-1} , where n is the measurement number). To assess the antitumor effect, a standard tumor growth inhibition rate was used - $T / C, \%$ (treatment / control), T / C criterion $\leq 42\%$.

After 15 days, it was noticeable that the tumor size in mice in the control group (without incubation with peptides) increased, and in the experimental group (pre-incubated with peptides), tumor growth slowed down. From the 20th day, it became noticeable that the tumor volume in the group of mice treated with peptides (concentration 40 μg / ml, the dose for inoculating mice 2.5 mg / kg) was significantly less than in the group of mice treated with peptides (control tumors). On the 35th day of the experiment, animals were killed using humane methods, tumors were excised and weighed. All results were expressed as mean \pm standard deviation. All experiments were carried out in strict accordance with the recommendations of the guidelines for the care and use of laboratory animals (National Institute of Health, USA).

VII Statistical Processing of Results

All *in vivo* experiments were duplicated three times, and the data are presented as the average of three repeated experiments. Statistical data analysis was performed using the GraphPad Prism, One-Way ANOVA software. To determine significant differences in the case of comparing two samples, Student t-test was used to compare the two groups. An asterisk indicates statistically significant differences between the positive control and all other types of treatment. The differences were considered significant at (* - $P < 0.05$).

VIII Ethical Considerations

All experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The protocol was approved by the Committee for the Ethics of Animal Experiments. Every effort was made in order to minimize animal suffering.

Results and Discussion

In this work, we investigated the effect of RHAMM target peptides on tumor growth in xenografts (immunodeficient mice). Immunodeficient animals are traditionally used to obtain models of various malignant tumors. We created such a model by implanting a transplantable cell culture in order to adapt it to growth in mice.

Prostate cancer cells (PC3mLN4) were grown to the logarithmic phase, then a cell suspension was prepared by trypsinization to a cell concentration of 2×10^7 / ml. Peptides (GEGEEGEE, DFGEEAEE and RYQLHPYR, final concentration 40 μg / ml) were added to the cells. An equal amount of growth medium was added to the control cells (without peptides). Then, a cell suspension (100 μl , cell concentration 2×10^7 / ml) was injected into the left subcutaneous anterior part, into the axilla of 4-week-old nude mice under aseptic conditions. After 1 week, all mice inoculated with prostate cancer cells (PC3mLN4) showed a slight bulge under the arm. After 2 weeks, tumors began to develop in all mice, and after that we measured the body weight of the mice and the volume of the tumor. After 15 days, it was noticeable that the tumor size in mice in the control group (without incubation with peptides) was increasing, and in the experimental group (cells previously incubated with peptides), tumor growth was slowed down.

From the 20th day, it became noticeable that the tumor volume in the group of mice treated with peptides was significantly less than in the group of mice inoculated with PC3mLN4 cells with untreated peptides (control tumors). On the 35th day of the experiment, the animals were euthanized, tumors were excised and weighed. The ratio of the size or mass of the tumor node per unit time in the treatment and control groups of animals, expressed as a percentage: $T / C \%$ (treatment / control), was used as a quantitative criterion for the effectiveness of the action of peptides. Minimum significant is $T / C \leq 42\%$. This means that such treatment was considered effective that allows to reduce the tumor by more than half.

It was shown that the average tumor weight was 0.226 g in the control group and 0.091 g in the experimental group (GEGEEGEE peptide), indicating a 58% inhibition of the tumor by the peptide (Table 1). Mice inoculated with cells previously incubated with the DFGEEAEE peptide had a tumor weight of 0.0825 g, indicating a 63.5% tumor inhibition by the peptide (Table 1). The tumor weight in the group of mice treated with the RYQLHPYR peptide was significantly less than in other groups and amounted to 0.0123 g, which indicates inhibition of tumor growth by 94.6%. (Table 1). All these values are statistically significant in relation to mice inoculated with cancer cells without prior incubation with peptides (* $P < 0.05$).

Table 1: Inhibition of the growth of subcutaneous xenografts of human prostate cancer under the action of RHAMM-targeted peptides. Differences are statistically significant * P < 0.05 compared with control tumors (without peptide treatment).

A reagent	Way introductions	Dose, mg / kg	Weight Tumors, g	Tumor inhibition, %	T / C %
Control group	subcutaneously	2,5	0,226	0,0	0,0
GEGEEGEE	subcutaneously	2,5	0,091	*59,8	*40,2
DFGEEAEE	subcutaneously	2,5	0,0825	*63,5	*36,5
RYQLHPYR	subcutaneously	2,5	0,0123	*94,6	*5,4

Targeted chemotherapy allows you to selectively and efficiently localize the drug to molecular targets in the cell (for example, receptors), at the same time limit its access to a normal cell and thus maximize the therapeutic effect and reduce the toxicity of the drug. Over the past years, the use of peptides as promising therapeutic agents for the treatment of cancer has been growing rapidly. Therapeutic peptides are gaining popularity for use in medicine in various aspects, including in the form of antitumor vaccines, antimicrobial therapy, cancer treatment, or nucleic acid delivery [29-36]. Recently, it was found that the antimicrobial peptide nisin has antitumor activity, nisin induces apoptosis and inhibits the proliferation of human astrocytoma cells (SW1088) [37]. In the course of studies, it was found that peptides have different physiological activity and different effects on tumor cells. Many natural and synthetic "proapoptotic" peptides induce apoptotic enzymes and cause cell death. For example, a cationic antimicrobial peptide isolated from a *Brazilian tarantula* possesses not only bacteriostatic properties, but also exhibits antitumor activity *in vitro* and *in vivo* [38].

The so-called "cell-penetrating peptides" enhance the effect of chemotherapeutic drugs, while "membrane-lytic", cationic antimicrobial peptides destroy the membranes of cancer cells [39-43]. Recently, peptides isolated from an Australian frog have been shown to inhibit breast cell division [44]. Chimeric peptides exhibit antitumor activity and decrease the multidrug resistance of tumors [45]. It is known that the cell-penetrating peptide dNP2 can accelerate the accumulation of antitumor drugs in the cell and thereby increase the effectiveness of cancer treatment [46]. Recent studies have shown that protein 2 (Grb2) bound to the growth factor receptor is an adapter protein that is significantly involved in tumor neoplasms and blocking this protein with peptides inhibits tumor growth [47]. Recently, the ephrin receptors of the kinase system have attracted increasing attention as the main class of potential drug targets [48]. Peptides that specifically bind to high affinity ephrin receptors have been identified [48].

These peptides, as a rule, are antagonists that inhibit the binding of ephrin and signaling to the ephrin receptor, but some are agonists that mimic the activation of the ephrin receptor [49]. In addition to modulating the function of the ephrin receptor, such peptides can serve as diagnostic and therapeutic agents, as well as for the delivery of various nanoparticles to tumors and other affected tissues representing ephrin-target receptors [50, 51]. Peptide antagonists have great prospects, because their main mechanism of action is aimed at a specific molecular target of tumor cells, which leads to their death. Therefore, the identification of such peptides, the study of their therapeutic potential for cancer treatment is relevant today.

It was previously shown that RHAMM-targeted peptides stimulated apoptosis, necrosis of cancer cells, and *in vitro* blocking tumor cell invasiveness [52-54]. The results of this study showed that RHAMM-targeted peptides significantly inhibit tumor growth in murine xenografts (from 60 to 94%). We first found that RHAMM-targeted peptides exhibit antitumor activity *in vivo* (mouse tumor model). These data serve as the basis for the development of a new targeted therapy approach in the treatment of cancer using RHAMM-target peptides as specific blockers for RHAMM signaling pathway. In future the RHAMM-target peptides can be used in practical oncology for diagnosis and therapy of cancer diseases.

Disclosure

The research work was done according to FASE (number of the state registration of research: # AAAA-A19-119071890015-6)".

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