Regulatory Role of Autophagy in Globular Adiponectin-Induced Apoptosis in Cancer Cells

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Abstract
Adiponectin, an adipokine predominantly secreted from adipose tissue, exhibits diverse biological responses, including metabolism of glucose and lipid, and apoptosis in cancer cells. Recently, adiponectin has been shown to modulate autophagy as well. While emerging evidence has demonstrated that autophagy plays a role in the modulation of proliferation and apoptosis of cancer cells, the role of autophagy in apoptosis of cancer cell caused by adiponectin has not been explored. In the present study, we demonstrated that globular adiponectin (gAcrp) induces both apoptosis and autophagy in human hepatoma cell line (HepG2 cells) and breast cancer cells (MCF-7), as evidenced by increase in caspase-3 activity, Bax, microtubule-associated protein light chain 3-II (LC3 II) protein levels, and autophagosome formation. Interestingly, gene silencing of LC3B, an autophagy marker, significantly enhanced gAcrp-induced apoptosis in both HepG2 and MCF-7 cell lines, whereas induction of autophagy by rapamycin, an mTOR inhibitor, significantly prevented gAcrp-induced apoptosis in hepatoma cells HepG2. Furthermore, modulation of autophagy produced similar effects on gAcrp-induced Bax expression in HepG2 cells. These results implicate that induction of autophagy plays a regulatory role in adiponectin-induced apoptosis of cancer cells, and thus inhibition of autophagy would be a novel promising target to enhance the efficiency of cancer cell apoptosis by adiponectin.

Key Words: Adiponectin, Apoptosis, Autophagy, Bax, HepG2, MCF-7

INTRODUCTION
Autophagy, a self-degradation process in eukaryotic cells, involves multiple steps, including initiation, vesicle formation, autophagosome-lysosome fusion and final degradation of dysfunctional proteins and/or organelles (Kroemer and Levine, 2008). Originally, autophagy was presumed simply as another cell death mechanism different from apoptosis. However, recent evidences highlight that autophagy plays diverse roles in regulation of many pathological conditions such as cancer, liver disease and neuronal disorders, and its modulation by various drugs/chemicals could impact the disease treatment outcome. In particular, even if autophagy was first used to describe the process of cell death, autophagy has also been shown to play a pro-survival function in many stressful conditions mainly via negative regulation of apoptosis (Gordy and He, 2012).

Adiponectin, the most abundant adipokine in the plasma, has been postulated to be involved in the regulation of diverse pathophysiological responses. For example, adiponectin possesses potent anti-tumor properties through various mechanisms. One of the most plausible mechanisms underlying is that adiponectin induces apoptosis of cancer cells in many in vitro and in vivo models (Kelesidis et al., 2006; Saxena et al., 2010). Recent studies have also implicated that adiponectin induces autophagy in various stressful conditions. Adiponectin activates autophagy particularly, by increasing the expression of autophagy related proteins, thereby inhibiting ethanol-induced cytotoxicity in liver cells (Nepal and Park, 2013; Nepal et al., 2014) and also support cell-survival in glucose deprived colorectal cancer cells (Habeeb et al., 2011).

Based on a variety of previous reports, it is clear that autophagy induction is an important target for modulation of apoptosis in cancer cells. Further, autophagy is also implicated in various adiponectin-induced biological responses. However, the molecular interplays between induction of autophagy and cancer cell death caused by adiponectin have not been reported. Thus, in an effort to understand the relationship between autophagy and apoptosis in cancer cells treated with adiponectin, we investigated the role of autophagy activation...
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in the regulation of apoptosis induced by globular adiponectin in cancer cell lines. Herein, we found that adiponectin induces both autophagy and apoptosis in HepG2 and MCF-7 cell line and further provide the first evidence that autophagy plays a critical regulatory role in apoptosis by globular adiponectin, demonstrating that autophagy process would be a novel mechanism modulating adiponectin-induced apoptosis of cancer cell.

MATERIALS AND METHODS

Materials
All the cell culture reagents were purchased from Hyclone laboratories (South Logan, UT, USA). Recombinant human globular adiponectin (gAcrp) was procured from Peprotech Inc. (Rocky Hill, NJ, USA). Caspase-3 activity assay kit and cell proliferation assay kit (MTS) were purchased from Promega Corporation (Madison, WI, USA). Antibodies against Bax, LC 3II and β-actin were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Cell culture
Human hepatoma cell line (HepG2) and breast cancer cell line (MCF-7) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and routinely cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin along with 0.1% amphotericin at 37°C.

Cell viability measurement (MTS assay)
For the determination of cell viability, MTS assay was performed as described previously. Briefly, cells were treated with gAcrp as indicated in figure legends. Cells were then incubated with 20 µl of MTS solution for 2 h at 37°C. Cell viability was then assessed via a microplate reader (Molecular Devices, CA, USA) by measuring absorbance at 490 nm.

Caspase-3 activity assay
Caspase-3 activity was assessed by using Caspase-Glo 3/7 assay kits (Promega Corporation, Madison, USA) according to the manufacturer’s instructions. Briefly, cells were treated with gAcrp for the indicated time points as mentioned in figure legends. Caspase-3 activity was determined by the measurement of luminescence from the cleavage of lumino-

Fig. 1. Globular adiponectin induces apoptosis in HepG2 cells. Cells were treated with gAcrp (1 µg/ml) for the indicated time points. Cell viability (A) and caspase-3 activity (B) were assessed by MTS assay and Caspase-3 activity assay, respectively. Values represent percentage of cell death or fold increase compared to control and expressed as mean ± SEM (n=3). *p<0.05 compared with control cells.

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Transgenic substrate Ac-DEVD-pNA with a micro-plate reader (Fluorostar Optima, BMG Labtech, Ortenberg, Germany).

Transient transfection with small interfering RNA (siRNA)
HepG2 and MCF-7 cells were transfected with LC3B siRNA or scrambled control siRNA with Hiperfect transfection reagent (Qiagen) according to the manufacturer’s instructions.

Preparation of cellular extracts and Western blot analysis
Bax and LC 3II protein expression were measured by Western blot analysis. Briefly, total cellular extracts were prepared by lysis of cells in RIPA buffer containing halprotease inhibitor cocktail, separated in 15% SDS-PAGE and transferred to PVDF membranes. The membrane was incubated with the designated primary antibodies, and incubated with secondary HRP-labeled antibodies and visualized using chemiluminescent substrate using a Fujifilm LAS-4000 mini (Fujifilm, Tokyo, Japan).

Confocal microscopic analysis
For confocal microscopic analysis, HepG2 and MCF-7 cells were transfected with enhanced green fluorescent protein (eGFP)-LC3 expression plasmid using Fugene HD transfection reagent (Promega) according to the manufacturer’s instructions, and treated with gAcrp for the indicated time points. The confocal images were captured using an A1 Confocal Laser Microscope System (Nikon Corp., Tokyo, Japan) followed by quantification of autophagic puncta (LC3 dots) with ImageJ software version 2.32 (Ehwa Optical Co., Seoul, Korea).

Statistical analysis
Values are expressed as mean ± SEM of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests using GraphPad prism software version 5.01 (CA, USA). Differences between groups were considered to be significant at p<0.05.

RESULTS

Globular adiponectin induces apoptosis in HepG2 cells
To investigate the role of autophagy in adiponectin-induced apoptosis of cancer cells, we first examined if adiponectin in-
duces apoptosis in cancer cells. For this, we examined the effect of adiponectin on cell viability and caspase-3 activity, a marker of cellular apoptosis, in human hepatoma cell line (HepG2 cells). As shown in Fig. 1A, globular adiponectin (gAcrp) treatment significantly decreased cell viability in a time dependent manner, but increased caspase-3 activity (Fig. 1B) with a pattern similar to the regulation of cell viability, confirming that adiponectin causes cell death in HepG2 cells via enhancing apoptosis in our experimental conditions.

Globular adiponectin induces autophagy in HepG2 and MCF-7 cells

Next, we determined the effect of adiponectin on autophagy in human hepatoma (HepG2) and breast cancer line (MCF-7 cells). As shown in Fig. 2A, globular adiponectin (gAcrp) treatment significantly decreased cell viability in a time dependent manner, but increased caspase-3 activity (Fig. 1B) with a pattern similar to the regulation of cell viability, confirming that adiponectin causes cell death in HepG2 cells via enhancing apoptosis in our experimental conditions.

Induction of autophagy negatively modulates globular adiponectin-induced apoptosis both in HepG2 and MCF-7 cells

Growing evidences have highlighted a cross-talk between autophagy and apoptosis (Gordy and He, 2012). We found that gAcrp induced both apoptosis and autophagy in HepG2 cells and autophagy in MCF-7 cells (Fig. 1 and 2), and speculated that globular adiponectin-induced autophagy could affect apoptosis of cancer cells. Thus, we next investigated the effect of autophagy on apoptosis induced by gAcrp in HepG2 cells. As shown in Fig. 3A and B, gAcrp-induced cell death and caspase-3 activation was further enhanced by transfection of cells with siRNA targeting LC3B, a marker of autophagy, whereas autophagy activation by pretreatment with rapamycin, an inhibitor of mTOR, significantly prevented adiponectin-induced apoptosis (Fig. 3C), indicating a possibility of negative modulation of gAcrp-induced apoptosis by autophagy. This regulatory role of autophagy induction in gAcrp-induced apoptosis was also confirmed in MCF-7 cells. As shown in Fig. 4A, inhibition of autophagy via transient transfection of
siRNA targeting LC3B gene resulted in enhancement of cell death. Since MCF-7 cells are deficient in caspase-3 and caspase-7 mediates apoptotic responses (Liang et al., 2001), we measured caspase-7 activation in MCF-7 cells after transfection of LC3B gene. As shown in Fig. 4B, autophagy inhibition via LC3B gene knock-down, significantly enhanced gAcrp-induced caspase-7 activation in MCF-7 cells, suggesting that gAcrp induced-autophagy possibly negatively modulates cancer cell death in MCF-7 cells.

**Globular adiponectin induced autophagy regulates Bax expression in HepG2 cells**

To identify the underlying mechanisms, we next examined modulatory role of autophagy induction in expression of Bax, as an apoptosis marker, in HepG2 cells. As shown in Fig. 5A, gAcrp increased Bax protein expression in a time dependent
manner. In addition, interestingly, gAcrp-induced Bax protein expression was prevented by autophagy activation (pretreatment with rapamycin, Fig. 5B), while it was further increased on autophagy inhibition (transfection with siRNA targeting LC3B, Fig. 5C). All these data imply that autophagy induction could affect Bax protein levels and thereby, impair gAcrp-induced apoptosis in HepG2 cells.

**DISCUSSION**

Autophagy is the process for the degradation of dysfunctional or unnecessary cellular components by double-membrane autophagosome fused with lysosomes and, is implicated in various human pathological disorders. The role of autophagy in cancer progression is controversial, showing that autophagy acts as a tumor suppressor, but it can be also used for cytoprotection of cancer cells probably depending on developmental stage of tumor (Mathew et al., 2007).

Adiponectin predominantly secreted from adipose tissue has been shown to induce autophagy in different experimental conditions. Recent studies have also demonstrated that autophagy is implicated in adiponectin-induced various biological responses. For example, autophagic process is induced and utilized for modulation of biological responses by adiponectin (Habeb et al., 2011; Guo et al., 2013). We have also shown that adiponectin restores ethanol-suppressed autophagy in HepG2 cells (Nepal and Park, 2013), whereas the other study has shown that adiponectin suppresses autophagy induced by oxidative stress in cardiomyocytes (Essick et al., 2013), indicating the differential effects of adiponectin on autophagy induction depending on experimental conditions.

It is well recognized that adiponectin treatment induces apoptosis in various types of cancer cells (Kelesidis et al., 2006). Results presented in this study (Fig. 1A, B) are also consistent with previous reports showing that adiponectin inhibits proliferation of cancer cells via inducing apoptosis of HepG2 cells. Based on previous reports, adiponectin is receiving much attention for the treatment of cancer and autophagy plays an important role in the development and progression of cancer. However, the role of autophagy in adiponectin-induced apoptosis in cancer cells has not been explored yet. In the present study, we investigated if autophagy induction could play any role in apoptosis of cancer cells treated with adiponectin. Here, we clearly showed that autophagy induction negatively regulates apoptosis of cancer cells by adiponectin.

Accumulating evidences reveal that important cross-talk exists between autophagy and apoptosis, which is critical for regulation of cell death or survival. For example, caspases cleave autophagy-related genes, including beclin-1, and autophagy also inhibits apoptosis through degradation of apoptotic proteins, including caspases and Bax, suggesting that autophagy and apoptosis mutually negatively regulate and these interactions would ultimately determine the fate of the cells (Gordy and He, 2012). Autophagy has been shown to protect cancer cells from stressful conditions and is associated with proliferation and growth of cancer tissue. For example, autophagy induction has been shown to cause some side-effects which may manifest as increased stress tolerance capacity to cancer cells, thereby maintaining cancer cell viability and enhancing cell proliferation when conditions become favorable (White and DiPaola, 2009). It has been also suggested that autophagy activation may suppress another protein degradation pathway, namely proteasomal degradation pathway. This may be problematic in conditions such as colon cancer where proteasomal degradation is required for effective suppression of colon cancer xenografts (Yang et al., 2011). Furthermore, autophagy inhibition has been shown to increase the apoptotic effects induced by various stimulants. For example, ursolic acid-induced apoptosis in prostatic cancer cells is increased by inhibition of autophagy (Shin et al., 2012) and autophagy inhibition up-regulates apoptotic effects caused by sulforaphane (Herman-Antosiewicz et al., 2006) as well as p53 or alkylating drugs (Amaravadi and Thompson, 2007), strongly indicating that inhibition of autophagy could be a novel therapeutic approach for enhancing anticancer effects of potential drugs/chemicals. In this regard, we speculated that adiponectin-induced autophagy may also impact the apoptosis of cancer cells treated with adiponectin. Our results indicated that adiponectin simultaneously induces autophagy and apoptosis both in hepatoma (HepG2) and breast cancer cells (MCF-7), in which autophagy acts as a negative regulator of apoptosis (Fig. 3, 4), providing clear evidence that autophagy process is tightly linked with apoptosis of the cancer cells.

A series of reports have shown that adiponectin-induced apoptosis is mediated via enhancing expression of Bax, which causes release of cytochrome c and finally activation of caspase-3 (Akifusa et al., 2009; Saxena et al., 2010). Moreover, it is also to be noted that autophagy activation degrades Bax protein in cardiac myocytes (Hamacher-Brady et al., 2006), and human glioma cells (Cheng et al., 2007). Therefore, Bax has been regarded as a target protein involved in interactions between autophagy and apoptosis (Gordy and He, 2012), and we speculated that autophagy induction negatively regulates apoptosis by gAcrp via down-regulation of Bax in HepG2 cells. Intriguingly, our findings (Fig. 5A-C) are in line with this hypothesis, and further characterized the role of autophagy activation by adiponectin on the regulation of Bax protein level thereby inhibiting apoptosis.

In conclusion, the data presented here demonstrate for the first time that induction of autophagy plays a modulatory role in adiponectin-induced apoptosis in cancer cells. Adiponectin has been shown to possess potent anti-tumor activities in various cancer cells and considered as a promising molecule for the treatment of cancer. Based on the data presented in the current study, we suggest that inhibition of autophagy would be an important therapeutic approach for enhancing the efficacy of treatment of cancer by adiponectin. Further studies to validate these findings in an in vivo model and to investigate the molecular nexus involved in autophagy regulation by adiponectin are now required.

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