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Adiponectin mediates antiproliferative and apoptotic responses in endometrial carcinoma by the AdipoRs/AMPK pathway

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HIGHLIGHTS

- Adiponectin plays antitumor role in endometrial cancer via AdipoRs/AMPK pathway.
- The effects of AdipoR1 and AdipoR2 on endometrial cancer cells are different.

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ABSTRACT

Objective. Determine the serum adiponectin levels in endometrial carcinoma (EC) cases and controls and explore the correlation between them. We assessed the functions of AdipoR1 and AdipoR2 in endometrial cancer cells to determine whether the AMPK/ERK and Akt pathways mediate the effects of adiponectin-induced apoptosis and anti-proliferation.

Material and methods. The serum adiponectin levels were measured via enzyme-linked immunosorbent assay (ELISA). The proliferation and apoptosis rates were determined with MTT and annexin V/PI assays. To evaluate the activation of AMPK, ERK, and Akt and the expression of Bcl-2 and Cyclin D1, western blot analysis was performed in Ishikawa 3-H-12 cells. We down-regulated AdipoRs by si-RNA to assess their functions.

Results. The serum adiponectin levels were significantly decreased in patients with EC compared to controls. The adiponectin-induced apoptosis and anti-proliferation effects in EC cells were blocked by Compound C. Ishikawa 3-H-12 cells exhibited time- and dose-dependent increases in the p-AMPK levels after treatment with adiponectin. Adiponectin treatment reduced the levels of ERK and Akt phosphorylations and cyclin D1 and Bcl-2 mRNA and protein expression. Compound C blocked the effects on ERK, Akt, cyclin D1, and Bcl-2. AdipoR1 and AdipoR2 were involved in adiponectin-induced growth inhibition and ERK activation inhibition. We speculated that AdipoR1 has a greater role than adipoR2 in apoptosis and Akt activation inhibition after adiponectin treatment.

Conclusion. Adiponectin was an apoptotic and anti-proliferation agent for EC cells, and these effects were dependent on the AMPK/ERK and Akt pathways. AdipoR1 and AdipoR2 may play different roles in this process.

Introduction

Endometrial carcinoma (EC) is the most common gynecologic malignancy [1]. In the United States, 49,560 new cases and 8190 deaths from EC were expected in 2013 [2]. The incidence of EC has also been increasing in China in the past 20 years, and there has been an increase of over 100% in the overall death rate from EC during this time [3]. Because the incidence of EC far exceeds the mortality from this disease, it is particularly important to identify the risk factors for mortality. Insulin resistance plays a central role in EC development [4]. Obesity and diabetes mellitus, both characterized by insulin resistance [5], are known risk factors for EC. Insulin resistance is characterized as a pathological state in which there are higher-than-normal levels of insulin circulating in the blood [6]. Low levels of adiponectin have a high correlation with hyperinsulinemia and the degree of insulin resistance [7]. In our previous studies, insulin promoted endometrial cancer cell proliferation, survival, and invasion and inhibited cell apoptosis [8,9]. In this study, we investigated the effect of adiponectin on endometrial cancer. Adiponectin, a hormone that is mainly produced in adipose tissue, is abundantly present in human plasma and has been described as an insulin-sensitizing adipocytokine [10,11]. However, unlike insulin, TNFa and leptin, the circulating adiponectin levels are strongly decreased in obese individuals [6,12,13]. Adiponectin has an anti-diabetic activity through modulating glucose, fatty acid metabolism and insulin

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sensitivity [14]. Moreover, adiponectin may influence cancer pathogenesis. In some previously reports, circulating adiponectin levels of endometrial cancer cases are lower than controls [15,16]. These findings raise the possibility that adiponectin may be functionally involved in cancer progression as well as carcinogenesis. Furthermore, adiponectin controls the cell number by inhibiting cell proliferation and inducing the apoptosis of breast cancer cells [17]. These observations have led us to investigate the hypothesis that adiponectin may play a negative role in the development and progression of EC by altering cell proliferation and/or apoptosis. The adiponectin receptors have recently been defined and designated as AdipoR1/R2 [18]. Although recent reports have shown that the AdipoR1/R2 mRNA were significantly expressed in EC [19], little is known about the roles of AdipoR1/R2 and their signal transduction in endometrial cells. Thus far, the major signaling pathway that has been identified for adiponectin is one involving AMP-activated protein kinase (AMPK) [20], a serine/threonine protein kinase that is the evolutionarily conserved energy sensor in eukaryotic cells [21]. Activation of AMPK is known to suppress cell growth and cause apoptosis in human cancer cells, such as prostate cancer cells [22] and glioma cells [23]. However, it is unknown whether the AdipoRs/AMPK pathway also plays a role in regulating these processes in EC cells in response to adiponectin stimulation. In previous studies, ERK and Akt phosphorylations were shown to be inhibited by AMPK activation and restored after si-AMPK [24,25], which suggested that AMPK plays an important role in ERK and Akt inactivation. It is still unclear whether ERK and Akt are downstream targets of adiponectin.

In this study, we investigated the role of adiponectin in EC. Specifically, we aimed to elucidate the mechanism by which adiponectin exerts its effects on EC cell proliferation and apoptosis. We hypothesized that adiponectin stimulates EC cell apoptosis and inhibits proliferation through its receptors and subsequent signal molecules. To test this hypothesis, we measured the serum adiponectin levels of the patients with EC and control subjects and evaluated the effects of adiponectin on proliferation, apoptosis, and AMPK pathway activation and its downstream targets in the Ishikawa 3-H-12 cell line, which is an endometrial cancer cell line. Then, we investigated whether AdipoR1 and R2 play different roles in this pathway.

Materials and methods

Serum adiponectin levels in EC

After approval from the Institutional Review Board, 88 women with EC (cases) and 90 women without a history of cancer (controls) were identified for participation in the study. Cases were identified through the Department of Gynecology, Tianjin First Centre Hospital, Tianjin, China. Control patients were obtained through a low-risk cancer screening program and collected at the time of enrollment in the program. Informed consent was obtained from participating subjects before the study. All patients were newly diagnosed and none had received any form of anticancer therapy before collection of the blood samples for biochemical analysis. The diagnosis of EC was established by biopsy confirmation. All tumors were staged according to the FIGO classification (2014). For all blood samples, serum was immediately separated by centrifugation and stored at −80 °C until further use. None of the samples had been previously thawed. Both case and control samples were run on the same microplate and the investigators were blinded to the study group. The serum adiponectin levels were measured using a commercially available quantitative sandwich enzyme-linked immunoabsorbent assay (ELISA) with a sensitivity of 0.246 ng/mL (R&D Systems, Minneapolis, MN). The coefficients of variance for intraassay and interassay precision were 2.5% to 4.7% and 5.8% to 6.9%, respectively, as reported by the manufacturer.

Reagents and cell lines

Recombinant human adiponectin was purchased from BioVendor Laboratory Medicine (Brno, Czech Republic) and R&D Systems (Minneapolis, MN, USA). Though not necessarily fully equivalent to the circulating forms in human blood, the full-length recombinant human adiponectin protein, after reconstitution, forms multimeric structures (described in BioVendor product data sheet). Six human endometrial cancer cell lines, AN3CA, RL95-2, Ishikawa3-H-12 (cell line of moderately differentiated adenocarcinoma cells provided by Professor Nishida of the Kasumigaura National Hospital, Japan), ECC-1, HEC1A and HEC1B were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from Ishikawa 3-H-12 cells with TRIzol reagent (Invitrogen). The RNA yield and purity were determined by spectrophotometry. Aliquots of 2 μg of total RNA were used for first strand cDNA synthesis in 40 μL reactions that included 200 units of M-MLV reverse transcriptase. The PCR primers are summarized in Table 1. After cDNA synthesis, the target genes were amplified. The PCR conditions were as follows: denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The PCR products were separated in 2% agarose gels, stained with ethidium bromide, and visualized under UV transillumination. β-actin was used as control.

Western blot

Ishikawa 3-H-12 cells were homogenized in lysis buffer containing 25 mM Tris/HCl (pH 8.0), 140 mM NaCl, 2 mM EDTA, 1 mM NaVO4, 1% Nonidet-P40, 1 mM PMSF, 5 μg/mL aprotinin, and 2 μg/mL leupeptin. Supernatant fractions were collected and the protein content was determined with the BCA Protein Assay Kit according to the manufacturer's instructions. Proteins were separated by electrophoresis in 12% SDS-polyacrylamide gels (PAGEs) and then transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies against AdipoR1, AdipoR2, AMPK, p-AMPKa (Thr172), ERK, p-ERK (Thr202/Tyr204), p-Akt(Ser473), cyclin D1, bcl-2, bax, caspase-3 or β-actin (all purchased from Santa Cruz) overnight at 4 °C. After washing

Table 1
List of primers used for PCR.

Primer sets	Sequence	PCR product(bp)
AdipoR1		
Sence	5′-TTCTTCCTCATGGCTGT GATGT-3′	148
Antisence	5′-AGTGGACAAAGGCTGCTGCCA-3′	
AdipoR2		
	5′-ATGAA CGAGCCAACAGAAAACCGA-3′	376
	5′-CGGTGTCCATGCAAGAGGAAGTCA-3′	
Cyclin D1		
	5′-CTGGCCATGAACTACCTGGA-3′	483
	5′-GCTACACTTGATCACTCTGG-3′	
Bcl-2		
	5′-TTGGATCAGGGAGTTG GAAG-3′	295
	5′-TGTCCCTACCAACCAGAAGG-3′	
Bax		
	5′-GGATGCGTCCACCAAGAA-3′	386
	5′-GCACTCCCGCCACA AAGA-3′	
Caspase-3		
	5′-TGGAAGCGAATCA ATGGACT-3′	346
	5′-TTTCAGGTCAACAGGTCCAT-3′	
β-Actin		
	5′-CCTGGGCATGGAGTCCTGTG-3′	305
	5′-AGGGGCCGGACTCGTCATAC-3′	

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