Inhibitory effects of resistin-13-peptide on the proliferation, adhesion, and invasion of MDA-MB-231 in human breast carcinoma cells

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Abstract

To investigate the potential effects of resistin-13-peptide on the growth, adhesion, and invasion in human breast carcinoma cells, MDA-MB-231. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay and colony-forming assay were used to assess the proliferation effects of resistin-13-peptide. The adhesive ability was investigated by cell adhesion assay, and the invasive potential was assessed using a transwell model. Activities of matrix metalloproteinase (MMP)-2 and MMP-9 were measured by zymography analysis and western blotting. Tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2 were determined by western blotting. In this study, we performed in vivo experiments and determined the effect of resistin-13-peptide on tumor growth and other organs, especially ovaries in a xenograft model using the cell line studied. Resistin-13-peptide inhibited MDA-MB-231 cell growth and colony formation in a dose- and time-dependent manner. Meanwhile, the invasive and adhesive abilities of MDA-MB-231 cells were yet cut down by resistin-13-peptide in a dose-dependent manner. Resistin-13-peptide decreased the gelatinolytic activities of both MMP-2 and MMP-9 and enhanced the protein expression of TIMP-1 and TIMP-2, which were secreted from the MDA-MB-231 cells. The animal experiments found that the growth of tumors was repressed by resistin-13-peptide, which affected other organs in the same time. Especially, ovaries did not have pathological changes yet. Treatment with resistin-13-peptide is effective in suppressing tumor proliferation, adhesion, and invasion. The possible mechanism is downregulation of MMPs and upregulation of TIMPs.

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Introduction

Obesity is a serious health problem, as it is not only associated with a variety of metabolic disorders but also with an increased risk of developing cancer. More specifically, obesity has been identified as a risk factor for breast cancer (Wolk et al. 2001). Unfortunately, the mechanism by which obesity increases cancer risk is not yet known. Insights into the mechanism(s) through which obesity increases the risk of cancer are urgently needed to develop new strategies for the prevention and treatment of certain cancers. Current epidemiological and experimental studies of the obesity cancer association focus on the role of increased adipose tissue, particularly the increase in circulating adipocyte-derived factors (adipokines). Multiple factors from the adipose tissue, such as leptin, adiponectin, cytokines, and other secreted products, influence the processes involved in carcinogenesis (Rajala & Scherer 2003, Fenton et al. 2005).

Resistin is known as an adipocyte-specific secretory cysteine-rich hormone, which can cause insulin resistance and decrease adipocyte differentiation (Steppan et al. 2001). Many studies have provided the evidence for a role of resistin in inflammatory processes...
that may be involved in atherosclerosis (Koemer et al. 2005). The aim of this study was to elucidate the potential of resistin-13-peptide, which contains 13 amino acids (from 22 to 34 of human resistin molecule), possesses some biological activity as the resistin molecule, and stimulates producing monoclonal rabbit antibody in accordance with Zhao et al. (2005).

Metastasis is one of the major causes of mortality in breast cancer patients and occurs as a complex multistep process that involves cancer cell adhesion, invasion, and migration (Liotta 1986). In the multiple stages of these processes, the degradation of environmental barriers, such as the extracellular matrix (ECM) and basement membrane, is the initial step, and several proteolytic enzymes participate in the degradation of these barriers (Kleiner & Stetler-Stevenson 1993, Aimes & Quigley 1995). Among these enzymes, matrix metalloproteinase (MMPs) play a major role. MMPs are a family of zinc-dependent proteinases, which hydrolyze most components of ECM and promote tumor invasion and metastasis (Sternlicht & Werb 2001). Tissue inhibitors of metalloproteinases (TIMPs) are natural inhibitors of MMPs found in most tissues and body fluids. By inhibiting MMPs’ activities, they participate in tissue remodeling of the ECM and inhibit tumor invasion and metastasis. Numerous investigations have pointed out that the MMPs and TIMPs play an important role in the invasion and metastasis of cancers (Beaudex et al. 2004). In addition, MMP-2 (Mr 72 000 type IV collagenase, gelatinase A) and MMP-9 (Mr 92 000 type IV collagenase, gelatinase B) degrade components of the basement membrane and are strongly implicated in the invasion and metastasis of malignant tumors (Cottam et al. 1992, Jiang et al. 2001, Sternlicht & Werb 2001). Therefore, the inhibition of invasion mediated by MMP-2 and MMP-9 may be crucial for the inhibition of cancer metastasis. Recent clinical studies have shown that MMP activity is required for rapidly proliferating and invading tumors rather than for already established tumors (Bergers et al. 1999, Coussens et al. 2002, Seiki 2003). Thus, the inhibition of MMP activity is important for the prevention of early-stage carcinogenesis, particularly the tumor promotion process.

The aim of this study was to elucidate the potential of resistin-13-peptide as an inhibitor of MMP-2 and MMP-9 activities using gelatin zymography, and to present the expression of TIMP-1 and TIMP-2 proteins using western blotting. We also investigated the influence of resistin-13-peptide on cancer cell viability, colony formation, adhesion, invasion, and animal experiments using MDA-MB-231 breast cancer cells.

Materials and methods

Cell culture and reagents

The MDA-MB-231 breast cancer cell lines were originally obtained from the IBMS cell center of PUMC. They were grown as a monolayer culture in RPMI 1640 media (Invitrogen) supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD, USA) in the presence of 5% CO₂. Resistin-13-peptide was provided by our own laboratory. Collagen and Matrigel were purchased from Collaborative Biomedical products, and fibronectin from BD Biosciences (Foster City, CA, USA). Boyden chambers were obtained from Becton Dickinson & Co. (San Jose, CA, USA).

Cell proliferation assay

Cell viability was measured by the 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were cultured for 24, 48, 72, or 96 h in 96-well plates at a concentration of 1000 cells/well. Each well was filled with fresh FBS-free medium that contained 5–10 000 ng/ml various concentrations of resistin-13-peptide. The cells were then incubated for 24, 48, 72, or 96 h at 37°C, and then with MTT for 4 h. The liquid was removed and dimethyl sulfoxide (DMSO) added to dissolve the solid residue. The optical density of each well at 570 nm was determined using a microplate reader (Molecular Devices, San Diego, CA, USA).

Colony-forming assay

To assay for contact-independent growth in soft agar, 92 mm gridded Petri dishes were first coated with 1.5 ml base agar consisting of 0.5% agar B (Difco, Lawrence, Kansas, USA) in the culture medium DMEM. Control and 50–10 000 ng/ml various concentrations of resistin-13-peptide cells were counted using a hemocytometer, and then resuspended at low density (1000 cells/1.5 ml) in 0.35% agar B culture medium kept at ~ 40°C. After pouring into the 92 mm dishes containing base agar, the immobilized cells were grown for 14 days in a humidified chamber at 37°C with 5% CO₂. Plates were stained with 0.005% crystal violet in PBS for 1 h. Colonies were photographed and then counted. The experiments were performed at least thrice.

Cell adhesion assay

The 96-well tissue culture plates were coated with 5 µg/well Matrigel or fibronectin, and left to air-dry for 40 min. Cells (5×10⁴) suspended in DMEM that contained 0.5% BSA were dispensed into each well of the 96-well culture plate, incubated in 5% CO₂ at 37°C.
for 60 min, and gently washed thrice with PBS to remove the unattached cells. Each well was then incubated with MTT for 4 h. The liquid was removed and DMSO added to dissolve the solid residue. The optical density of each well at 570 nm was determined using a microplate reader (Molecular Devices). The experiments were performed at least thrice.

**Cell invasion assay**

MDA-MB-231 cells invasion was determined in vitro, using a method described previously by Dewhurst *et al.* (1997). Transwell inserts with polycarbonate filters were obtained from Costar UK (High Wycombe, Bucks, UK). For each short-term culture to be tested, three filters with 8 μm pores were coated with 50 μl human fibronectin solution (10 μg/ml). The ability of the cells to permeate through Matrigel-coated filters was measured in a Boyden chamber. Transwell filters (8 μm) coated with 50 μg Matrigel were used according to the standard protocols. Fibroblast conditioned medium, which was obtained by a 24 h incubation of NIH-3T3 cells with 8 mM EDTA. Cells were washed and 10^5 cells were removed from the tissue culture flasks using 0.5% trypsin solution and centrifuged at 250 g for 10 min, after which they were resuspended in serum-free medium supplemented with 0.1% BSA. Single-cell suspensions of control incubated overnight in the medium alone or in the media containing resistin-13-peptide at concentrations of 5, 50, 500, 5000, 10,000 ng/ml were obtained by the treatment with PBS containing 5 mM EDTA. Cells were washed and 10^5 cells per well were placed in the upper chamber in 0.1 ml DMEM and 0.1% BSA in the presence or absence of resistin-13-peptide for an indicated period of time. MDA-MB-231 cell suspensions (100 μl) were seeded at 1.2 × 10^5 cells/well in the upper compartment of each invasion chamber. After 19 h at 37°C in a 5% carbon dioxide/95% air atmosphere, the top surface of the membrane was gently scrubbed with a cotton bud, and cells on the undersurface were fixed and stained with the Diff-Quick staining kit, examined by bright field microscopy, and photographed. Values for invasion were expressed as the average number of migrated cells bound per microscopic field over four fields per assay, and expressed as averages for triplicate experiments. The assay was repeated thrice for each culture.

**Gelatin zymography**

The identification of proteolytic enzymes expressed was performed by the electrophoresis of serum-free conditioned medium taken from confluent MDA-MB-231 cells growing in tissue culture, based on the method of Laemmli (1970). The conditioned media were collected and the cell numbers determined. Gelatin/SDS-polyacrylamide gel was used to detect the presence of gelatinolytic 72 kDa MMP-2 and 92 kDa MMP-9. Both active forms and pro-enzymes are revealed by this technique as the exposure of pro-MMPs to SDS during SDS-PAGE leads to activation without proteolytic cleavage. Electrophoresis was carried out at room temperature at a constant voltage of 110 V. When the tracking dye reached the bottom of the gel, it was washed twice with 2.5% Triton X-100 (Sigma) for 60 min to remove the SDS. The gel was subsequently incubated for 48 h at room temperature in a buffer that contained 50 mM Tris–HCl (pH 7.6), 200 mM NaCl, 5 mM CaCl₂. The gel was stained with 0.5% Coomassie blue for 2 h and then destained with water containing 10% glacial acetic acid and 30% methanol. Areas of gelatinolytic degradation appeared as transparent bands on the blue stained background of the gel, and the intensities of the bands were measured. A wide range molecular weight marker (M4038; Sigma) was used to estimate the apparent molecular weights for the bands of substrate degradation, and a negative sample of serum-free media was run in one lane.

**Western blotting**

MDA-MB-231 cells were treated with indicated concentrations of resistin-13-peptide for 24 h. The conditioned media were collected and analyzed by Western blotting. The total protein of supernatant was separated by SDS-PAGE using 10% gel and transferred to PVDF membranes (Bio-Rad), the blots were blocked overnight in a blocking buffer, and then incubated with the primary antibody. The immunoreactive bands were revealed by means of an enhanced chemiluminescence kit. The resultant blots were scanned using an imaging densitometer.

**Animal experiments**

Animal studies were conducted in accordance with the Institutional Animal Care and Use Committee. MDA-MB-231 cells (8 × 10⁶) in PBS were injected subcutaneously into the right flanks of 5- to 6-week-old female athymic nude mice using a 27 gauge needle. The mice were randomly assigned to one of the three groups: sodium chloride, 2.5 mg/kg resistin-13-peptide, or 5 mg/kg resistin-13-peptide. Mice were weighed and tumor sizes measured with a caliper once in 4 days. The volume of the tumor was determined using the following formula: tumor volume = 0.523 × width² × length. Mice with weight
loss over 20% and tumor size larger than 1 cm in any dimension were terminated. At the end of the experiment, animals were terminally anesthetized and primary tumors dissected, weighed, and fixed in 10% formalin.

**Statistical analysis**

All of the experiments were replicated thrice. The mean standard deviation, mean square errors, two-factor ANOVA, correlation, and interaction of main effects were calculated using the GraphPad Prism 4.0. Appropriate comparisons were made using Dunnett’s method for multiple comparisons. $P < 0.05$ was considered statistically significant.

**Results**

In the MTT assay, we treated MDA-MB-231 human breast cells with concentrations of resistin-13-peptide ranging from 5 ng/ml to 10 μg/ml for four different time intervals: 24, 48, 72, and 96 h. For the four treatment time periods, dose-dependent and time-dependent inhibition was observed (Fig. 1). With the treatment period of 24 h, we observed weak growth inhibition even at the maximum concentration of 10 μg/ml, with values not much greater than those observed for the untreated control. On the contrary, we could observe a significant growth inhibition of over 40% at 5.0 μg/ml after 48 h of treatment, at 0.5 μg/ml after 72 h, and at 50 ng/ml after 96 h. These results imply that multistep molecular events are necessary for resistin-13-peptide’s function of switching MDA-MB-231 cells from a proliferative state to an inhibited state of cell growth. It appears that resistin-13-peptide requires more than 24 h to significantly affect MDA-MB-231 cell proliferation.

Soft agar colony-forming assays were performed as described in the experimental procedures. The 92 mm plates were seeded with $1 \times 10^3$ MDA-MB-231 cells, and allowed to grow for 2 weeks. After 2 weeks, the colonies were stained with crystal violet and counted whether the colonies exceeded 50 cells. Results showed that resistin-13-peptide inhibited colony formation of MDA-231 cells in a dose-dependent manner.
colonies were stained with crystal violet, and counted whether the colonies exceeded 50 cells. The results showed that resistin-13-peptide inhibited colony formation of MDA-MB-231 cells in a dose-dependent manner (Fig. 2). This result also indicates that resistin-13-peptide can inhibit the proliferation of MDA-MB-231 cells.

Tumor metastasis comprises multiple steps. Therefore, tumor cells need to express a variety of properties, including altered adhesiveness, increased motility, and invasive capacity, to complete the metastatic process. Because the adhesion and motility of tumor cells in the ECM are considered important steps in the invasive processes of metastatic tumor cells, the effects of resistin-13-peptide on cell adhesion were examined. Incubation of MDA-MB-231 cells with 5–10 000 ng/ml resistin-13-peptide for 60 min significantly inhibited cell adhesion to the Matrigel- and fibronectin-coated substrate in a concentration-dependent manner (Fig. 3).

We further evaluated the anti-metastatic activity of resistin-13-peptide using the transwell assay. We tested the ability of MDA-MB-231 cells to permeate through a reconstituted basement membrane barrier (Matrigel) with or without resistin-13-peptide. Similar to the migration assay, resistin-13-peptide inhibited the invasion of MDA-MB-231 cells in a dose-dependent manner in the range of 5–10 000 ng/ml. When the MDA-MB-231 cells were grown on Matrigel, a significant reduction in the number of invasive cells was seen when the cells were treated with 5–10 000 ng/ml resistin-13-peptide for 16 h, when compared with the control (fresh medium alone), with the levels of invasion being reduced from 24.91% to 83.17% of the control levels at 5–10 000 ng/ml resistin-13-peptide (Fig. 4). No significant reduction in invasiveness was observed when the cells were treated with the lower dose of 5 ng/ml resistin-13-peptide.

Gelatinases/type IV collagenase play a major role in the facilitation of cancer metastasis. Thus, gelatin zymography was used to study the effects of resistin-13-peptide on MMP-2 and MMP-9 activities.

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The activities of MMPs are regulated at various levels, such as during secretion and the activation of pro-MMP to the enzymatically active MMP. To examine the inhibitory effects of resistin-13-peptide on pro-MMP-2, MMP-2, and MMP-9, conditioned media from MDA-MB-231 cells cultures were subjected to gelatin zymography in the presence of various concentrations of resistin-13-peptide for 24 h. The conditioned media were analyzed by the zymogram. Representative zymogram shows gelatinolytic activity of MMP-9 and MMP-2 in the conditioned media from the invasion assays and the corresponding data analysis of MMP-9 from three separate experiments. Gelatin zymogram shows examples of MMP-2 (72 kDa) and MMP-9 (92 kDa) secretion by cultured MDA-MB-231 cells. A molecular weight marker was run on each gel (not shown). Resistin-13-peptide at 50 ng/ml and higher concentrations clearly suppressed pro-MMP-2, MMP-2 activity, and MMP-9 activity in a dose-dependent manner. Thus, in the MDA-MB-231 cell system, both MMP-2 and MMP-9 were downregulated by resistin-13-peptide treatment (**$P<0.01$).

Meanwhile, western blot assay also confirms these results that the protein expression of MMP-2 and MMP-9 were inhibited in a dose-dependent manner (Fig. 6). We further examine the effects of resistin-13-peptide on MDA-MB-231 secreting TIMP-1 and TIMP-2 by western blot analysis. We observed that the expression of TIMP-1 and TIMP-2 increased in the conditioned media (Fig. 7). Especially, ovaries did not have pathological changes yet.
Discussion

Epidemiological studies show a positive association between obesity and cancer risk. In addition to increased body adiposity and secretion of fat-derived hormones, obesity is also linked to insulin resistance and chronic inflammation. Insulin resistance and inflammation are responsible for the positive correlation with cancer (Nunez et al. 2006). Resistin is a recently discovered novel peptide hormone secreted by human adipocyte-specific and mononuclear cells. It is reported that resistin is related to insulin sensitivity and inflammation (McTernan et al. 2002a,b). In the present study, we tried to reveal the relationship between resistin and cancer using MDA-MB-231 breast cancer cells treated with resistin-13-peptide. But our observations disagree with the results of Di Simone et al. (2006), which indicated that resistin can enhance both MMP-2 protein and mRNA expression, significantly reducing TIMP-1 and TIMP-2 and increasing trophoblast-like cell invasion in human choriocarcinoma cells (BeWo). Our results demonstrated that resistin-13-peptide inhibits the invasive capacity of MDA-MB-231 cells in vitro and in vivo through decreased expression of MMP-2 and MMP-9 protein and enhanced secretion of TIMP-1 and TIMP-2 protein in a dose-dependent manner. Meanwhile, resistin-13-peptide can arrest the proliferation and colony formation of MDA-MB-231 breast cancer cells in vitro, and repress the growth of tumor of MDA-MB-231 cells in vivo.

Invasion and metastasis of tumor is the major cause of morbidity and mortality worldwide. Metastasis is an active process involving the altered attachment of the tumor cells to the basement membrane, localized degradation of connective tissue, and migration through stromal tissue (Curran & Murray 2000). In this study, using adhesion and invasion assays, we have shown that resistin-13-peptide effectively inhibits the migration and invasion of MDA-MB-231 cells in vitro. These results suggest that the resistin-13-peptide is linked to its anti-invasive actions. Our studies in vitro or in vivo support this hypothesis because resistin-13-peptide regulates MMP-2, MMP-9, and TIMPs. The balance between MMPs and TIMPs activities is involved in both invasion and metastasis. The MMPs constitute a large family of structurally related matrix degrading proteases that have pivotal roles in development, tissue remodeling, and cancer (Duffy et al. 2000, John & Tuszynski 2001, Murphy et al. 2002), and their levels of...
expression increase with the progression of the tumor. We showed that resistin-13-peptide decreased the secretion of MMP-2 and MMP-9 protein and enhanced the expression of TIMP-1 and TIMP-2 protein in a dose-dependent manner in the MDA-MD-231 cells. The activity of MMPs is physiologically regulated by the TIMPs. Our results indicate that resistin-13-peptide act as an important regulator of ECM breakdown during tumor invasion and metastasis as a result of their ability to regulate MMP and TIMP production. Obviously, tumor expansion in the surrounding tissues requires the removal of the basement membrane and ECM. The MMPs have the combined ability to degrade the major components of the ECM including type I collagen. Experimental studies support the idea that they play an important role in metastasis of tumor cells.

In addition, we observed the effect of resistin-13-peptide on tumor growth and other organs, especially ovaries in a xenograft model using the MDA-MB-231 cell line studied. The results showed that the growth of tumors was repressed by resistin-13-peptide, but the mice weights and other organs did not contrast between control and experiment classes. Especially, ovaries did not have pathological changes yet.

Meanwhile, metastasis is one of the most important factors related to cancer therapeutic efficacy and prognostic survival (Sugiyama & Sadzuka 1999). In cancer research, one of the most active fields is the development of novel anti-metastatic drugs with low toxicity and high efficacy. In this study, using adhesion, invasion assays, and animal experiments, we have shown that resistin-13-peptide effectively inhibits the invasion of tumor cells in vitro and in vivo. In addition, we have demonstrated that resistin-13-peptide suppresses the activities of MMP-2 and MMP-9 and enhances TIMP-1 and TIMP-2, which may have implications for the pathogenesis of breast cancer. Nevertheless, the underlying molecular mechanisms of this action of resistin-13-peptide remain unknown and require further research.

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