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Adiponectin Promotes Autophagy and Apoptosis in Endometrial Cancer Cell Lines

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Abstract

Obesity is a current pandemic and an established risk factor for endometrial cancer. There is emerging evidence that obesity is linked to malignancy development *via* dysregulation of adipose tissue-secreted hormones, known as adipokines. Adiponectin is one of the most abundant circulating adipokines and has anti-inflammatory and anti-neoplastic properties. The aim of our study is to evaluate the role of adiponectin in autophagy and apoptosis in endometrial cancer cells. We treated KLE endometrial cancer cell lines with 1 μg/ml of adiponectin for 24 h. We demonstrated that the levels of all three major autophagy markers (ATG5, Beclin-1 and LC3) increase with adiponectin treatment, suggesting activation of autophagy. In contrast, treatment with adiponectin reduced the protein levels of the anti-apoptotic protein Bcl-2 and augmented nuclear localization of Bcl-2, suggesting promotion of apoptosis. Last treatment with adiponectin increased reactive oxygen species production. The results of this study suggest that the anti-neoplastic effects of adiponectin on endometrial cancer cell lines are at least partially-mediated *via* a dual induction of autophagy and apoptosis and increased production of reactive oxygen species.

Introduction

Obesity is a current pandemic and an established risk factor for endometrial cancer [1-3]. The association between obesity and endometrial cancer has been traditionally attributed to elevated estrogen levels in obese women [4,5]. However, there is emerging evidence that obesity is linked with malignancy development *via* dysregulation of adipose tissue-secreted hormones, known as adipokines [6,7]. Adiponectin is one of the most abundant circulating adipokines and has anti-inflammatory and anti-neoplastic properties [8,9]. Adiponectin levels are reduced in patients with obesity, with lower levels in patients with higher body-mass index or central obesity [10]. Low adiponectin levels have been associated with increased risk of endometrial cancer primarily in post-menopausal obese women [11,12]. Adiponectin exerts its antineoplastic effects *via* two main mechanisms; (i) acting directly on cancer cells through receptor-mediated pathways and (ii) indirectly influencing cancer biology by modulating insulin sensitivity, inflammation and tumor angiogenesis [13]. At the molecular level, both healthy and neoplastic endometrial cells express adiponectin receptors (AdipoR1 and AdipoR2) on their surface [14-16]. Administration of adiponectin to endometrial cancer cell lines results in reduced proliferation, survival, adhesion, tissue invasion, and promotes apoptosis [14-17]. However, the molecular mechanisms linking low adiponectin levels and endometrial cancer are not completely understood.

Autophagy is a cellular homeostatic mechanism necessary for cellular survival *via* recycling of cellular organelles and macro-molecules. The exact role of autophagy in oncogenesis remains unknown, while a dual pro-neoplastic and anti-neoplastic role has been proposed [18]. In endometrial cancer, autophagy promotes the initiation of endometrial carcinoma and the survival of tumor cells under stress, but excessive autophagy leads to apoptosis and cell death [19,20]. Adiponectin induces autophagy in several healthy and neoplastic cell types [21,22]. However, the outcome of adiponectin-activated autophagy remains controversial. Adiponectin-induced autophagy can suppress tumor growth but can also support cell survival in glucose-deprived media conditions [23]. The role of adiponectin in regulation of the autophagy pathway in endometrial cancer cells is not known to date.

The primary aim of our study is to evaluate the role of adiponectin in autophagy and apoptosis in endometrial cancer cells. Since oxidative stress is implicated in both autophagy and apoptosis [24,25], the secondary aim of our study is to evaluate the role of adiponectin in inducing Reactive Oxygen Species (ROS) as a marker of oxidative stress. The significance of this study is that establishing the

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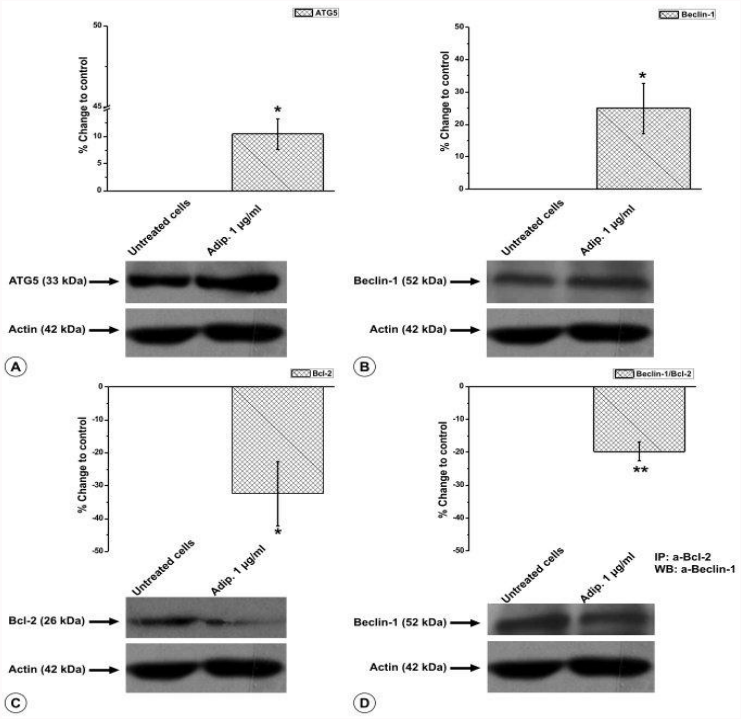


Figure 1: The effect of adiponectin on proteins implicated in autophagy, in KLE endometrial cancer cells. After adiponectin treatment, A) ATG5 protein levels were increased (10.4 ± 2.8%), B) Beclin-1 protein levels were increased (25 ± 7.8%), C) Bcl-2 protein levels were decreased (32.5 ± 9,7%), and D) The amount of Beclin-1 protein bound to Bcl-2 was decreased (19.8 ± 2.7%). The Western Blot images are representative of three independent experiments. The asterisks denote statistically significant differences between untreated cells and cells treated with adiponectin 1 μg/ml for 24 h (*p<0.05 and **p<0.001).

regulatory effects of adiponectin on autophagy in endometrial cancer cells can help elucidate the molecular mechanisms that link obesity with endometrial cancer.

Materials and Methods

Cell culture and reagents

In this study we used the KLE endometrial cancer cell line (American Type Culture Collection, LGC Standards, Wesel, Germany). We cultured KLE cells as recommended by the manufacturer in DMEM: F12 supplemented with 100 μg/mL penicillin G/streptomycin, 50 μg/mL gentamycin and 10% fetal bovine serum (Biochrom, Berlin, Germany). We cultured KLE cells at 37ºC, 5% CO_2, and 100% humidity. To study the effect of adiponectin on KLE cells we treated the cultured cell lines with adiponectin (10772-H02H, Sigma-Aldrich, Inc., USA) at a concentration of 1 μg/ml for 24 h. We performed each experiment at least in triplicate to assure reproducibility of our results.

Immunoblotting

We plated the KLE cells on Petri dishes. After reaching 80% confluency, we collected cells with scrapper and we lysed them using lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 10% glycerol, 1 mM phenylmethyl-sulphonyl-fluoride, 2 mM Na-orthovanadate and 10 mM leupeptin). We determined protein concentration with the Bradford assay. We analyzed lysates by immunoblotting as previously described [26]. We used a goat polyclonal anti-Beclin-1 (dilution 1:500, Santa Cruz, CA, USA), a rabbit monoclonal anti-Bcl-2 (dilution 1:1000, Cell Signaling, Technology, Leiden, The Netherlands), a rabbit monoclonal anti-ATG5 (dilution 1:500, Cell Signaling, Technology, Leiden, The Netherlands) and a mouse monoclonal anti-actin antibody (dilution 1:5000, Chemicon, Millipore, Temecula, CA, USA). We detected immunoreactive proteins by chemiluminescence using horseradish peroxidase substrate SuperSignal (Pierce, Rockford, IL, USA), according to the manufacturer's instructions.

Autophagy

We plated cells in a 24-well plate at a concentration of 40,000 cells per well in 500 μl of medium and we incubated the cells overnight at 37°C with 5% CO_2. We treated the cells with adiponectin at a concentration of 1 μg/ml for 24 h as described above. After treatment, we removed the medium from the cells and washed the cells once with PBS. We then treated the cells according to the manufacturer's instruction using the Muse™ Autophagy LC3-antibody based assay (Catalog No. MCH200109, Muse, Millipore). Briefly, we replaced the culture media with 200 μl of PBS with 0.2 μl Autophagy Reagent A (1:1000 dilution) and incubated the cells at 37°C for ~6 h to induce autophagy under starvation conditions. We cultured untreated cells with culture media supplemented with FBS in the 96-well plate. After 6 h of treatment, we aspirated wells to remove culture supernatants and washed once with PBS. We detached cells using 100 μl trypsin for 5 min at 37°C. We then transferred cells to the Muse sample tubes and we added 100 μl of PBS to each well. We spun the sample tubes at 300x g for 5 min at 4ºC and we removed the supernatant. We then added 5 μl of Anti-LC3 Alexa Fluor® 555 and 95 μl of Autophagy Reagent B to each sample. We preserved the samples for 30 min on ice in dark conditions. We spun the sample tubes at 300x g for 5 min at 4ºC and removed the supernatants. We then washed the cells once with Assay Buffer. We resuspended cells in each sample tube in 200 μl Assay Buffer and immediately analyzed them with the Muse Cell Analyzer.

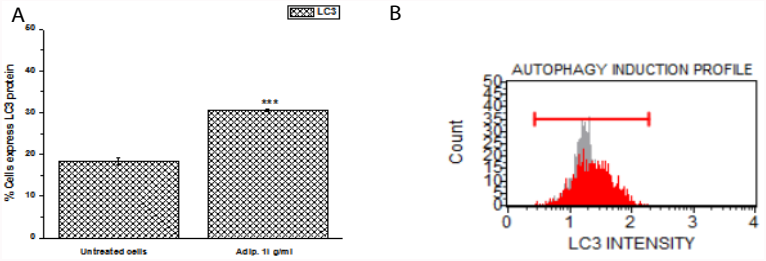


Figure 2: The effect of adiponectin on LC3 protein implicated in autophagy, in KLE endometrial cancer cells. A) Adiponectin increased the protein expression of LC3. B) Muse analyzer was used and a representative plot from three independent experiments is presented. Gray and red areas signify the untreated and adiponectin-treated KLE cells respectively. The asterisks denote statistically significant differences between untreated cells and cells treated with adiponectin 1 μg/ml for 24 h (***p<0.0001).

Oxidative stress

We plated cells in 10 mm petri dish until 80% to 90% confluency was achieved using medium supplemented with 10% FBS and incubated overnight in a 37°C with 5% CO_2. We treated cells with adiponectin at a concentration of 1 μg/ml for 24 h. After treatment, we removed the medium from the cells and we diluted the cells in Assay Buffer according to the manufacturer's instructions using the Muse Oxidative stress kit (Catalog No MCH100111, Muse, Millipore). Briefly, we added 10 μl of cells in suspension into each tube containing 190 μl of Muse Oxidative Stress Reagent. We thoroughly mixed the cells in the tubes by pipetting for 3 sec to 5 sec and we incubated the samples for 3 min at 37°C. The samples were then analyzed with the Muse Cell Analyzer.

Statistical analysis

We express all results as mean ± Standard Error of the Mean (SEM) from at least three independent experiments. We evaluated for differences between adiponectin-treated cells and controls with the Student's t-test. P-values are two-sided, and the statistical significance was defined at an alpha criterion of 0.05.

Results

Adiponectin promotes autophagy *via* increase of Beclin-1 and ATG5 protein levels in endometrial cancer cell lines

Adiponectin increased ATG5 protein levels by 10.4 ± 2.8% (p<0.05, Figure 1A, 1B) and Beclin-1 protein levels by 25 ± 7.8% (p<0.05,). Increased protein levels of ATG5 and Beclin-1 indicate activation of the autophagy process. Furthermore, adiponectin decreased the amount of Beclin-1 protein bound to Bcl-2 by 19.8 ± 2.7% (p<0.001, Figure 1D). Activation of autophagy requires release of Beclin-1 from Bcl-2, and the decrease of Beclin-1 conjugated with Bcl-2 is consistent with autophagy activation.

Adiponectin promotes autophagy *via* increase of key protein LC3 levels

Adiponectin increased the intensity of LC3 (Figure 2A), although fewer cells (red area, Figure 2B) expressed this protein compared to untreated cells (gray area, Figure 2B). LC3 is a protein responsible for the elongation of the double membrane that forms the autophagosome in the autophagy process.

Adiponectin promotes apoptosis *via* decrease and nuclear localization of Bcl-2 and production of ROS

To assess the relation between autophagy and apoptosis *vs.* survival, we evaluated the effect of adiponectin on the levels of the anti-apoptotic cell protein Bcl-2. Adiponectin decreased the protein levels of Bcl-2 by 32.5 ± 9.7% (p<0.05, Figure 1C). Furthermore, adiponectin treatment resulted in increased localization of Bcl-2 in the endometrial cancer cell nucleus (Figure 3). Treatment of KLE cells with adiponectin resulted in a 9-fold increase in ROS production (p<0.001, Figure 4).

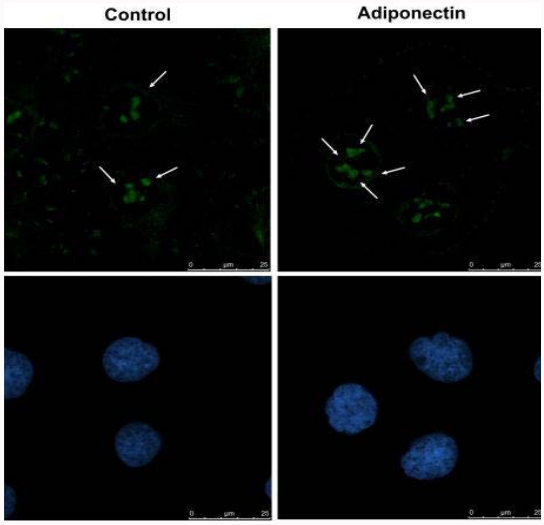
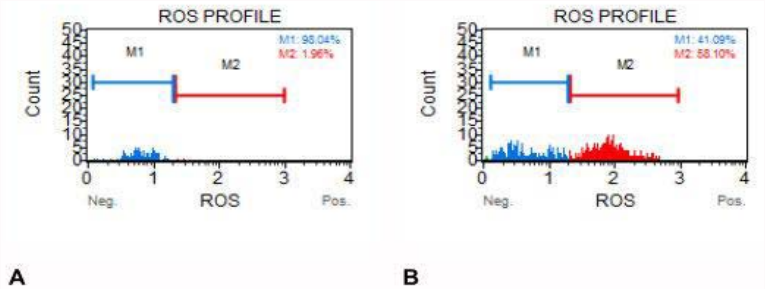


Figure 3: Localization of Bcl-2 inside the nucleus of KLE cells. Bcl-2 protein and the nucleus are illustrated by green and blue fluorescence respectively. The images from the immunofluorescence assay are representative of three independent experiments using a confocal microscope (Leica, Germany) at 63x magnification.

Discussion

The goal of our study was to evaluate the role of adiponectin in autophagy and apoptosis in endometrial cancer cells. We demonstrated that administration of adiponectin to endometrial cancer cells (a) causes activation of the autophagy mechanism by increasing ATG5, Beclin-1 and LC3 protein levels, and, (b) Promotes apoptosis by decreasing Bcl-2 protein levels, while increasing nuclear localization of Bcl-2 and production of ROS.

In the present study, we examined the effect of adiponectin on autophagy and apoptosis in an endometrial cancer cell line. Autophagy is a homeostatic mechanism that occurs at a constitutive level in every cell to maintain cell survival by recycling organelles and macromolecules. It is required for physiologic cellular responses to stress and is implicated in oncogenesis [27]. Limited data support that autophagy modulates both cytoprotection and cell death, and its role in determining cell death or survival depends on the specific



	Control	Adiponectin 1 μg/ml
% cells ROS (+)	5.9 ± 1.5	53.9 ± 1.7 (p<0.001)

Figure 4: The effect of adiponectin on ROS production, in KLE endometrial cancer cells. (A) Untreated cells *vs.* (B) Adiponectin-treated cells. Blue (M1) represents ROS (-) cells, red (M2) represents ROS (+) cells.

cellular context [28]. Recent studies have demonstrated that the cytoprotective effects of adiponectin are mediated, at least in part, *via* the induction of autophagy [23]. Our data show that treatment with adiponectin induces autophagy by increasing ATG5, Beclin-1 and LC3 while at the same promotes apoptosis by reducing the levels of the anti-apoptotic protein Bcl-2. These findings are in agreement with studies of human breast cancer and human hepatoma cells, which demonstrate that adiponectin augments the expression of LC3B and ATG5 by activating FoxO3A transcription factor. These studies however fail to demonstrate a significant effect of adiponectin on Beclin-1 expression [29,30].

Studies evaluating the mechanisms *via* which adiponectin increases Beclin-1 levels suggest that it does not directly affect Beclin-1 expression, but rather modulates the biological activity of Beclin-1 required for autophagy induction. More specifically, in order for autophagy to occur, Beclin-1 needs to bind with class III PI3K (VPS34). If during this process Beclin-1 binds to Bcl-2 instead, the binding of Beclin-1 to class III PI3K is inhibited [31]. Therefore, the formation of the Beclin-1 to Bcl-2 complex is a critical negative regulator of autophagy induction. Our data supports that adiponectin reduced the protein levels of Beclin-1 bound to Bcl-2. We can thus postulate that the increase we observed in Beclin-1 protein levels is not because of new protein synthesis but due to Beclin-1 protein being released from Bcl-2 binding. Recent data support that adiponectin inhibits the association of Beclin-1 with Bcl-2 *via* multiple mechanisms. It suppresses Bcl-2 expression by inducing expression of mRNA binding proteins that target Bcl-2, such as TTP, ZFP36L1, and AUF1, which in turn induces destabilization of Bcl-2 mRNA in macrophages [23,32]. The decrease in Bcl-2 expression and subsequent increase in the free form of Beclin-1 leads to initiation of the autophagic process. Furthermore, phosphorylation of Beclin-1 at Thr119 causes dissociation from Bcl-2, which in turn leads to complex formation of Beclin-1 with class III PI3K and further autophagy induction. Adiponectin also induces Beclin-1 phosphorylation in a death-associated protein kinase-1 (DAPK-1)-dependent manner [23,32].

Recent data from various cancer cell lines suggests that there is a significant interplay between autophagy and apoptosis, namely that apoptosis inhibits the cytoprotective effects of autophagy in human cancer [33]. Adiponectin-induced autophagy has been shown to negatively regulate apoptosis of cancer cells, indicating that inhibition of autophagy could enhance the efficiency of apoptosis in cancer cells by adiponectin [29]. The controversial effect of adiponectin in apoptosis prompted us to study the localization of Bcl-2 and we demonstrated that Bcl-2 protein was increased in the nucleus of the endometrial cancer cells, which supports the activation of apoptosis. Furthermore, we studied the effect of adiponectin on ROS levels, an oxidative stress marker that directly correlates to autophagy. Existing data suggests that ROS production can mediate autophagy initiation, or it can be the result of protein degradation through the autophagy process. Conversely, autophagy can act as a tumor suppressor mechanism by eliminating damaged mitochondria, which are known to be the major source of ROS, thus preventing ROS accumulation [34]. We found that adiponectin induces ROS production although this was only observed in a cell subpopulation (KLE). In our cell lines, it appears that ROS production correlates with autophagy in a manner that leads endometrial cells to death and this effect is enhanced by the induction of apoptosis.

To summarize, the results of this study suggest that the anti-neoplastic effects of adiponectin on endometrial cancer cell lines are -at least partially-mediated *via* a dual induction of autophagy and apoptosis as well as increased production of reactive oxygen species. Our understanding of the differential roles of autophagy in endometrial cancer cells is limited. Future research is required to understand the exact mechanisms through which adiponectin regulates autophagy and endometrial cancer cell survival, before translation of these results in clinical practice is feasible.

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