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Research paper

Increased antitumor activity of tumor-specific peptide modified thymopentin

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ABSTRACT

Thymopoietin pentapeptide (thymopentin, TP5), an immunomodulatory peptide, has been successfully used as an immune system enhancer for treating immune deficiency, cancer, and infectious diseases. However, poor penetration into tumors remains a key limitation to the efficacy and application of TP5. iRGD (CRGDK/RGPD/EC) has been introduced to certain anticancer agents, and increased specific tumor penetrability of drugs and cell internalization have been observed. In the present study, we fused this iRGD fragment with the C-terminal of TP5 to yield a new product, TP5−iRGD. Cell attachment assay showed that TP5−iRGD exhibits more extensive attachment to the melanoma cell line B16F10 than wild-type TP5. Tumor cell viability assay showed that iRGD conjugation with the TP5 C-terminus increases the basal antiproliferative activity of the pentapeptide against the melanoma cell line B16F10, the human lung cancer cell line H460, and the human breast cancer cell line MCF-7. Subsequent injections of TP5−iRGD inhibited in vivo melanoma progression more efficiently than the native TP5. Murine spleen lymphocyte proliferation assay also showed that TP5−iRGD and the parent pentapeptide feature nearly identical spleen lymphocyte proliferation activities. We built an integrin αvβ3 and TP5−iRGD computational binding model to investigate the mechanism by which TP5−iRGD promotes increased activity further. Conjugation with iRGD promotes binding to integrin αvβ3, thereby increasing the tumor-homing efficiency of the resultant peptide. These experimental and computational observations of increased TP5−iRGD activity help broaden the usage of TP5 and reflect the great application potential of the peptide as an anticancer agent.

1. Introduction

Thymopoietin pentapeptide (thymopentin, TP5), a biologically active fragment of thymopoietin (residues 32−36, Arg-Lys-Asp-Val-Tyr) [1,2], exerts subtle immunoregulatory effects on a host and relieves immunosuppression in tumor-bearing mice [3,4]. TP5 has been considered a potential agent for treating immune deficiencies and cancers for many years. Uptake of anticancer agents in solid tumors is an ongoing issue in cancer therapy research. Many anticancer drugs, such as doxorubicin, only migrate 40−50 μm (3−5 cells in diameter) from the vasculature, resulting in minimal efficacy and drug resistance [5−7]. Pleiotropy and limited activity of TP5 in cancer therapy could be caused by poor TP5 penetration into tumor cells. Thus, more effective means of delivering TP5 to target tumor cells must be developed to utilize the potent effects of the peptide on tumor cells fully.

To address the poor penetration of TP5 into tumor cells, the use of RGD tripeptide (arginine-glycine-aspartic) has gained increased attention in recent research on anticancer drugs. RGD exists in many extracellular matrix proteins, such as vitronectin, fibronectin, fibrinogen, lamin, and collagen, and exposed RGD motifs can bind to integrin αvβ3 [8−10]. Although integrin αvβ3 is expressed at low levels in the blood vessel cells of normal tissues, it is expressed at relatively high levels in certain tumor cells [11,12]. Therefore, integrin αvβ3 is considered an excellent target for cellular adhesion and migration in the development of the tumor neovasculature [13−16].

The RGD motif increases the activity of antitumor polypeptide drugs, such as endostatin, through its specific binding to integrin αvβ3 [17]. However, most endogenous linear RGD peptides have very short half-lives in plasma. Cyclic RGD exhibits better biological stability and receptor affinity than linear RGD and thus has a relatively long half-life in plasma. Cyclic RGD has increasingly been

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used in tumor-targeting therapy [18,19]. A highly effective tumor-penetrating peptide, iRGD (CRGDK/RGPD/EC), was recently identified at the base of RGD sequences through the phage display method; this peptide is capable of homing and penetrating into tumor cells [20–22]. iRGD homes in on tumors through three steps [21]. First, iRGD binds to αv integrins, which are highly and specifically expressed within tumors but not in normal tissues. Second, iRGD is proteolytically cleaved to generate CRGDK/R, a truncated peptide with a conditional C-end Rule (CendR) motif (R/KXXR/K). Third, CRGDK/R can bind to neuropilin-1 (NRP-1), activate the CendR pathway, and trigger tissue penetration, acting similarly to VEGF-A165 and other ligands of NRP-1 [23]. iRGD (CRGDK/RGPD/EC) has been introduced to certain anticancer agents and shows increased specific tumor penetrability of drugs and cell internalization [20,21]. For example, a proapoptotic peptide was previously designed to fuse with iRGD; this peptide became highly active when the tumor-penetrating and cell-internalizing peptide iRGD was fused to it [22]. In another example, a recent study showed that adding iRGD significantly improves the antitumor activity of endostatin [24]. In the present study, we conjugated iRGD with TP5 by introducing the iRGD sequence to the TP5 C-terminus. The conjugate exhibited significantly higher bioactivity than wild-type TP5, as determined by various experimental assays. We also performed computational modeling to determine whether or not interactions between the iRGD fragment and integrin αvβ3 could account for the increased activity of the conjugate.

2. Materials and methods

2.1. Materials

The mouse melanoma cell line B16F10, the human lung cancer cell line H460, and the human breast cancer cell line MCF-7 were purchased from American Type Cell Culture (Shanghai, China). Concanavalin A (ConA) was purchased from Sigma–Aldrich Company (USA). Paclitaxel (Taxol) was provided by Jiangsu Yew Pharmaceutical Company Limited (Wuxi, Jiangsu Province, China). ICR (SPF) and C57BL/6 mice were purchased from the Comparative Medicine Center of Yangzhou University (China). TP5 and TP5–iRGD were synthesized by Apeptide Company Limited (Shanghai, China).

2.2. Methods

2.2.1. Tumor cell viability assay

The effects of TP5 or TP5–iRGD on tumor cell growth inhibition were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method (MTT) assay, as described previously [25–27]. The MTT assay depends on MTT cellular reduction through mitochondrial dehydrogenase in viable cells to produce blue formazan, which can be measured by spectrometry. The mouse melanoma cell line B16F10, the human breast cancer cell line MCF-7, and the human lung cancer cell line H460 were used in this assay. Tumor cell suspensions were first obtained using 0.25% trypsin-EDTA. Cell concentrations were determined by using a hemocytometer. Approximately 5×10^3 cells per well (100 μL) were seeded in a 96-well plate and incubated for 8 h. TP5 or TP5–iRGD at concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 μmol/mL were then added to the wells. The plate was incubated in a humidified atmosphere of 5% CO_2 and air at 37 °C for another 36 h; each drug concentration was replicated in four wells. RPMI 1640 was used as a negative control and paclitaxel was used as a positive control.

Twenty microliters of MTT solution (5 mg/mL) was added to each well, and the plate was incubated once more at 37 °C for another 4 h. After careful removal of the supernatant, the formazan crystals produced over 4 h in each well were dissolved by 150 μL of dimethyl sulfoxide. The plate was then shaken for 10 min on a plate shaker at room temperature, and the absorbance of the solutions in the wells was read immediately at 570 nm on an ELISA plate reader (Thermo Electron Corporation, USA). The results are reported as percentage of cell growth inhibition; the optical density (OD) measured from RPMI 1640-treated cells was considered the negative control. The percentage of cell growth inhibition was calculated using the equation $(OD_N - OD_T)/OD_N \times 100\%$, where OD_T refers to the absorbance of the treatment group and OD_N refers to the absorbance of the negative control group.

2.2.2. Tumor growth measurement

Suspensions of B16F10 cells [5×10^5 cells in 0.1 mL phosphate-buffered saline (PBS)] were implanted subcutaneously on the mid-left side of C57BL/6 mice. After the average tumor volume of B16F10 mouse melanoma reached 100 mm^3, the mice were randomly divided into four groups with seven mice per group. Group 1 was subsequently injected with 0.1 mL PBS. Group 2 received TP5 at a dose of 0.22 μmol/kg in 0.1 mL PBS. Group 3 was injected with TP5–iRGD at the same dose of 0.22 μmol/kg in 0.1 mL PBS. Paclitaxel [10 mg/(kg.3d)] was set as a positive control and PBS was used as a negative control. All of the groups, except for the paclitaxel group, were subcutaneously injected once daily for 13 d at a site distant from the tumor. The tumor volume was measured every 2 d and determined according to the formula tumor volume $= A \times B^2 \times 0.50$, where A denotes the largest dimension of the tumor and B represents the smallest dimension.

2.2.3. Histochemistry and immunohistochemistry

For immunohistochemical analysis, the tumors were fixed with 4% formaldehyde, embedded in paraffin, and then sectioned for hematoxylin and eosin (H&E) staining as well as immunohistochemical staining for CD8 or CD86. H&E staining was used to detect necrosis in tumor tissues under a light microscope. Immunohistochemical staining of CD8 or CD86 was used to evaluate lymphocyte infiltration and CD86 expression in the tumor tissues. In brief, staining for CD8 or CD86 was performed on sections using their specific primary antibodies and horseradish peroxidase-tagged goat anti-mouse secondary antibody, visualized with diaminobenzidine chromogen as a peroxidase substrate, and then counterstained with hematoxylin. The sections were mounted, dehydrated using alcohol washes of increasing concentration, and cleared using xylene before imaging under a microscope.

2.2.4. Cell attachment assay

Cell attachment experiments were conducted as previously described [28]. In detail, TP5 or TP5–iRGD at doses ranging from 0.008 μmol/mL to 1.0 μmol/mL were incubated in a 96-well ELISA plate (100 μL/well) (Costar, USA) at 4 °C overnight. PBS was used as a negative control. A solution of 2% bovine serum albumin in 1640 medium was used to block each well in the plate at 37 °C for 2 h. The wells were then washed twice with isotonic-buffered saline. Exactly 100 μL of B16F10 melanoma cells (3×10^4/100 μL) was added to each well of the pre-coated plate, and the plate was incubated at 37 °C for 1 h. Unbound cells were removed by washing twice with isotonic-buffered saline. The attached cells were fixed, stained with 0.5% crystal violet staining buffer, and then photographed. The crystal violet in the cells was extracted using 10% acetic acid (100 μL per well). The absorbance of the remaining adherent B16F10 melanoma cells was measured at 595 nm using a microplate reader. Relative cell adhesion (%) was calculated using the equation $(OD_T - OD_C)/OD_C \times 100\%$, where OD_T refers to the absorbance of the treatment group and OD_C refers to the absorbance of the negative control group.

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