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Inhibition of proliferative and invasive capacities of breast cancer cells by arginine-glycine-aspartic acid peptide *in vitro*

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Abstract. The Arg-Gly-Asp (RGD) sequence was selected by using phage-display peptides to target tumors, focusing on targeting α(v) integrins in tumor blood vessels. Recent studies suggest that peptides containing the RGD sequence can bind to tumor cells, as well as tumor endothelial cells. To investigate whether the RGD peptide has other effects on tumor cells expressing α(v) integrins, besides its tumor targeting capability, we designed and synthesized a 10-amino peptide that contained the RGD sequence in a cyclic conformation with a disulfide bond, which specifically bound to breast cancer cell lines MDA-MB-231 and MCF-7. We found that this RGD peptide, GCGGRGDGGC, inhibited tumor cell proliferation in a dose-dependent manner, and also induced apoptosis and G1-phase cell cycle arrest in both of the cell lines that bound and internalized the peptide. Normal ovarian epithelial cells, which did not bind the RGD peptide, were unaffected. RGD peptide treatment also reduced cell invasiveness in both cell lines *in vitro*. This study suggests that the RGD peptide not only possesses tumor targeting capacity, but also has direct tumor cytotoxic and invasiveness inhibition effects dependent on the blockage of α(v) integrin activity, which would make it more efficient in tumor targeting therapy.

Introduction

Targeting agents specifically to tumors and their metastases is a central challenge in improving existing cancer detection and therapy. Directing therapeutic agents to tumor cells, tumor blood vessels or tumor lymphatic vasculature is likely to enhance the efficacy of anti-cancer drugs and decrease side effects (1). Different strategies have been pursued to achieve this goal, among which 'homing' peptides are promising alternatives because they bind to surface molecules specific to the tumor, and tumor endothelial and tumor lymphatic cells (2-4), but are smaller than other agents such as antibody fragments, which allow them to easily penetrate tumor tissues. A prominent example for homing peptides is an Arg-Gly-Asp (RGD) sequence that was selected by using phage-display peptides to target tumors, focusing on targeting α(v) integrins in tumor blood vessels (5). The RGD motif is present in many extracellular matrix components, such as fibronectin and vitronectin, and binds to integrins. Peptides containing the RGD sequence are commonly employed in tumor imaging (6,7) and tumor targeting therapy while coupled with therapeutic agents such as radionucleotides (8,9) and chemotherapeutic drugs (10,11).

The RGD-peptide receptor-α(v) integrins are also expressed on the surface of many kinds of tumor cells. Studies indicate that the RGD sequence can bind not only to tumor endothelial cells, but also to tumor cells *in vivo* (3,12). And *in vitro*, the RGD peptide can be internalized by tumor cells (13). It is known that α(v) integrins can mediate adhesion and migration in some tumor cell lines (13) and regulate cell proliferation and survival (14). However, whether the RGD peptide has any direct anti-tumor effects on tumor cells expressing α(v) integrins, besides its tumor targeting capability by inhibiting integrin ligand binding, remains unclear.

In this study, we designed and synthesized a 10-amino peptide containing the RGD sequence in a cyclic conformation with a disulfide bond, which specifically bound to tumor cells. We found that this RGD peptide, GCGGRGDGGC, inhibited tumor cell proliferation in a dose-dependent manner, induced apoptosis and G1-phase cell cycle arrest, and reduced cell invasiveness *in vitro*. This study suggests that the RGD peptide possesses not only tumor targeting capacity, but also direct tumor cytotoxic and invasiveness inhibition effects dependent on the blockage of α(v) integrin activity.

Materials and methods

Cell line. MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection

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Abbreviations: RGD, Arg-Gly-Asp; FACS, fluorescence-activated cell sorting

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and cultured in DMEM containing 10% fetal calf serum (FCS) at 37˚C in 5% CO_2 humidified air.

Primary tissue culture (15). Primary ovarian epithelial cell cultures were prepared from normal adult ovaries. The tissues from all patients in this study were reviewed by a specialized pathologist, and the diagnosis was reconfirmed histologically. The ovarian tissues were aseptically dissected, followed by squeezing through a cell strainer (FALCON) in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. The single cell suspension was pelleted by centrifugation at 1,000 rpm for 5 min and plated in fresh DMEM medium.

Peptide. The RGD and polyG peptides were synthesized (Xi'an Huachen Bio-tech Ltd., Xi'an, China) with the sequences GCGGRGDGGC and GCGGGGGGGC. These two peptides contained flanking cysteines, which reformed a disulfide loop for each. Glycine residues provide a 'space' function, permitting the physical formation of a loop structure. These two cyclic peptides were conjugated with biotin at the N-terminus, which aimed to be detected easily. PolyG peptide served as the negative control peptide.

Immunofluorescence. MDA-MB-231, MCF-7 and normal ovarian epithelial cells were seeded onto glass coverslips, grown to 60% confluence, fixed with methanol/acetone (1:1), blocked in PBS with 10% FCS at room temperature for 30 min, incubated in PBS with 10% FCS and the RGD peptide (30 μM) overnight at 4˚C, followed by incubation with FITC-conjugated avidin (1:64) for 45 min at 37˚C, then examined with a fluorescence microscope. The negative control was incubated with 30 μM polyG peptide overnight.

Cell proliferation assay. Cells were plated on a 96-well plate (10,000 cells/well) and incubated overnight at 37˚C to allow for attachment and spreading. Cells were washed once with PBS, and 50 μl of 10% FCS in DMEM and 50 μl PBS with different concentrations of peptide (5, 15, 50, 150 and 500 μM) were added to the cells. After incubation for 24 h at 37˚C, 20 μl 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/ml, Sigma Chemical Co.) was added to the wells, which was followed by incubation at 37˚C for 4 h, aspirating the supernatants, adding 100 μl DMSO and incubation at 37˚C for an additional 20 min before absorbance was read at 490 nm. All experiments were performed in triplicate and repeated 3 times.

FACS analysis. MDA-MB-231 and MCF-7 cells were seeded on a 6-well plate (1x10^6 cells/well), incubated with 150 μM RGD peptide (RGD^+) or equal volume of PBS (RGD^-) in DMEM containing 10% FCS for 24 h, harvested and fixed with 70% ethanol in PBS at 4˚C overnight, stained with a solution containing 15 μg/ml propidium iodide (PI), 0.5% Tween-20, and 0.1% RNaseA, and incubated for an additional 30 min at room temperature. Cells were sorted using a FACScan (BD Biosciences) and analyzed with CellQuest version 3.3 software. The apoptosis and subpopulation of cell cycles were calculated after FACS analyses were repeated 3 times.

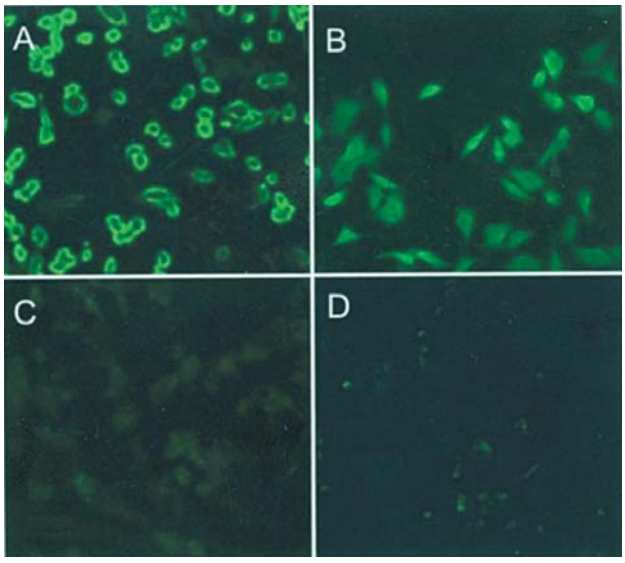

Figure 1. Specific binding of the RGD peptide to MDA-MB-231 and MCF-7 cell lines. MDA-MB-231, MCF-7 and normal ovarian epithelial cells were incubated with 30 μM RGD peptide overnight, and the negative control was incubated with 30 μM polyG peptide overnight. The samples were then stained and examined according to the manufacturer's protocol. (A and B) Bright membrane staining of the RGD peptide for MDA-MB-231 and MCF-7 cells. (C) Normal primary ovarian epithelial cells showed only background staining. (D) The polyG peptide showed only background staining for MCF-7 cells. Original magnification, x100.

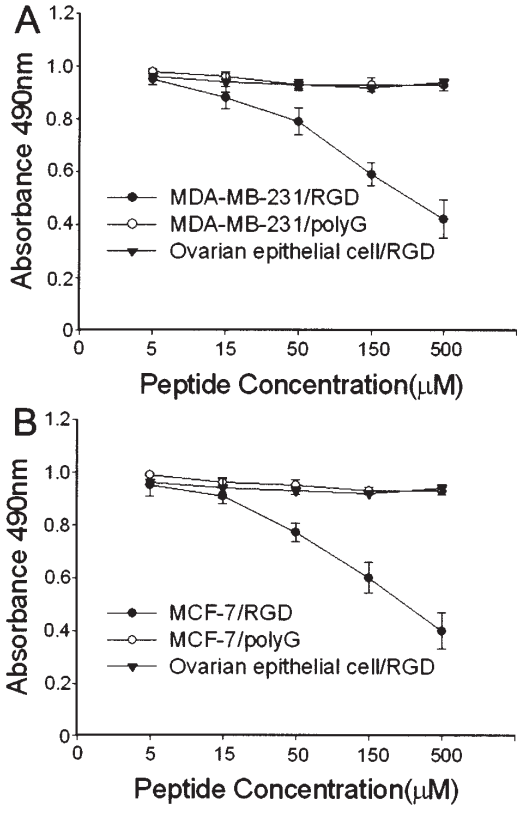

Figure 2. Effect of the RGD peptide on MDA-MB-231 and MCF-7 cell proliferation *in vitro*. MDA-MB-231 and MCF-7 cells were incubated with different concentrations of the RGD peptide at 37˚C for 24 h, and cell proliferation was monitored by MTT. (A and B) *In vitro* cell proliferation assay demonstrated a dose-response inhibition effect of RGD on MDA-MB-231 and MCF-7 cell lines. Primary ovarian epithelial cells, which do not bind the RGD peptide, were not affected. The polyG peptide had no cell binding activity or effect on either cell line. Each point represents the mean of 3 independent experiments performed in triplicate. Bars, ± SD.

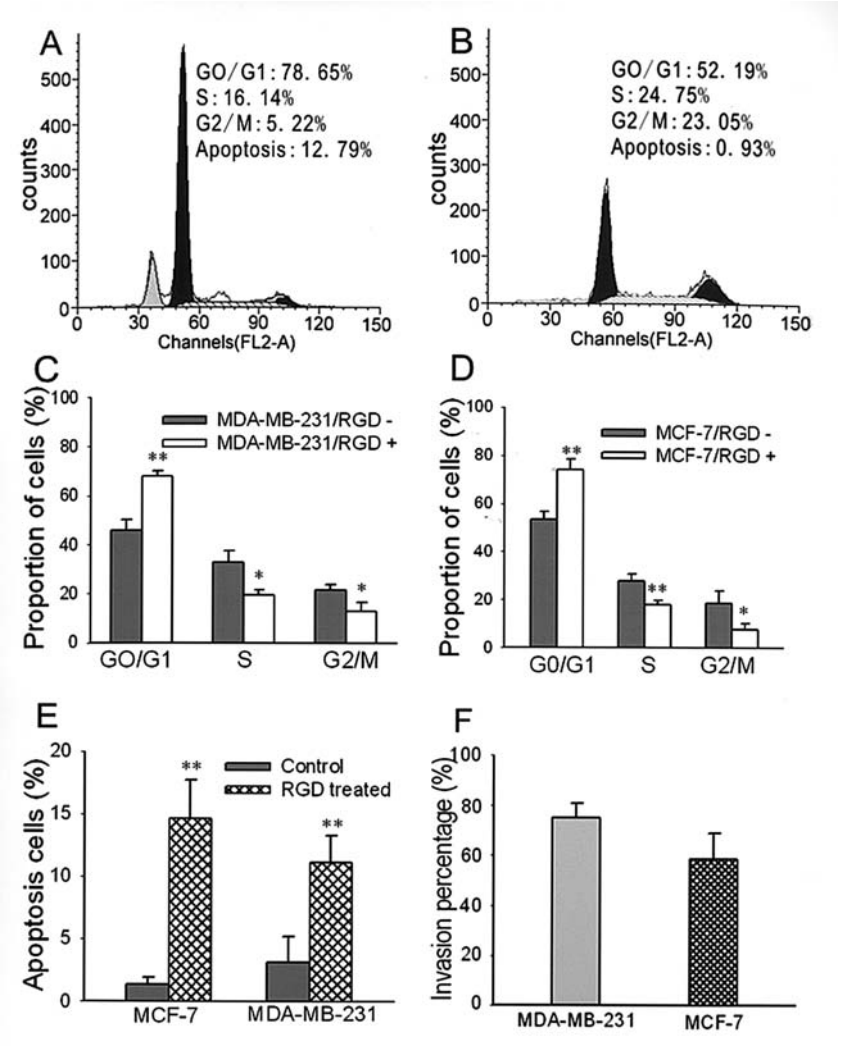

Figure 3. Cell cycle analysis of RGD-treated MDA-MB-231 and MCF-7 cell lines. Both cell lines were incubated with 150 μM RGD peptide (RGD^+) or an equal volume of PBS (RGD^-) in DMEM containing 10% FCS for 24 h and analyzed by flow cytometry. (A and B) Flow cytometric analysis showed increased apoptosis and G0/G1 cell cycle arrest in RGD^+ MCF-7 cells (A) compared to that in RGD^- cells (B). (C and D) Treatment with the RGD peptide caused a significant increase in the proportion of cells in G1 phase and a significant decrease in that of S and G2/M phase in MDA-MB-231 and MCF-7 cell lines. (E) A significant increase of apoptosis in both cell lines after RGD treatment. Data represent the average of 3 independent experiments. (F) Cell invasiveness inhibition with RGD treatment. MDA-MB-231 or MCF-7 cells were treated with 150 μM RGD peptide for 30 min, then plated in the upper chamber in serum-free medium. Data represent the average of 3 independent experiments performed in triplicate. Bars, ± SD. $^*P<0.05$ and $^{**}P<0.01$ vs. RGD^- cells.

Matrigel invasion assay. The possible role played by the RGD peptide in the invasive ability of MDA-MB-231 and MCF-7 cells was assessed by comparing the invasiveness of cells treated with 150 μM RGD peptide for 30 min (RGD^+) with that of the control (RGD^-). *In vitro* invasiveness was measured using a modified Boyden chamber. The polycarbonate filters, 8-μm pore size (Costar), were coated with an extract of basement membrane component (Matrigel, 5 μg/filter; Collaborative Research Co.) in serum-free DMEM medium. Cells ($2x10^5$ cells/chamber) were plated in the upper compartment of the Boyden chamber in serum-free medium. NIH3T3 fibroblast-conditioned medium was used as a chemoattractant in the lower compartment of the chamber. After 16 h incubation, cells in the upper compartment were removed by wiping with a cotton swab, and invasive cells in the bottom chamber were fixed with methanol, stained with hematoxylin and counted in 5 randomly selected fields (original magnification, x400). Invasive ability of each cell line was determined in 3 independent experiments in triplicate.

Statistical analysis. Values were expressed as mean ± SD of at least 3 samples. Differences between groups of data were analyzed using the Student's t-test. $P<0.05$ was considered statistically significant.

Results

The RGD peptide specifically binds to tumor cells. Many kinds of tumor cells, including MDA-MB-231 and MCF-7 (14), express RGD peptide receptor-α(v) integrins on their surface. To determine whether there were enough α(v) integrins on the tumor cell surface to be specifically recognized by the RGD peptide, we initially carried out immunofluorescence *in vitro* binding experiments using the RGD peptide and MDA-MB-231 and MCF-7 cells. Results of this binding experiment are depicted in Fig. 1. The RGD peptide showed bright membrane staining and specificity for MDA-MB-231 and MCF-7 cells. The PolyG peptide is not expected to have any specific binding to membrane proteins (16), and therefore

showed only background staining. In these experiments, normal primary ovarian epithelial cells were used as the negative control. Although α(v) integrins were expressed on the surface (17), there were too few to be detected by the RGD peptide. Thus, the cells showed only background staining.

Inhibition of cell proliferation by RGB after binding and being internalized. After adding the RGD peptide to cultured MDA-MB-231 and MCF-7 cells, we noticed that high concentrations of RGD-treated cells tended to round up 24 h later, and the morphology of their nuclei frequently suggested apoptosis. To investigate whether the RGD peptide inhibits cell proliferation and causes cell death after binding and being internalized by cells, we incubated MDA-MB-231 and MCF-7 cells with different concentrations of RGD peptide at 37˚C for 24 h and monitored cell proliferation. We found that MDA-MB-231 and MCF-7 cells had a significant dose-dependent inhibition of cell proliferation when treated with a 15 μM or higher concentrations RGD peptide ($P<0.01$). Primary ovarian epithelial cells, which do not bind the RGD peptide, were not affected (Fig. 2). The polyG peptide, acting as a control peptide, had no cell binding activity and no effect on either cell line. Thus, the RGD peptide inhibits cell proliferation, specifically after binding and being internalized by tumor cells.

RGD induces apoptosis and G1-phase cell cycle arrest. Given that RGD had an *in vitro* cytotoxic effect on MDA-MB-231 and MCF-7 cells, we examined whether the RGD peptide caused cell apoptosis and cell cycle arrest. After incubation with 150 μM RGD at 37˚C for 24 h, MDA-MB-231 and MCF-7 cells underwent apoptosis as visualized by changes in morphology. Staining with PI and flow cytometric analysis revealed that the percentage of apoptotic cells increased from 3.2±2.0% to 11.1±2.1% or 1.3±0.6% to 14.6±3.0% in MDA-MB-231 and MCF-7 cells, respectively. In addition to inducing apoptosis, as denoted by an increase in the percentage of the sub-G1 population of cells, treatment with the RGD peptide caused a significant increase in the proportion of cells in G1 phase and a significant decrease in that of S and G2/M phase, indicating arrest in the G1 to S transition (Fig. 3A-E).

RGD reduces MDA-MB-231 and MCF-7 cell invasiveness in vitro. To investigate whether RGD-integrin binding affected MDA-MB-231 and MCF-7 cell invasiveness *in vitro*, we incubated cells with 150 μM RGD at 37˚C for 30 min. The cell invasiveness was then measured using a modified Boyden chamber. The number of RGD^+ cells reaching the lower chamber within 16 h was significantly decreased compared to that of RGD^- cells in both cell lines (Fig. 3F). This agent-related decrement in cell transmigration was not secondary to decreased proliferation, as the RGD-treated duration used in this assay did not block cell proliferation (data not shown). Thus, we had evidence of RGD treatment limiting tumor cell invasiveness *in vitro*.

Discussion

We report here that a cyclic RGD peptide, GCGGRGDGGC, can specifically bind to tumor cells expressing α(v) integrins on their surface, inhibit their proliferation in a dose-dependent manner, and induce apoptosis and G1-phase cell cycle arrest after binding and being internalized by the cells. We also show that the RGD peptide-integrin binding can reduce tumor cell invasiveness *in vitro*. These results suggest that the RGD peptide has direct anti-tumor effects due to its integrin ligand-binding blockage capacity.

The RGD peptide was used in this study because it is a promising candidate for both tumor targeting therapy and tumor imaging. In initial studies, it was regarded as a tool to deliver various image or therapeutic agents to the blood vessels of tumors or their metastases. Other studies have found that preventing α(v)ß(3) integrin and, in some cases, α(v)ß(5) integrin in endothelial cells from binding to their ligands using RGD analogues caused apoptosis of newly formed blood vessels (18-20). Studies have found that the RGD peptide can bind not only to tumor endothelial cells, but also tumor cells. Therefore, we aimed to elucidate whether the RGD peptide has other mechanisms in tumor targeting therapy besides those described above.

A previous study has shown that many kinds of tumor cells, including MDA-MB-231 and MCF-7 cells, expressed RGD-receptor α(v) integrins on their surface (13). In order to determine whether there were sufficient α(v) integrins to be specifically recognized by the RGD peptide in both cell lines, we performed an immunofluorescence study. The results indicated that the RGD peptide could specifically bind to MDA-MB-231 and MCF-7 cells. The binding was specific because the negative polyG peptide, which had the same structure and only three different amino acids, did not bind to either cell line.Although normal ovarian epithelial cells also express α(v) integrins on their surface, the amount was insufficient to bind the RGD peptide.

Furthermore, we found that the RGD peptide could not only bind to tumor cells, but also be internalized. Internalization is likely to contribute to the dose-dependent effect of the peptide on inhibition of cell proliferation. In this regard, the RGD peptide is similar to the Tat peptide and other cell penetrating peptides, which are also taken up by cells (21). Moreover, this cytotoxic effect is specific because normal ovarian epithelial cells that do not bind the RGD peptide were not affected by the peptide, and the negative polyG peptide, which cannot bind and be internalized by tumor cells, did not cause cell death in either cell line.

In addition, FACS analysis also indicated that MDA-MB-231 and MCF-7 cells treated with the RGD peptide underwent increased apoptosis and arrest in the G1 to S phase. This is due to the fact that the RGD peptide acts as an α(v) integrin analogue, inhibits ligand-binding, and then blocks the function of integrins. The mechanism of α(v) integrins regulating cell proliferation differs according to the kind of tumor. In breast cancer, a positive correlation was reported between α(v)ß(3) integrin and the overexpression of heregulin (HRG), a growth factor associated with breast cancer aggressiveness (14). Mechanistically, the functional blockade of α(v)ß(3) impaired HRG-promoted hyperactivation of ERK1/ERK2 MAPK in breast cancer. An α(v)ß(3) blockage could significantly decrease S- and G2/M-phase subpopulations of cells, which was linked to an increase in the level and nuclear translocation of the CDK inhibitor

In human ovarian cancer, it was found that α(v) regulate cell proliferation through activation of integrin-linked kinase (ILK) (22). The α(v) integrin blockade by a neutralizing antibody caused the inhibition of ILK activity, and was accompanied by an increase in $p27^{Kip1}$ expression, which resulted in a reduced growth rate and G1/S cell cycle arrest. However, α(v) integrins control melanoma cell survival through a pathway involving p53 regulation of MEK1 signaling (23). The α(v) integrins inactivated p53 and were required for MAPK kinase (MEK) 1 and extracellular signal-regulated kinase (ERK)1/2 activity in melanoma cells, whereas inhibition of MEK1 activity induced apoptosis.

Besides the cytotoxic effect of the RGD peptide on tumor cells, we found a significantly decreased cell invasiveness when incubating MDA-MB-231 and MCF-7 cells with 150 μM RGD at 37˚C for 30 min. This agent-related decrement in cell transmigration was not secondary to decreased proliferation, as the RGD-treated duration used in this assay did not block cell proliferation (data not shown). It was reported previously that down-regulating or functionally blocking α(v) integrins inhibits endogenous p38 mitogen-activated protein kinase (MAPK) activity and urokinase plasminogen activator (uPA) expression in invasive MDA-MB-231 breast cancer cells, and results in reducing cell invasiveness (24). The results suggest that it may be related to blocking the function of α(v) integrins by the RGD peptide mediating decreased adhesion and migration of the cells (14).

In conclusion, our study suggests that the RGD peptide has a direct cytotoxic effect and invasiveness inhibition effect on tumor cells *in vitro,* besides its agent delivery and anti-angiogenesis capacity. Having both the targeting and direct 'killing' effects would make it a more efficient tool for tumor targeting therapy.

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