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Adiponectin induces growth inhibition and apoptosis in cervical cancer HeLa cells

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Abstract: Obesity is a known risk factor for postmenopausal cervical cancer. In human, plasma adiponectin (ADPN) levels are inversely associated with obesity. ADPN may influence the pathogenesis of cervical cancer. In this paper, the effects of ADPN on the proliferation of the cervical cancer HeLa cells and its underlying mechanism were studied. We discovered that two ADPN receptors were expressed in HeLa cells and ADPN inhibited the proliferation of HeLa cells. Western-blotting showed that ADPN activated AMP kinase in HeLa cells. Reverse transcriptase-polymerase chain reaction revealed ADPN down-regulated the expression of cell cycle regulators cyclin D1 and c-myc, and induced the expression of $p21^{WAF1/CIP1}$ and p53. These results indicate that ADPN inhibits proliferation and induces apoptosis of HeLa cells by altering the expression of cell cycle regulators.

Key words: adiponectin; adiponectin receptors; HeLa cell; proliferation; apoptosis.

Abbreviations: AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; ADPN, adiponectin; AI, apoptotic index; AMPK, AMP-activated protein kinase; f-Ad, full-length adiponectin; FITC, fluorescein isothiocyanate; g-Ad, globular adiponectin; GAPDH, glyceraldehyde-phosphate dehydrogenase; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; PI, proliferation index; RT-PCR, reverse transcriptase-polymerase chain reaction.

Introduction

Adiponectin (ADPN), a hormone secreted by adipose tissue, circulates at high concentrations in blood plasma (5–30 μg/mL) (Arita et al. 1999). ADPN can exist as full-length ADPN (f-Ad) and as a proteolytic form in the plasma. The proteolytic product of f-Ad contains the globular domain, which is called globular ADPN (g-Ad). f-Ad is a 30–kDa polypeptide and is comprised of four parts: an amino-terminal signal sequence, a variable domain, a collagen-like domain, and a carboxyl-terminal globular domain (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996). There are two ADPN receptors, AdipoR1 and AdipoR2. AdipoR1 is abundantly expressed in skeletal muscle whilst AdipoR2 occurs mainly in the liver (Yamauchi et al. 2003). AdipoR1 is a high-affinity receptor for g-Ad with very low affinity for f-Ad, whereas AdipoR2 has intermediate affinity for both forms of ADPN (Yamauchi et al. 2003). Different biological functions have been reported for ADPN following binding with its receptors. Several studies have suggested that low plasma levels of ADPN correlate with the pathogenesis of obesity-related diseases: type 2 diabetes, hypertension and atherosclerosis (Kiris et al. 2006; Ekmekçi et al. 2009; Li et al. 2009).

Recently, a number of studies have also suggested that ADPN may influence the pathogenesis of cancer. Circulating ADPN levels have been reported to be inversely associated with an increased risk of breast cancer (Mantzoros et al. 2004; Chen et al. 2006), endometrial cancer (Takemura et al. 2005; Cust et al. 2007), prostate cancer (Sher et al. 2008; Li et al. 2010), colorectal cancer (Ferroni et al. 2007; Otake et al. 2010), and gastric cancer (Ishikawa et al. 2005). Thus, low plasma ADPN levels may provide an important index for the pathogenesis of cancers. Furthermore, ADPN has also been shown to inhibit cell proliferation and induce apoptosis of leukemia and endothelial cells (Yokota et al. 2010; Brĺkenhielm et al. 2004). Studies have demonstrated that prostate cancer cells such as DU145, PC-3 and LNCaP-FGC express ADPN receptors (Miyazaki et al. 2005) and ADPN can also inhibit prostate cancer cell growth (Miyazaki et al. 2005). In addition, some research groups have reported that breast cancer cells also

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express AdipoR1 and AdipoR2 and ADPN is able to mediate the proliferation of breast cancer cells directly through its receptors (Kang et al. 2005; Dieudonne et al. 2006; Arditi et al. 2007; Körner et al. 2007; Nakayama et al. 2008; Pfeiler et al. 2010).

Cervical cancer is the second most common malignant tumor amongst women in China just next to breast cancer. Obesity is another serious health problem worldwide with extensive evidence demonstrating that it is not only associated with a variety of metabolic disorders but also with an increased risk of developing cancer. For example, various studies have shown that obesity is a risk factor for postmenopausal cervical cancer (Maruthur et al. 2009). Obese women have higher mortality rates for cervical cancer than non-obese women. As noted above, plasma ADPN levels are inversely associated with obesity and this hormone may influence the pathogenesis of cervical cancers. However, there have, as yet, been no studies about the effects of ADPN on cervical cancer. Therefore, we studied whether ADPN influences the proliferation of cervical cancer HeLa cells and the underlying mechanism thereof.

Material and methods

Reagents and cells

Recombinant full-length human ADPN was produced and purified as described previously (do Carmo Avides et al. 2008). The purity of ADPN reached 98.5%, and purified ADPN samples were stored frozen in aliquots of 1.5 mg/mL at −20 ℃. Cervical cancer HeLa cells were preserved in Cancer Research Center (CRC), Medical College of Xiamen University, China. Reverse transcriptase-polymerase chain reaction (RT-PCR) reagent kits were provided by Shenggong Ltd., Shanghai, China. Rabbit polyclonal anti-phospho-AMP-activated protein kinase (AMPK) α antibody and anti-AMPK antibody were obtained from Santa Cruz Biotechnology, Inc. HRP-conjugated sheep anti-rabbit IgG were purchased from Promega. Primers were synthesized by Shenggong Ltd., Shanghai, China.

Fluorescein isothiocyanate (FITC) fluorescence labeling analysis

HeLa cells were harvested and rinsed in ice-cold phosphate-buffered saline (PBS) (pH 7.4) twice before being fixed with methanol at 4 ℃ for 30 min. After fixing, cells were rinsed with ice-cold FACS buffer (PBS with 20 mL/L calf-serum and 1 g/L NaN_3) twice and blocked with PBS containing 20 mL/L calf-serum at 4 ℃ overnight. Thereafter, cells were stained with FITC-labeled ADPN and incubated in the dark at 37 ℃ for 1 h. After rinsing with FACS buffer, cells were counterstained with Hoechst 33342 for 10 min, rinsed three-times with FACS buffer, spread the suspension of cell to slides and observed under the fluoromicroscope.

RT-PCR analysis

Cells, seeded in 6-well plates (25,000 cells/well), were treated with recombinant human ADPN (0, 1.0, 10 μg/mL) and harvested after 48 h. Total RNA was extracted using TRIzol® Reagent and stored at −20 ℃. RNA (1 μg) was reverse-transcribed using random primers in a 20 μL-reaction system according to the manufacturer's instructions (RT-PCR reagent kits, Shenggong Ltd., China). cDNA (5 μL) from the above reaction was further PCR-amplified by specific AdipoR primers. AdipoR1 forward: 5'-AGG ACA ACG ACT ATC TGC TAC-3'; reverse: 5'-CAT CCC AAA AAC CAC CTT CTC-3'. AdipoR2 forward: 5'-AGA GAA AGT GGT GGG GAA AG-3'; reverse: 5'-GGG CGA GGG AGG AAA ATA AC-3'. The glyceraldehyde-phosphate dehydrogenase (GAPDH) genes served as the loading control.

In order to analyze the effects of ADPN on cell cycle regulators and apoptosis-related genes, HeLa cells were treated with various concentrations of ADPN (0, 1.0, 10 μg/mL) for 48 h and then harvested for RNA extraction. 2 μg RT-synthesized cDNAs were further PCR-amplified using specific primers (Table 1) for cyclin D1, c-myc, $p21^{WAF1/CIP1}$, p53, bax, and bcl-2. The PCR conditions used were 1 cycle at 95 ℃ for 5 min followed by 30 cycles at 95 ℃ for 45 s, 53 ℃ for 45 s, and 72 ℃ for 1 min. PCR products were electrophoresed on 1.5% agarose in Tris-borate buffer, visualized by ethidium bromide staining, and measured by densitometry.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

The effects of ADPN on the proliferation of HeLa cells were examined using the MTT assay. Briefly, HeLa cells (5,000/mL) were treated with various concentrations of ADPN for 48 h and then were incubated in medium containing 5 mg/mL of MTT reagent at 37 ℃ incubator for 4 h, after which the tetrazolium salts were converted into water insoluble formazan crystals. The formazan crystals were dissolved in dimethyl sulfoxide (200 μL/well) and their absorbance at 570 nm was measured by a microplate spectrophotometer.

Flow cytometric analysis

HeLa cells were seeded (25,000 cells/well) in 6-well plates and cultured in the presence of various concentrations of ADPN (0, 1.0, 10 μg/mL) for 48 h. The harvested cells were washed with cold PBS twice, and fixed in 75% ethanol at 4 ℃ refrigerator overnight. Cells were then washed and resuspended in PBS containing RNase (0.01 mg/mL). After centrifugation at 2,500 rpm for 10 min at 4 ℃, cells were stained with propidium iodide (50 μg/mL) at 37 ℃ for 30 min, and then the samples were analyzed by flow cytometry. Proliferation index (PI) was calculated according to the equation: PI = (S + G2/M)/ total cells × 100%. Apoptotic index (AI) = (apoptotic cells/ total cells) × 100%.

DNA fragmentation assay

HeLa cells were treated with ADPN (0, 1.0, 10 μg/mL) for 48 h. According to the manufacturer's protocol, DNA fragments were extracted using a DNA Mini-Prep Kit (V-Gene Biotechnology, Ltd.). Then 10 μL DNA samples were mixed with 4 μL loading dye and analyzed by a 1.5% agarose gel.

Western blotting analysis

After treatment with 1.0 μg/mL ADPN for 0, 5, 10, 15, 30 min, HeLa cells were harvested in the cell lysate buffer and separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The electrophoresed proteins in gel were transferred onto the nitrocellulose membrane and probed with rabbit polyclonal anti-phospho-AMPK-α antibody at 4 ℃ for 2 h. After washing with PBS plus 0.1% Tween-20 three times, the probed blot was bound with a peroxidase-conjugated secondary antibody. After washing with PBS plus 0.1% Tween-20 for a further three times, the membrane was developed using diaminobenzidine as chromogen. Meanwhile, cell lysates

Table 1. Primers used in the present study.

Genes		Primer sequences	PCR product (bp)
cyclin D1	Sense:	5'-TCTAAGATGAAGGAGACCATC-3'	353
	Antisense:	5'-GCGGTAGTAGGACAGGAAGTTG-3'	
c-myc	Sense:	5'-CCAGCTTGTACCTGCAGGATC-3'	664
	Antisense:	5'-ACCTTGGGGGCCTTTTCATTG-3'	
$p21^{WAF1/CIP1}$	Sense:	5'-GGGGGCATCATCAAAAACTTG-3'	347
	Antisense:	5'-ACTGAAGGGAAAGACAAGGGG-3'	
p53	Sense:	5'-ACTAAGCGAGCACTGCCCAA-3'	231
	Antisense:	5'-ATGGCGGGAGGTAGACTGAC-3'	
Bax	Sense:	5'-GCGTCCACCAAGAAGCTGAG-3'	311
	Antisense:	5'-ACCACCCTGGTCTTGGATCC-3'	
bcl-2	Sense:	5'-TGTGGCCTTCTTTGAGTTCG-3'	280
	Antisense:	5'-TCACTTGTGGCCCAGATAGG-3'	
GAPDH	Sense:	5'-ACCCACTCCTCCACCTTTG-3'	178
	Antisense:	5'-CTCTTGTGCTCTTGCTGGG-3'	

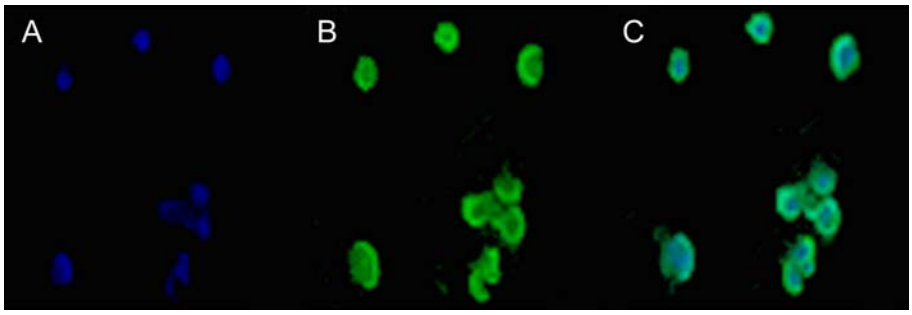

Fig. 1. ADPN binding to membrane receptors in HeLa cells. (A) Hoechst 33342 stained HeLa cells; nuclei of HeLa cells stained with Hoechst 33342 was blue. (B) FITC-ADPN labeled HeLa cells; the membrane of HeLa cells stained with FITC-ADPN was green. (C) A merged picture of (A) and (B). It shows that FITC-ADPN were mainly localized on the membrane of HeLa cells. Fluorescence was visualized by a vertical fluorescence microscopy.

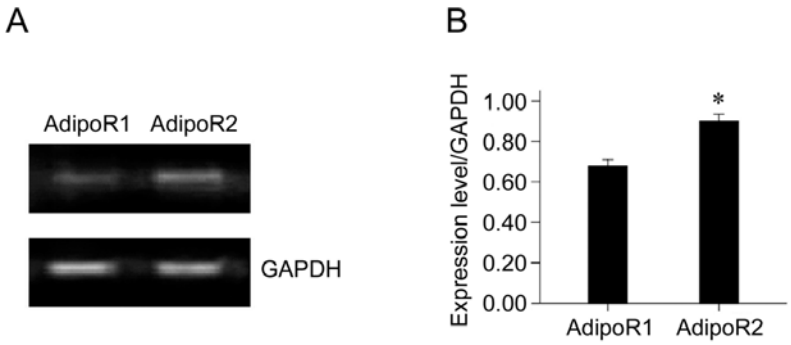

Fig. 2. The mRNA expressions of AdipoR1 and AdipoR2 in HeLa cells detected by RT-PCR. (A) Representative PCR gels stained with ethidium bromide. Lane 1, AdipoR1; lane 2, AdipoR2. (B) Densitometric analysis of RT-PCR products. Values are normalized to GAPDH in the corresponding samples. Asterisk indicates $P < 0.05$ for Student's t-test comparing AdipoR2 to AdipoR1.

were probed with specific anti-AMPK antibodies as indicated, and relative phosphorylation levels of AMPK were normalized to its own total AMPK in Western blotting.

Statistical analysis

Statistical analysis was performed using SPSS10.0 software. Differences between groups were analyzed by Student's t-test. The results shown are from one single representative experiment (of at least three experiments) and the data are reported as mean ± SD. A value of $P < 0.05$ was considered to be statistically significant.

Results

ADPN binding to membrane receptors in HeLa cells

Firstly, we detected the expression of ADPN receptors by FITC fluorescence labeling analysis. Bright fluorescence was observed on the membranes of HeLa cells (Fig. 1). The results showed the nuclei of HeLa cells stained with Hoechst 33342 was blue (Fig. 1A) whilst the membrane of HeLa cells stained with FITC-ADPN was green (Fig. 1B). Figure 1C exhibited a merged picture of Figure 1A and Figure 1B, which reveals that all HeLa cells expressed ADPN receptors.

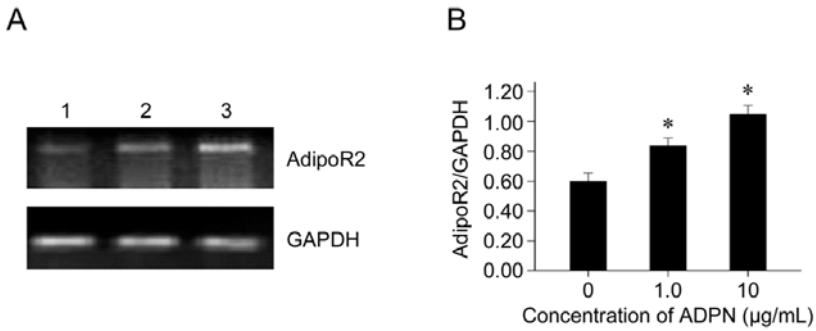

Fig. 3. ADPN stimulates the expression of adipoR2. (A) Representative PCR gels stained with ethidium bromide. Lane 1, PBS; lane 2, 1.0 μg/mL ADPN; lane 3, 10 μg/mL ADPN. (B) Densitometric analysis of RT-PCR products. Values are normalized to GAPDH in the corresponding samples. Asterisks indicate $P < 0.05$ for Student's t-test, compared to the control group.

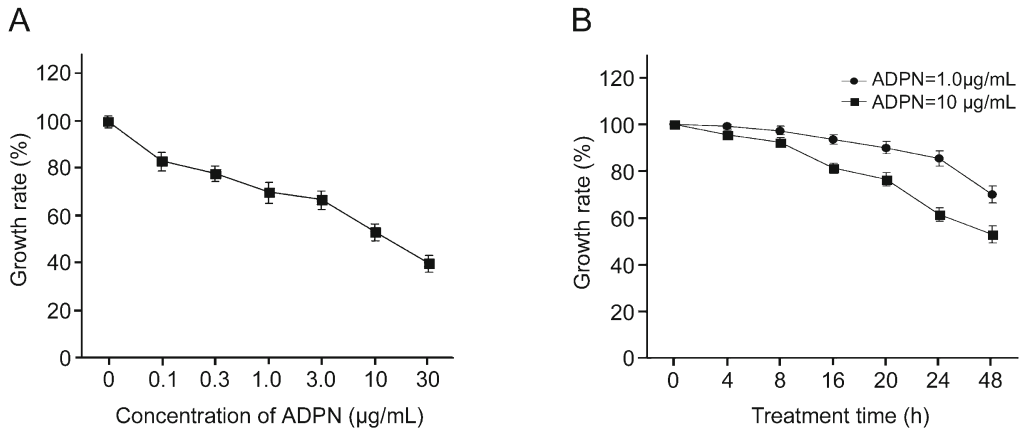

Fig. 4. The inhibition of ADPN on HeLa cell proliferation at subphysiological concentrations. (A) ADPN inhibits proliferation of HeLa cells in a concentration-dependent manner. (B) ADPN inhibits HeLa cells growth in a time-dependent manner.

We also demonstrated that ADPN receptor 1 and 2 mRNAs are expressed in HeLa cells using semi-quantitative RT-PCR analysis (Fig. 2). These results indicated that the mRNA expression level of AdipoR2 was higher than that of AdipoR1 in HeLa cells. Furthermore, we found that the mRNA expression level of AdipoR2 was significantly increased when treated with 10 μg/mL ADPN, comparing to 1.0 μg/mL ADPN treatment and PBS control (Fig. 3). However, no significant changes were observed in the mRNA expression level of AdipoR1 (data not shown). These results suggested that ADPN may be able to mediate the expression of ADPN receptors automatically.

ADPN inhibited the proliferation of HeLa cells

Because HeLa cells expressed AdipoR1 and AdipoR2 (Fig. 2), we postulated that ADPN may regulate the growth of HeLa cells. Thus, we employed the MTT assay to determine whether ADPN influences HeLa cell growth. The results from the MTT assay showed a concentration-dependent inhibition of ADPN on HeLa cell growth after treatment for 48 h (Fig. 4A); when the concentration of ADPN was 30 μg/mL, the growth rate was reduced by 60% ($P < 0.01$). Figure 4B demonstrated that ADPN inhibited HeLa cells growth in a time-dependent manner.

Table 2. Effects of ADPN on cell cycle and apoptosis index of HeLa cells.[a]

	Adiponectin (μg/mL)		
	Control	1.0	10
G0/G1 (%)	42.2 ± 5.3	59.2 ± 1.5*	51.5 ± 4.9*
PI [S+G2/M] (%)	57.8 ± 5.3	40.8 ± 1.5*	48.5 ± 4.9*
AI (%)	1.56 ± 4.8	4.02 ± 2.3*	14.86 ± 3.6*
PI/AI	37.1 ± 2.5	10.1 ± 2.6*	3.26 ± 4.2*

[a] PI: proliferation index; AI: apoptotic index. Asterisks indicate $P < 0.05$ for Student's t-test, compared to the control group.

ADPN increased the proportion of G0/G1 phase cells

Since ADPN inhibited the growth of HeLa cells, we next needed to determine whether ADPN influences HeLa cell cycle progression. After treatment with ADPN for 48 h, HeLa cells were stained with propidium iodide and analyzed by flow cytometry. The results (Fig. 5, Table 2) showed that ADPN (1.0 μg/mL) significantly increased the number of G0/G1 phase cells (from 42.2 ± 5.3% to 59.2 ± 1.5%, $n = 3$, $P < 0.05$) accompanied by a reduction in the cell population in both S and G2/M phases (from 57.8 ± 5.3% to 40.8 ± 1.5%, $n = 3$, $P < 0.05$), when compared with the control. These results suggest that ADPN inhibits HeLa cell

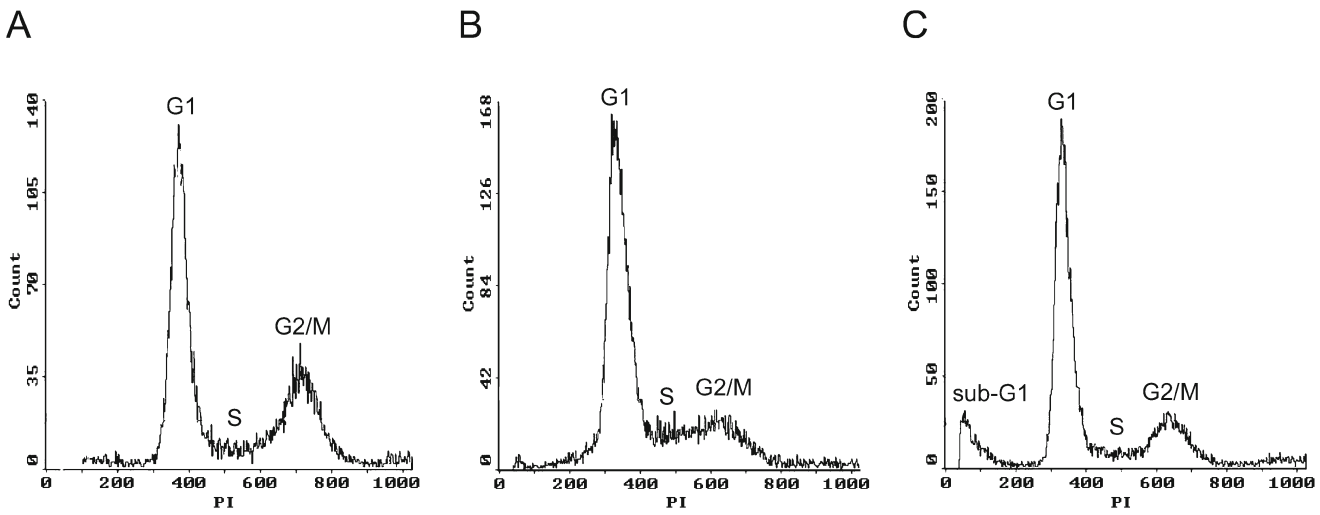

Fig. 5. ADPN increases the proportion of G0/G1 phase cells by flow-cytometry analysis. After treatment with ADPN for 48 h, HeLa cells were stained with PI and analyzed by flow cytometry. (A) PBS control. (B) 1.0 μg/mL ADPN significantly increased the number of G0/G1 phase cells (from 42.2 ± 5.3% to 59.2 ± 1.5%, $n = 3$, $P < 0.05$). (C) The apoptotic peak (AP, sub-G1 phase) appeared before the G1 phase after 10 μg/mL ADPN treatment.

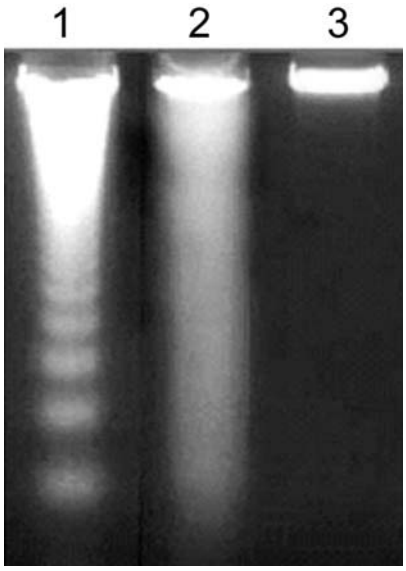

Fig. 6. ADPN-induced DNA fragmentation of HeLa cells detected with agarose gel electrophoresis. HeLa cells were exposed to ADPN for 48 h, apoptotic DNA was prepared as described in the text and separated on a 1.5% agarose gel. The DNA was visualized by staining with ethidium bromide. Lane 1, 10 μg/mL ADPN; lane 2, 1.0 μg/mL ADPN; lane 3, control.

growth by arresting cell cycle progression at G0/G1 phase. Cell apoptosis was induced and increased AI (from 1.56 ± 4.8 to 14.86 ± 3.6, $n = 3$, $P < 0.05$, compared to the control group) was observed when HeLa cells were treated with ADPN (10 μg/mL) for 48 h. A DNA ladder experiment (Fig. 6) further demonstrated that ADPN (10 μg/mL) significantly caused apoptosis in these cells.

Modulation of AMPK pathways by ADPN

The activation of AMPK through phosphorylation is the major transduction pathway for ADPN signaling. Therefore, we investigated the phosphorylation levels of AMPK stimulated by ADPN in HeLa cells. Data showed that ADPN induced a rapid increase in the phosphorylated form of AMPK (Fig. 7). The phosphorylated state appeared after 5 min and reached a maximal effect after 10 min thereafter declining after 15 min exposure to ADPN.

The effects of ADPN on expression of cell cycle regulators in HeLa cells

To gain further information about the molecular basis underlying the ADPN-inhibited cell proliferation, expression of some cell cycle key regulatory genes were studied in HeLa cells. The transcription factor cyclin D1 and c-myc play an important role in mediating cell entry into the S phase. Therefore, we used RT-PCR to measure the influence of ADPN on the expression of cyclin D1 and c-myc in HeLa cells. RT-PCR results show that ADPN decreased cyclin D1 and c-myc mRNA expressions by 38% and by 53%, respectively (Fig. 8A,B). These observations indicate that ADPN inhibited expression of the positive regulator cyclin D1 and c-myc, perhaps in turn to arrest the entry of G1 into S phase in cell cycle progression.

In order to study further the involvement of ADPN in cell cycle progression, we detected the expression of $p21^{WAF1/CIP1}$ by RT-PCR, which plays a role as a major negative regulator in the G1 checkpoint. Our results demonstrate that ADPN increases expression of cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ by 43% after treatment with ADPN 10 μg/mL, compared to the control group (Fig. 8C). Our data indicate that ADPN may stimulate the braking of cell cycle progression through increasing the expression of the negative regulator $p21^{WAF1/CIP1}$.

p53 is a tumor suppressor and is also positive regulator of $p21^{WAF1/CIP1}$. To evaluate whether ADPN influences cell cycle progression of HeLa cells by p53

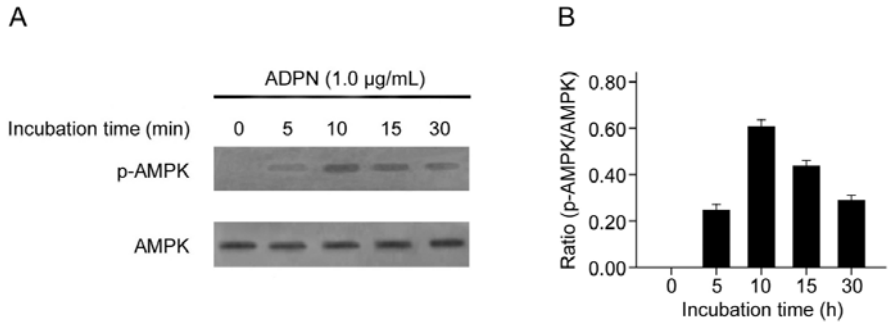

Fig. 7. Influence of ADPN on AMPK phosphorylation in HeLa cells. HeLa cells were incubated with ADPN (1.0 μg/mL) for 30 min. At the indicated times (0, 5, 10, 15, 30 min), total cell lysates were subjected to Western-blotting analysis. (A) AMPK phosphorylation was detected with rabbit polyclonal anti-phospho-AMPK-α antibody, and total AMPK proteins were probed with specific anti-AMPK antibody. (B) Relative phosphorylation levels of AMPK were normalized to its total AMPK and the ratio of the phosphorylated AMPK to total AMPK were measured. Each bar represents means ± SD of triplicates.

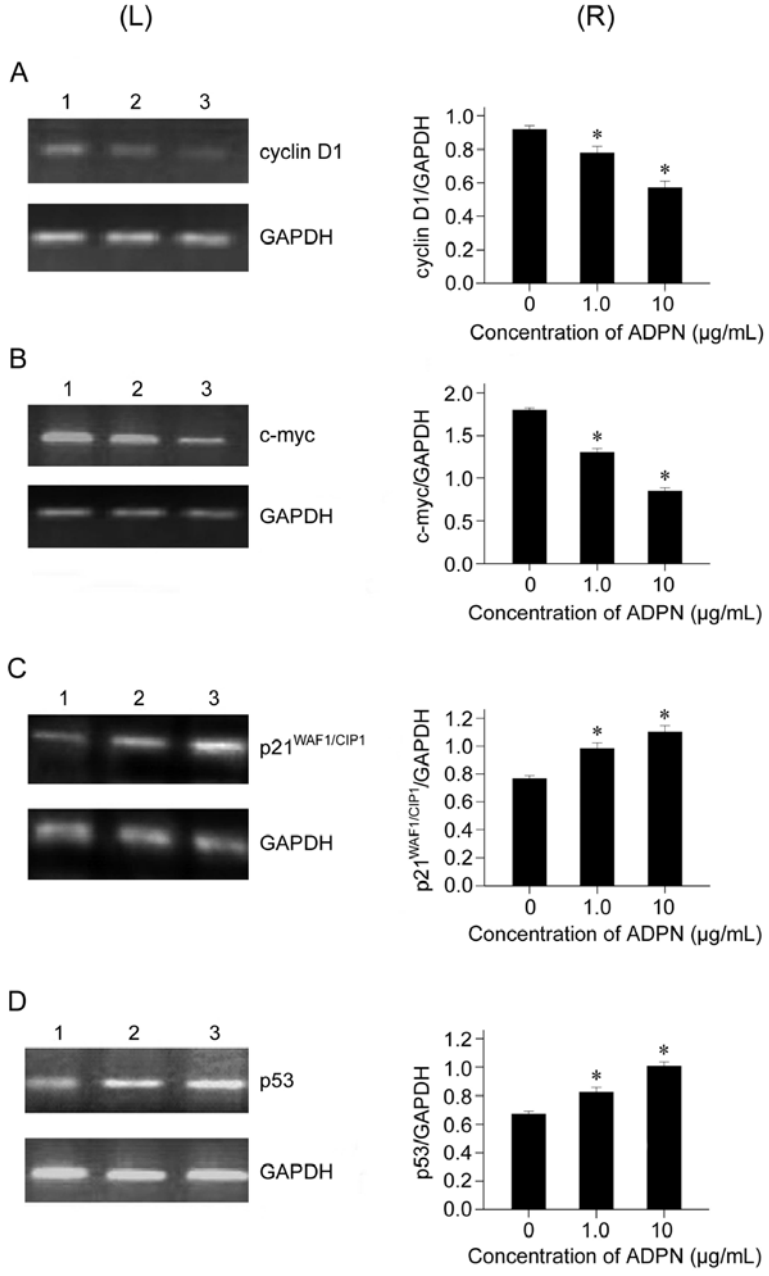

Fig. 8. Effects of ADPN on cyclin D1, c-myc, $p21^{WAF1/CIP1}$, and p53 mRNA expressions. (A) ADPN inhibits the expression of cyclin D1. (B) ADPN down-regulates the expression of c-myc. (C) ADPN induces the expression of $p21^{WAF1/CIP1}$. (D) ADPN up-regulates the expression of p53. (L) Representative PCR gels stained with ethidium bromide. Lane 1, PBS; lane 2, 1.0 μg/mL ADPN; lane 3, 10 μg/mL ADPN. (R) Densitometric analysis of RT-PCR products. Values are normalized to GAPDH in the corresponding samples. Asterisks indicate $P < 0.05$ for Student's t-test, compared to the control group.

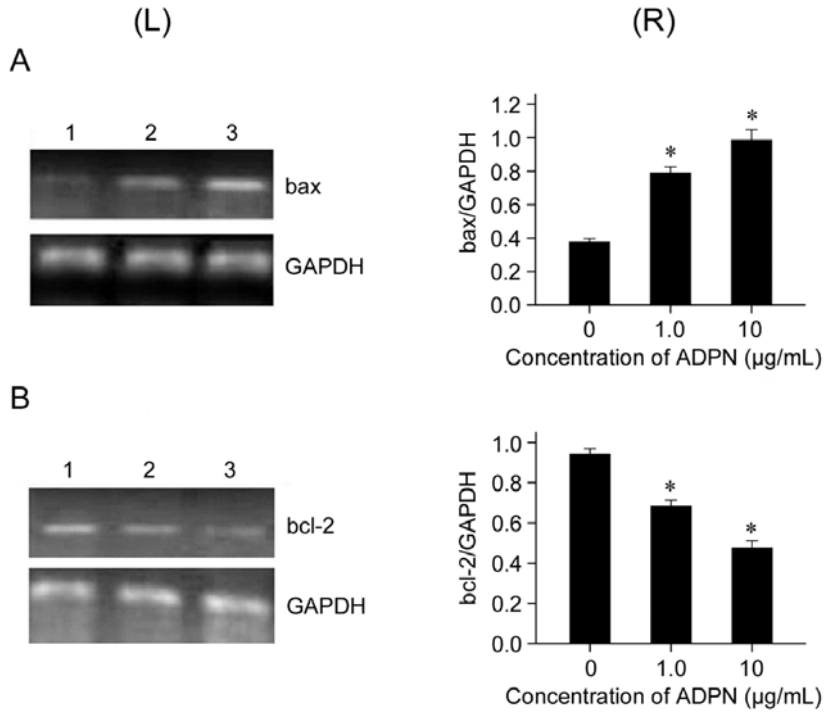

Fig. 9. Effects of ADPN on bax and bcl-2 mRNA expressions detected by RT-PCR. (A) ADPN stimulates the expression of the apoptotic genes bax after 48 h exposure. (B) ADPN inhibits the expression of the anti-apoptotic gene bcl-2. (L) Representative PCR gels stained with ethidium bromide. Lane 1, PBS; lane 2, 1.0 μg/mL ADPN; lane 3, 10 μg/mL ADPN. (R) Densitometric analysis of RT-PCR products. Values are normalized to GAPDH in the corresponding samples. Asterisks indicate $P < 0.05$ for Student's t-test, compared to the control group.

pathway, we detected the expression of p53 mRNA by RT-PCR. The result shows that the abundance of p53 mRNA increased by 51% after treatment with ADPN 10μg/mL, compared to the control group (Fig. 8D). The results suggested that the ADPN signal is involved in the pathway of p53–p21$^{WAF1/CIP1}$.

Expression of apoptosis-related genes in HeLa cells was regulated by ADPN

To investigate the molecular basis underlying the ADPN-induced cell apoptosis, the expression of a range of apoptotic genes were studied in HeLa cells. We used semi-quantitative RT-PCR to study the influence of ADPN on bax and bcl-2 mRNA expressions. As shown in Figure 9, ADPN stimulated the expression of the apoptotic gene bax after 48 h exposure (Fig. 9A). Under the same conditions, expression of the anti-apoptotic gene bcl-2 was clearly reduced (Fig. 9B). These data suggest that ADPN can induce HeLa cells to undergo apoptosis by promoting the expression of apoptotic related genes.

Discussion

An increasing number of reports suggest that ADPN inhibits the proliferation of tumor cells and induces their apoptosis. ADPN was demonstrated to inhibit the growth of breast cancer cell and induce their apoptosis (Kang et al. 2005; Dieudonne et al. 2006). These studies found that ADPN activated the AMPK and p42/44 MAPK signal pathways and mediated the expression of cell cycle regulators cyclin D1, c-myc and apoptosis related genes p53, bax, bcl-2 (Dieudonne et al. 2006). However, at least one report suggested that ADPN only diminished the proliferation rate of MCF7 cells but did not cause apoptosis (Arditi et al. 2007). Furthermore, ADPN reportedly reduced the growth of prostate cancer cell (Miyazaki et al. 2005; Bub et al. 2006). These various studies have showed that f-Ad and g-Ad can activate JNK in prostate cancer cell Du145, PC-3, LNCaP-FGC and inactivated the STAT3 in DU145 and liver cell HepG2. Constitutive STAT3 activation is able to induce malignant transformation and stimulate cancer progression including cell growth promotion. Therefore, ADPN may inhibit the growth of prostate cancer cells through STAT3 inactivation. All of these results suggested that ADPN is able to mediate the proliferation of many cancer cells by selectively interfering with a range of signaling pathways.

It is clear that ADPN exerts its biological activities through its receptors. Therefore, in order to detect the effects of ADPN on HeLa cells, we examined the expression of ADPN receptors in these cells. FITC fluorescence labeling assay showed that FITC-ADPN were mainly localized on the membrane of HeLa cells, so we speculated HeLa cells express ADPN receptors in the membrane. RT-PCR confirmed that HeLa cells express ADPN receptors, and further demonstrated that the expression level of AdipoR2 mRNA was up-regulated in HeLa cells treated with ADPN. This is similar to reports that g-Ad stimulated the expression of AdipoR2 mRNA in prostate cancer cells (Mistry et al. 2006). The

MTT assay showed that ADPN inhibits proliferation of HeLa cells in a concentration- and time-dependent manner. Moreover, flow cytometric analysis demonstrated that low-dose ADPN can arrest cells at G0/G1-phase and delay the cells entering into S-phase and inhibit the proliferation of HeLa cells, whereas high-dose ADPN can induce the apoptosis of HeLa cells.

To further investigate the molecular basis underlying the effects of ADPN on inhibiting cell proliferation, expression of phosphorylated AMPK and cell cycle key regulatory genes and apoptosis-related genes were studied in HeLa cells. As a metabolic sensing protein kinase, AMPK is known to play a major protective role in conditions of metabolic stress. However, AMPK has recently been implicated in the negative control of cell cycle progression via up-regulating the expression of p53 and p21 (Igata et al. 2005), and AMPK activation was found to induce the apoptosis in various cell types (Hardie 2004; Saitoh et al. 2004; Dagon et al. 2006). We observed that ADPN can activate AMPK within a short-term period (< 30 min) and down-regulate the expression of cell cycle regulators cyclin D1 and c-myc. Moreover, ADPN stimulates expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and tumor suppressor p53. c-myc was reported to mediate the expression of $p21^{WAF1/CIP1}$ in p53 dependent or independent manner (Seoane et al. 2002; Vaqué et al. 2005), p53 can activate $p21^{WAF1/CIP1}$ via interacting with transcription factor SP1 (Oswald et al. 1994). In addition, cyclin D1 expression could be induced by c-myc; on the other hand, expression of cyclin D1 led to an E2F-dependent transactivation of c-myc promoter (Lagger et al. 2003). Therefore, we postulated that ADPN enlarges their effects on the growth of HeLa cells via AMPK activation and meditating the expression of cell cycle regulators and their reciprocal activation.

To investigate the molecular basis of the apoptotic action of ADPN, we studied the influence of ADPN on bax and bcl-2 mRNA expression. The results suggested that ADPN promotes apoptosis of HeLa cells through up-regulating the expression of the apoptotic gene bax and down-regulating the anti-apoptotic gene bcl-2 mRNA expression.

In conclusion, our results suggested that ADPN reduces the proliferation of HeLa cells and induces apoptosis in these cells. These data provide a possible basis for the use of ADPN in the therapeutic intervention of cervical cancer.

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