Adiponectin inhibits colorectal cancer cell growth through the AMPK/mTOR pathway

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Abstract. Adiponectin is a peptide hormone secreted by adipose tissue. It is a key hormone responsible for insulin sensitization, and its circulating level is inversely associated with abdominal obesity. Recent studies have shown that a reduced plasma adiponectin level is significantly correlated with the risk of various cancers. However, there are few studies regarding the association of adiponectin and colorectal cancer. To address this issue, we investigated the effect of adiponectin on colorectal cancer cells. Three colorectal cancer cell lines express both AdipoR1 and AdipoR2 receptors. MTT assay revealed that adiponectin inhibited human colorectal cancer cell growth. Furthermore, Western blot analysis revealed that adiponectin activated adenosine monophosphate-activated protein kinase (AMPK) and suppressed mammalian target of rapamycin (mTOR) pathways. Selective AMPK inhibitor compound C abrogated the inhibitory effect of adiponectin on cell growth. Our results clearly demonstrate the novel findings that adiponectin inhibits colorectal cancer cell growth via activation of AMPK, thereby down-regulating the mTOR pathway.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies. Obesity, especially visceral obesity, has been reported to be associated with CRC (1,2). Adipose tissue is not only a fat storage organ, but it secretes several bioactive

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substances known as adipocytokines (3,4). Adiponectin is secreted from adipocytes and is a key hormone responsible for insulin sensitization (5-12). Its plasma level is dramatically decreased in patients with obesity and type 2 diabetes mellitus (DM) (4,5,13). Since both obesity and type 2 DM have been reported to be associated with an elevated risk of CRC (14), it is speculated that the plasma level of adiponectin may be related to the risk of CRC. However, several contradictory results have been reported from human clinical studies on the relationship between the plasma levels of adiponectin and the risk of CRC (15,16).

It is well known that the adiponectin receptor exists in two isoforms: adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) (17). These receptors mediate cellular functions by activating intracellular signaling pathways (17). The molecular pathways downstream of AdipoRs remain to be fully elucidated, but studies in metabolically-responsive cells have shown that activation of the pleiotropic adenosine monophosphate-activated protein kinase (AMPK) is involved in the signaling cascade downstream of adiponectin receptors (18,19). AMPK plays a key role in the regulation of energy homeostasis and acts as a 'metabolic sensor' to regulate adenosine triphosphate (ATP) concentrations (20). It is also associated with cell growth; phosphorylated AMPK suppresses mammalian target of rapamycin (mTOR) signaling pathway (21,22). mTOR plays a central role in the regulation of cell proliferation, growth, differentiation, migration and survival (23-26), and may be abnormally regulated in tumors (23,27-29). The 70-kDa ribosomal protein S6 kinase (p70S6K) and S6 ribosomal protein (S6P) are part of the signaling cascade downstream of mTOR; they are activated via phosphorylation by mTOR (28,30,31). Non-cleaved adiponectin (full-length adiponectin; f-adiponectin) and proteolytically-cleaved adiponectin containing a C-terminal globular region (globular adiponectin; g-adiponectin) were reported to have different affinities to AdipoR1 and AdipoR2 (17). In this study, we only examined the g-adiponectin because this isoform binds both receptors, while f-adiponectin has low affinity to AdipoR1 (17), and it exerts more potent effect than f-adiponectin (5). However, the expression levels of AdipoR1 and AdipoR2, the affinity of the different forms of

adiponectin to those receptors, and the associated intracellular signaling pathways in the colorectum remain unclear. In this study, we investigated the effect of adiponectin on cell growth and the intracellular signaling pathway involved in CRC cell lines.

Materials and methods

Reagents and antibodies. Human globular adiponectin was purchased from BioVendor Laboratory Medicine Inc. (Brno, Czech Republic). Compound C was purchased from Calbiochem (La Jolla, CA) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-total and -phosphorylated (Thr172) AMPK, anti-total and -phosphorylated (Ser2448) mTOR, anti-total and -phosphorylated (Thr421/Ser424) p70S6 kinase, anti-total and -phosphorylated (Ser240/244) S6 ribosomal protein, and anti-rabbit horseradishperoxidase-conjugated IgG antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-AdipoR1 (C-14), anti-AdipoR2 (N-19), and anti-goat horseradishperoxidase-conjugated IgG antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antibody was from Trevigen, Inc. (Gaithersburg, MD).

Cell lines. The human colon adenocarcinoma cell lines, HT-29, Lovo, and HCT116 were used for this study. Lovo was obtained from Health Science Research Resources Bank (Osaka, Japan), while HT-29 and HCT116 were obtained from American Type Culture Collection (Manassas, VA). HCT116 and HT-29 were cultured in McCoy's 5A, and Lovo was cultured in Ham's F12, supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml) (all from Invitrogen, Carlsbad, CA) at 37°C under a humidified atmosphere of 5% CO₂.

Western blot analysis. Cultured cells treated with the test compound for indicated time periods were rinsed with phosphate-buffer saline (PBS). For obtaining the total cell extracts, cells were harvested in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1% TritonX-100) containing a cocktail of protease inhibitors (Sigma). The lysates were incubated on ice for 30 min and centrifuged at 15,000 rpm. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA). Proteins were separated by SDS/PAGE (7.5-12.5% gels) and transferred onto a Hybond-P PVDF membrane (Amersham Biosciences, Little Chalfont, UK). After the transfer, the membranes were blocked with Blocking One-P (Nacalai Tesque, Kyoto, Japan) and probed with the primary antibodies specified below. Horseradish-peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham Biosciences, Little Chalfont, UK) were used for the detection of specific proteins. Images were captured and analyzed by LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Cell growth assay. Cells were seeded in 96-well, flat-bottom microtiter plates at a density of 5x10³ cells per well and incubated in medium containing 1% FBS. After 24 h, the

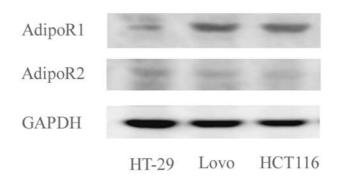


Figure 1. Expression of adiponectin receptors in colorectal cancer cells. Western blot analysis revealed that three kinds of colorectal cancer cell lines HCT116, HT-29 and Lovo cells, expressed both AdipoR1 and AdipoR2 receptors.

complete medium was replaced with test medium for 24 h at 37°C. After incubating the plates for an additional 4 h with MTT solution (0.5%), sodium dodecylsulfate was added to a final concentration of 10% and absorbance at 595 nm was determined for each well using a microplate reader (Model 550; Bio-Rad). Three independent experiments were carried out for each cell line. Annexin V-FITC and PI double staining with the Annexin V-FITC apoptosis detection kit I (Becton-Dickinson, San Jose, CA, USA) followed by FACScan flow cytometry (Becton-Dickinson) was used to identify apoptotic cells. Cell fluorescence was measured with a FACScan flow cytometer from BD Biosciences (San Jose, CA, USA). Dual parameter cytometric data were analyzed by using CellQuest software from BD Biosciences. Apoptosis measures were performed in triplicate.

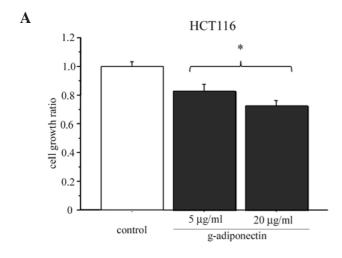
Statistical analysis. All results are expressed as mean \pm SEM. Statistical analyses were performed using Student's t-test after analysis of variance (ANOVA). The results were considered to be statistically significant at p<0.05.

Results

Expression of adiponectin receptors on colorectal cancer cells. Western blot analysis revealed that three kinds of colorectal cancer cell lines, HCT116, HT-29 and Lovo, expressed both AdipoR1 and AdipoR2 receptors (Fig. 1). In HT-29, weak expressions of both of AdipoRs were observed. In Lovo and HCT116, strong expression of AdipoR1, and weak expression of AdipoR2 were observed.

Globular adiponectin (g-adiponectin) inhibited human colorectal cancer cell growth. To determine the effect of g-adiponectin on colorectal cancer cell growth, MTT assay was performed using HCT116, HT-29 and Lovo cells. To reduce the effect of adiponectin in serum, all experiments were conducted using the culture medium containing 1% FBS. G-adiponectin significantly inhibited colorectal cancer cell growth in all examined cells in a dose-dependent manner (Fig. 2).

G-adiponectin up-regulates AMPK activity in colorectal cancer cells. The effect of g-adiponectin on the phosphorylation of



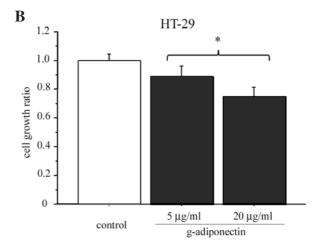
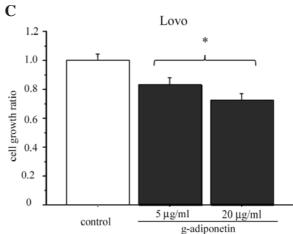


Figure 2. Globular adiponectin inhibited human colorectal cancer cell growth. HCT116 (A), HT-29 (B), and Lovo (C) cells were plated onto 96-well flatbottom plates in culture medium containing 1% FBS at a density of $5x10^3$ cells per well. After 24 h, culture medium containing 1% FBS and g-adiponectin (5 mg ml or 20 mg/ml) was added, and incubated for 24 h. Cell growth in each well was measured by MTT assay. Cell growth ratio (Y-axis) is represented by the ratio of treated to untreated cells via the MTT readings. Columns indicate means of three independent experiments carried out in triplicate (n=8); Values are means \pm SEM. $^{\circ}P<0.05$.



AMPK and mTOR signaling pathway was examined. Western blot analysis revealed that g-adiponectin significantly phosphorylated AMPK and its effect on AMPK phosphorylation was maximal at 6 h after treatment (Fig. 3A). We also observed the significant phosphorylation of mTOR, p70S6K, and S6 proteins by the treatment with g-adiponectin (Fig. 3B-D). These results suggest that g-adiponectin inhibits colorectal cancer cell growth via AMPK activation and mTOR signaling pathway suppression. Selective AMPK inhibitor compound C reversed the g-adiponectin induced cell growth inhibition in HCT116 colorectal cancer cells, as detected by MTT assay (Fig. 3E). This indicates that g-adiponectin-induced cell growth inhibition is mediated by activation of AMPK.

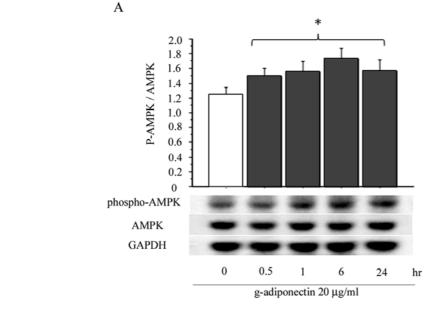
G-adiponectin has no effect on apoptosis. The effect of gadiponectin on apoptosis in HCT116 and HT-29 was evaluated using annexin V-FITC and PI double staining. There was no apoptotic effect in HCT116 and HT-29 treated with 5 μ g/ml g-adiponectin or 20 μ g/ml g-adiponectin in basal medium containing 1% FBS (Fig. 4).

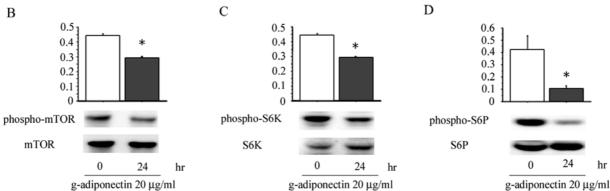
Discussion

The association of low plasma adiponectin level and cancer risk was previously reported (32-35). However, there are few studies regarding the association of adiponectin and colorectal cancer (15,36). In the present study, we demonstrated both of

the AdipoR expression levels and g-adiponectin activation of the AMPK, resulting in the suppression of mTOR signaling pathway in colorectal cancer cell lines. Our recent study demonstrated that AdipoRs were expressed in normal colon epithelial and colorectal cancer cells in human (37). In this study, three kinds of colorectal cancer cell lines HCT116, HT-29 and Lovo cells were shown to express AdipoRs. Furthermore, we clearly demonstrated that globular adiponectin inhibited colorectal cancer cell growth and activated AMPK, while selective AMPK inhibitor compound C reversed the effect of g-adiponectin-induced cell growth inhibition, which indicates that g-adiponectin inhibits cell growth via regulation of AMPK. mTOR is one of the enzymes downstream of AMPK. AMPK activation acts as an inhibitor of mTOR pathway and suppresses tumor development (38,39). In this study, we demonstrated that g-adiponectin suppressed the mTOR pathway following the activation of AMPK. These results suggest that g-adiponectin suppresses cancer cell growth through AMPK activation and subsequent inhibition of mTOR pathway. However, the mechanisms through which adiponectin affects cancer cells are not completely elucidated, thus there is a possibility that suppression of mTOR pathway by adiponectin is mediated through other enzymes (40,41). Further studies are needed to evaluate the molecular pathways downstream of each AdipoR.

In conclusion, this study clearly demonstrates the novel findings that g-adiponectin inhibits colorectal cancer cell





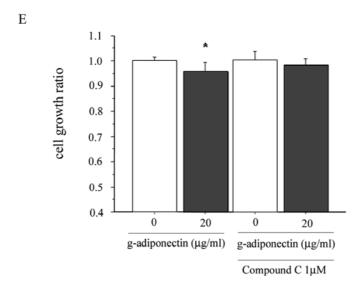


Figure 3. G-adiponectin up-regulates AMPK activity in colorectal cancer cell and selective AMPK inhibitor compound C reversed the g-adiponectin-induced cell growth. Western blot analysis was performed. HCT116 cells were treated with culture medium containing 1% FBS and g-adiponectin (20 μ g/ml) for 24 h. After harvesting, cells were lysed and prepared for immunoblot analysis. (A) G-adiponectin increases AMPK phosphorylation and its effect on AMPK phosphorylation was maximal at 6 h after treatment. (B-D) G-adiponectin reduced phosphorylation of mTOR, p70S6K and S6P in HCT116 cells. Western blot analysis was performed in triplicate. Values are means \pm SEM. *P<0.05. (E) To confirm the effect of adiponectin-induced growth inhibition via AMPK, HCT116 incubated in culture medium containing 1% FBS and adiponectin (20 μ g/ml) alone or with the addition of compound C (1 μ M) for 24 h were used for MTT assay. Columns indicate means of four independent experiments carried out in triplicate (n=8); Values are means \pm SEM. *P<0.05.

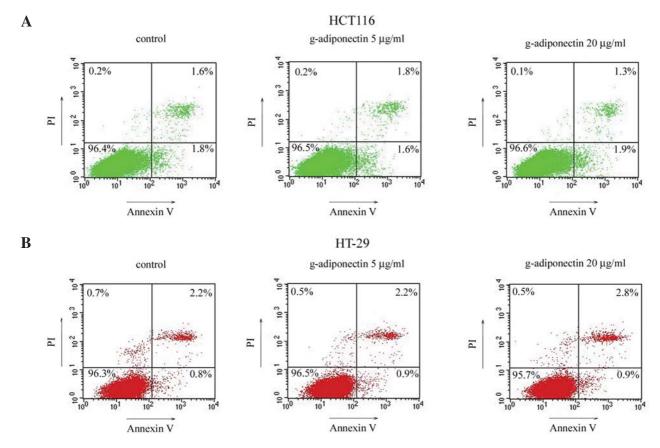


Figure 4. G-adiponectin has no effect on apoptosis. The apoptotic effect of g-adiponectin in HCT116 (A) and HT-29 (B) colorectal cancer cells incubated with culture medium containing 1% FBS and g-adiponectin (5 μ g/ml) or 20 μ g/ml) were evaluated via annexin V-FITC and PI double staining. Apoptosis measures were performed in triplicate.

growth through the activation of AMPK and subsequent suppression of mTOR pathway. This may be a key step in the elucidation of the effect of adiponectin on colorectal cancer. Further studies are required to elucidate the function of adiponectin in colorectal cancer.

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