

Apelin-13 induces autophagy in hepatoma HepG2 cells through ERK1/2 signaling pathway-dependent upregulation of Beclin1

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Received August 26, 2014; Accepted June 11, 2015

DOI: 10.3892/ol.2015.3991

Abstract. The aim of the present study was to investigate the effect of Apelin-13 on autophagy in hepatocellular carcinoma HepG2 cells and the underlying mechanism of the effect. The HepG2 cells were treated with Apelin-13 at a final concentration of 0.0001, 0.001, 0.01 and 0.1 μ mol/l for 24 h. Cells were also treated with 10 μ mol/l PD98059 for 24 h. The expression of the extracellular signal-regulated kinase (ERK)1/2, phosphorylated ERK1/2 (pERK1/2) and Beclin1 proteins were detected by western blot analysis. Beclin1 mRNA expression was also detected by reverse transcription-polymerase chain reaction. Autophagy was observed using fluorescence microscopy subsequent to monodansylcadaverine (MDC) staining. Following treatment with the various concentrations of Apelin-13, the expression of the ERK1/2 protein remained at a similar level, whereas the expression of pERK1/2 increased in a dose-dependent manner. Compared with the control group, the increase was significant ($P<0.05$). Similarly, Beclin1 expression was upregulated at the protein and mRNA levels by Apelin-13 treatment in a dose-dependent manner and was significantly increased compared with the control group. However, following treatment with the Apelin-13 inhibitor PD98059, the expression of pERK1/2, Beclin1 protein and Beclin1 mRNA were significantly decreased ($P<0.05$). In addition, Apelin-13 induced the autophagy of HepG2 cells in a dose-dependent manner, as revealed by MDC staining. PD98059 inhibited autophagy of HepG2 cells induced by Apelin-13. Therefore, Apelin-13 may

promote autophagy in HepG2 cells by inducing the phosphorylation of ERK1/2 and upregulating the expression of Beclin1.

Introduction

In previous years, the association between tumor development and autophagy has received increasing attention. As the first gene identified to inhibit tumorigenesis through lysosome degradation, Beclin1 has been widely studied. The mammalian Beclin1 gene encodes a B-cell lymphoma 2-interacting coiled-coil protein that possesses structural similarities to the yeast autophagy protein. The human Beclin1 gene, which contains 12 exons, is located on human chromosome 17q21. It has previously been reported that Beclin1 is a mammalian autophagy gene that inhibits tumorigenesis (1-3). Beclin1, as a key factor that mediates the localization of other autophagy proteins into the precursor of autophagic bodies, is involved in the regulation of mammalian autophagy formation (4).

Extracellular signal-regulated kinase (ERK)1/2 is a protein involved in the Ras/Raf-1/ERK1/2 signaling pathway that is important for autophagy (5). Sodium arsenite induces autophagy through ERK1/2 phosphorylation in human uroepithelial cells (6). Apelin is isolated from an endogenous ligand from bovine gastric secretion (7). Apelin-13 is one of the most active protein fragments in the Apelin/Apelin receptor system, and promotes the phosphorylation of ERK1/2 in rat vascular smooth muscle cells in a concentration-dependent manner (8).

In the present study, the effect of Apelin-13 on the protein and mRNA expression of ERK1/2, phosphorylated ERK (pERK)1/2 and Beclin1 was determined. The effect of Apelin-13 on autophagy in HepG2 cells was also investigated.

Materials and methods

Reagents and instruments. RPMI-1640, fetal bovine serum (FBS), trypsin and dimethyl sulfoxide (DMSO) were purchased from Beyotime Institute of Biotechnology (Hangzhou, Zhejiang, China). Apelin-13 and PD98059 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Monodansylcadaverine (MDC) and mouse monoclonal β -tubulin (D-10; sc-5274), rabbit polyclonal pERK (Thr 177/Thr 160; sc-23759-R), mouse monoclonal ERK (C-9;

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Key words: extracellular signal-regulated kinase 1/2, expression levels, phosphorylation

sc-514302) and mouse monoclonal Beclin-1 (G-11; sc-48381) antibodies were purchased from Santa Cruz Biotechnology, Inc. Horseradish peroxidase-conjugated rabbit anti-mouse and mouse anti-rabbit secondary antibodies, and the hyper-pure RNA, PrimeScript reverse transcription-polymerase chain reaction (RT-PCR), enhanced chemiluminescence western blot and bicinchoninic acid (BCA) protein assay kits were purchased from Beijing ComWin Biotech Co., Ltd. (Beijing, China). The primers for RT-PCR were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

An IX73 inverted and reflected fluorescent microscope was purchased from Olympus Optical Co., Ltd. (Tokyo, Japan). The FlourChem E HD2 and AlphaImager 2200 gel imaging systems were purchased from ProteinSimple (San Jose, CA, USA).

Cell lines and culture. Hepatocarcinoma HepG2 cells were provided by the Tumor Research Institute of University of South China. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, and maintained in a humidified incubator at 37°C and in a 5% CO₂ atmosphere. Adherent cells were trypsinized using 0.25% trypsin-EDTA, harvested and re-plated into flasks every 2-3 days. Cells in the logarithmic phase of growth were used in the subsequent analyses.

Experimental groups and treatments. The cells were treated with Apelin-13 and PD98059. Apelin-13 was dissolved in phosphate-buffered saline (PBS) and PD98059 was dissolved in DMSO. In the Apelin-13 group, the cells were treated with Apelin-13 at final concentrations of 0.0001, 0.001, 0.01 and 0.1 µmol/l for 24 h. In the PD98059 group, the cells were treated with 10 µmol/l PD98059 for 24 h. In the Apelin-13 and PD98059 groups, the cells were treated with 0.1 µmol/l Apelin-13 and 10 µmol/l PD98059 for 24 h, respectively. The cells treated with 10% FBS and DMSO were used as the control groups.

Western blot analysis. The samples were lysed in 100 µl of lysis buffer (RIPA:PMSF ratio, 94:6). Subsequent to incubating the samples on ice for 30 min, the cell lysate was centrifuged at 18,000 x g for 8 min at 4°C and the supernatant was collected. The protein concentration in the supernatant was quantified using the BCA kit. The proteins were then separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Subsequent to blocking, the membrane was incubated at 21°C overnight with mouse anti-β-tubulin (dilution, 1:250), mouse anti-ERK1/2 (dilution, 1:250), rabbit anti-pERK1/2 (dilution, 1:250) and mouse anti-Beclin1 (dilution, 1:250) monoclonal primary antibodies. Subsequent to washing, the mouse anti-rabbit and rabbit anti-mouse secondary antibodies were added at dilutions of 1:10,000 and 1:5,000, respectively, and the membrane was incubated at 21°C for 4 h. Finally, the membrane was developed using enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). The developed film was scanned using the AlphaImager 2200 gel imaging system. The western blot images were analyzed using GraphPad Prism 5, which was purchased from GraphPad Software, Inc., (La Jolla, CA, USA). β-tubulin was used as an internal control. The relative expression of ERK1/2, pERK1/2 and Beclin1 was calculated based on the grayscale value of β-tubulin.

RT-PCR assay. Total RNA was extracted from HepG2 cells, and the expression of Beclin1 mRNA was detected using the PrimeScript RT-PCR kit. According to the manufacturer's instructions, the purity and concentration of the RNA was calculated using a DNA/RNA calculator (Pharmacia, Fairfield, CT, USA). For the amplification of Beclin1, PCR was performed with pre-denaturation step at 95°C for 5 min, 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 40 sec and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. GAPDH was used as the internal reference. The PCR products were separated by electrophoresis under a constant voltage of 80 V in a 12 g/l agarose gel for 30 min. The gel was then observed and images of the gel were captured using the AlphaImager 2200 gel imaging analysis system. This reaction was conducted three times. The primer sequences used were as follows: GAPDH sense, 5'-ACCACAGTCCATGCCATCAC-3' and anti-sense, 5'-TCCACCACCCTGTTGCTGTA-3'; Beclin1 sense, 5'-CTA AGTCGTCCAACAACAGCAC-3' and anti-sense, 5'-CGATGT CAAAAAGGTCCC-3'. The primers were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The relative expression level of Beclin1 was determined by the grayscale ratio of Beclin1 to GAPDH.

MDC staining and autophagy detection. Based on the method described by Biederbick *et al*, MDC was used to stain the autophagosomes (9). Briefly, the cells were placed on six-well plates and treated with Apelin-13 and PD98059, as aforementioned. Following incubation for 24 h, 0.05 mmol/l MDC was added to the plates and the cells were incubated at 37°C for 60 min. The cells were then fixed with 4% paraformaldehyde for 15 min. Subsequent to rinsing twice with cold PBS, the cells were observed and photographed by inverted fluorescence microscopy.

Statistical analysis. The data were expressed as the mean ± standard deviation and were analyzed using SPSS software, version 13 (SPSS, Inc., Chicago, IL, USA). The differences between groups were compared using one-way analysis of variance. Multiple comparison between the groups was performed using the S-N-K method. The variables in the two groups were compared by Student's *t*-test. *P* < 0.05 was considered to indicate a statistically significant difference.

Results

Apelin-13 upregulates the expression of pERK1/2 in HepG2 cells. To determine the effect of Apelin-13 on the expression of ERK1/2 and pERK1/2, western blot analysis was performed. The cells were treated with Apelin-13 at final concentrations of 0.0001, 0.001, 0.01 and 0.1 µmol/l for 24 h. The cells treated with 10% FBS were used as a negative control. Representative western blot results are presented in Fig. 1A and quantitative results are reported in Fig. 1B. As indicated in Fig. 1, following treatment with Apelin-13, the expression level of ERK1/2 was not evidently changed. However, the level of pERK1/2 was increased in a dose-dependent manner. The relative expression levels of pERK1/2 in cells treated with 10% FBS or 0.0001, 0.001, 0.01 and 0.1 µmol/l Apelin-13 were 0.445±0.021, 0.764±0.013, 0.857±0.018, 0.906±0.072 and

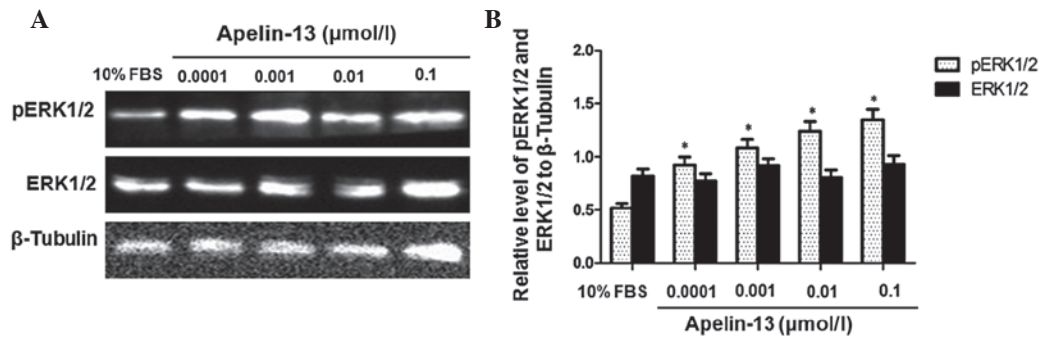


Figure 1. Analysis of the expression of ERK1/2 and pERK1/2 subsequent to treatment with Apelin-13. The cells were treated with 0.0001, 0.001, 0.01 or 0.1 $\mu\text{mol/l}$ Apelin-13 for 24 h. ERK1/2 and pERK1/2 expression was then detected by western blot analysis. β -tubulin was used as an internal control. The cells treated with 10% FBS were used as negative control. (A) Representative western blotting results. The relative expression level of ERK1/2 and pERK1/2 was the ratio of ERK1/2 to β -tubulin and the ratio of pERK1/2 to β -tubulin, respectively. The data were presented as the mean \pm standard deviation of three independent experiments. * $P < 0.01$ vs. cells treated with 10% FBS. ERK1/2, extracellular signal-regulated kinase 1/2; pERK1/2, phosphorylated ERK1/2; FBS, fetal bovine serum.

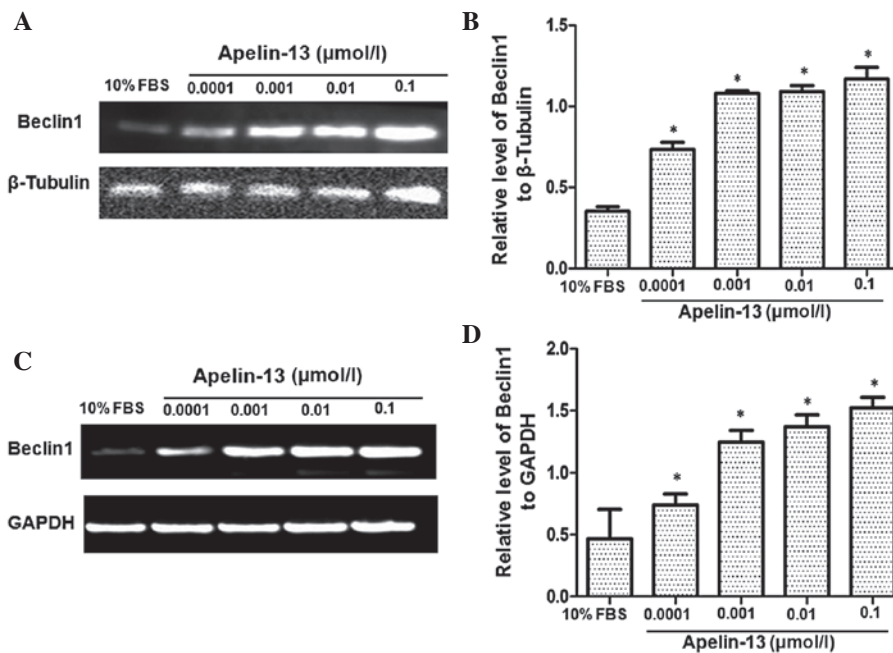


Figure 2. Analysis of the expression of Beclin1 subsequent to treatment with Apelin-13. The cells were treated with 0.0001, 0.001, 0.01 or 0.1 $\mu\text{mol/l}$ Apelin-13 for 24 h. The cells treated with 10% FBS were used as a negative control. Beclin1 protein expression was detected by western blot analysis. β -tubulin was used as an internal control. (A) Representative western blot results. (B) Quantitative western blot results. The relative expression level of the Beclin1 protein was the ratio of Beclin1 to β -tubulin. Beclin1 mRNA expression was analyzed by RT-PCR. GAPDH was used as an internal control. (C) Representative RT-PCR results. (D) Quantitative RT-PCR results. The relative expression level of Beclin1 mRNA was the ratio of Beclin1 to GAPDH. The data were expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.01$ vs. cells treated with 10% FBS. RT-PCR, reverse transcription-polymerase chain reaction; FBS, fetal bovine serum.

1.019 \pm 0.041, respectively. Following treatment with Apelin-13, the expression levels of pERK1/2 at concentrations of 0.0001, 0.001, 0.01 and 0.1 $\mu\text{mol/l}$ were significantly increased compared with the negative control group ($P < 0.05$). These data indicate that Apelin-13 induced the phosphorylation of ERK1/2 and upregulated the level of pERK1/2 in HepG2 cells in a dose-dependent manner.

Apelin-13 upregulates the expression of Beclin1 protein and mRNA in HepG2 cells. To analyze the effect of Apelin-13 on the expression of Beclin1 protein, western blot analysis was performed using HepG2 cells treated with 0.0001, 0.001, 0.01 and 0.1 $\mu\text{mol/l}$ Apelin-13. The negative control was

performed using 10% FBS. Fig 2A presents the representative western blot results and Fig. 2B reports the quantitative western blot results. In the cells treated with 10% FBS, Beclin1 protein expression was extremely low. However, the protein expression of Beclin1 increased significantly subsequent to Apelin-13 treatment. The increase was dose-dependent. Subsequent to quantification, the relative expression of the Beclin1 protein in cells treated with 10% FBS and with 0.0001, 0.001, 0.01 and 0.1 $\mu\text{mol/l}$ Apelin-13 were 0.354 \pm 0.010, 0.734 \pm 0.017, 1.080 \pm 0.068, 1.092 \pm 0.014 and 1.168 \pm 0.024, respectively. Compared with the cells treated with 10% FBS, the cells treated with Apelin-13 demonstrated significantly increased levels of Beclin1 expression ($P < 0.05$).

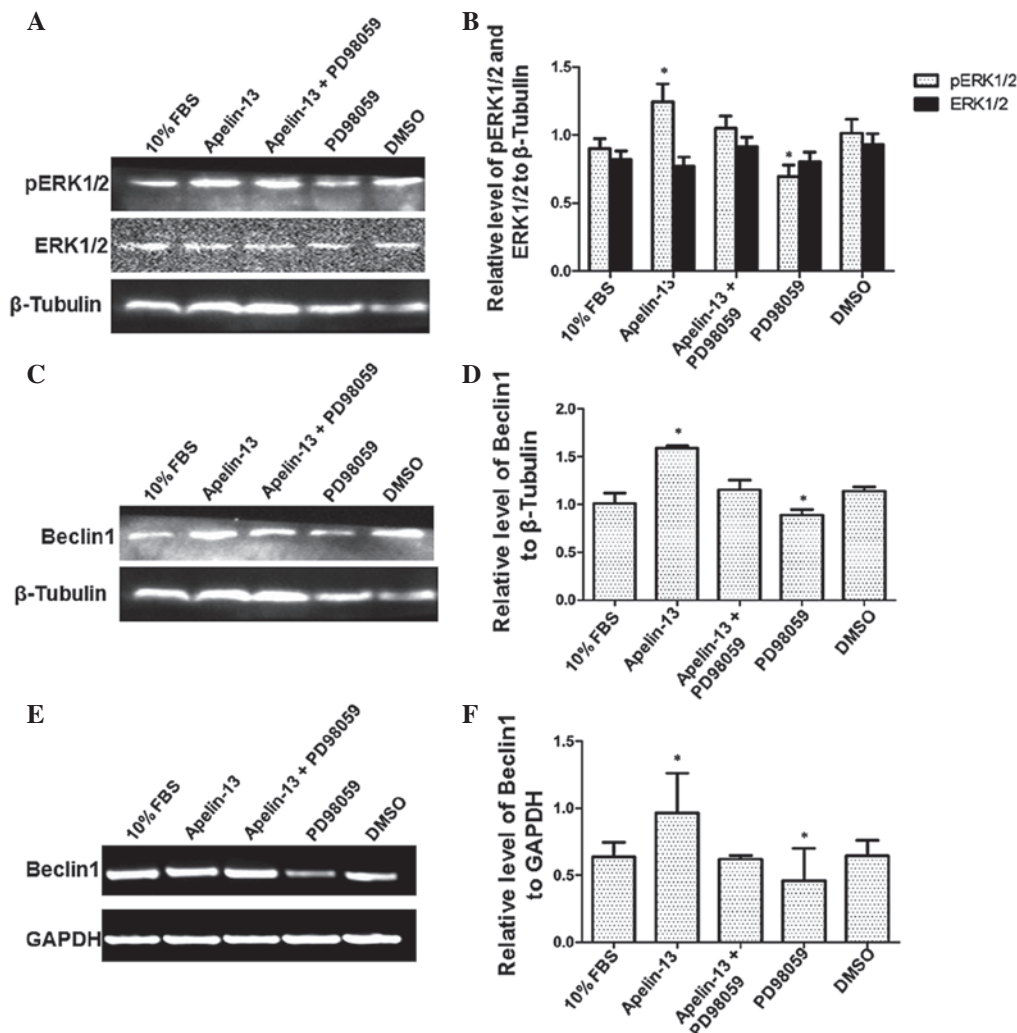


Figure 3. Analysis of ERK1/2, pERK1/2 and Beclin1 expression subsequent to treatment with Apelin-13 and PD98059. The cells were treated with 0.1 μ mol/l Apelin-13, 0.1 μ mol/l Apelin-13 combined with 10 μ mol/l PD98059 or 10 μ mol/l PD98059 for 24 h. The cells treated with 10% FBS and DMSO were used as negative controls. (A and B) The expression of ERK1/2 and pERK1/2 was detected by western blot analysis. (A) Representative and (B) quantitative western blot results are shown. β -tubulin was used as an internal control. The relative expression levels of ERK1/2 and pERK1/2 were calculated as the ratio of ERK1/2 to β -tubulin and the ratio of pERK1/2 to β -tubulin, respectively. (C and D) Beclin1 protein expression was detected by western blot analysis. (C) Representative and (D) quantitative western blot results are shown. β -tubulin was used as an internal control. The relative expression level of Beclin1 protein was calculated as the ratio of Beclin1 to β -tubulin. (E and F) Beclin1 mRNA expression was analyzed by RT-PCR. GAPDH was used as an internal control. (E) Representative and (F) quantitative RT-PCR results are shown. The relative expression level of Beclin1 mRNA was calculated as the ratio of Beclin1 to GAPDH. The data were expressed as the mean \pm standard deviation of three independent experiments. * P <0.01 vs. cells treated with 10% FBS and DMSO. ERK1/2, extracellular signal-regulated kinase 1/2; pERK1/2, phosphorylated ERK1/2; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; RT-PCR, reverse transcription-polymerase chain reaction.

In addition, the effect of Apelin-13 on Beclin1 mRNA expression was also investigated by RT-PCR. GAPDH was used as an internal control. Similarly, Beclin1 mRNA was barely detectable in cells treated with 10% FBS, whereas the expression became gradually upregulated in cells treated with various concentrations of Apelin-13 (Fig. 2C and D). Quantitatively, the Beclin1 mRNA level was 0.466 ± 0.095 in cells treated with 10% FBS, and the Beclin1 mRNA levels were 0.737 ± 0.038 , 1.246 ± 0.038 , 1.370 ± 0.039 and 1.522 ± 0.034 in cells treated with 0.0001, 0.001, 0.01 and 0.1 μ mol/l Apelin-13, respectively. Statistically, there were significantly higher levels of Beclin1 mRNA in cells treated with Apelin-13 compared with the cells treated with 10% FBS (P <0.05). Overall, these results suggest that Apelin-13 stimulated the expression of Beclin1 at the protein and mRNA level in a dose-dependent manner.

Apelin-13 inhibitor PD98059 suppresses the phosphorylation of pERK1/2 and expression of Beclin1 induced by Apelin-13. To further verify the effect of Apelin-13 on pERK1/2 and Beclin1, the HepG2 cells were treated with the Apelin-13 inhibitor PD98059. As aforementioned, the most notable effect of Apelin-13 was observed at the concentration of 0.1 μ mol/l. Therefore, 0.1 μ mol/l Apelin-13 was used in the present study. The concentration of PD98059 used was 10 μ mol/l. First, western blot analysis was performed to assess the effect of Apelin-13 and PD98059 on pERK1/2 expression (Fig. 3A and B). Consistently, cells treated with Apelin-13 alone demonstrated increased levels of pERK1/2. The relative levels of pERK1/2 in the control cells treated with 10% FBS and DMSO were 1.169 ± 0.010 and 1.087 ± 0.053 , respectively. The relative pERK1/2 level was 1.511 ± 0.044 in cells treated with Apelin-13 alone. However, the relative pERK1/2 level decreased to 1.1435 ± 0.041 in cells

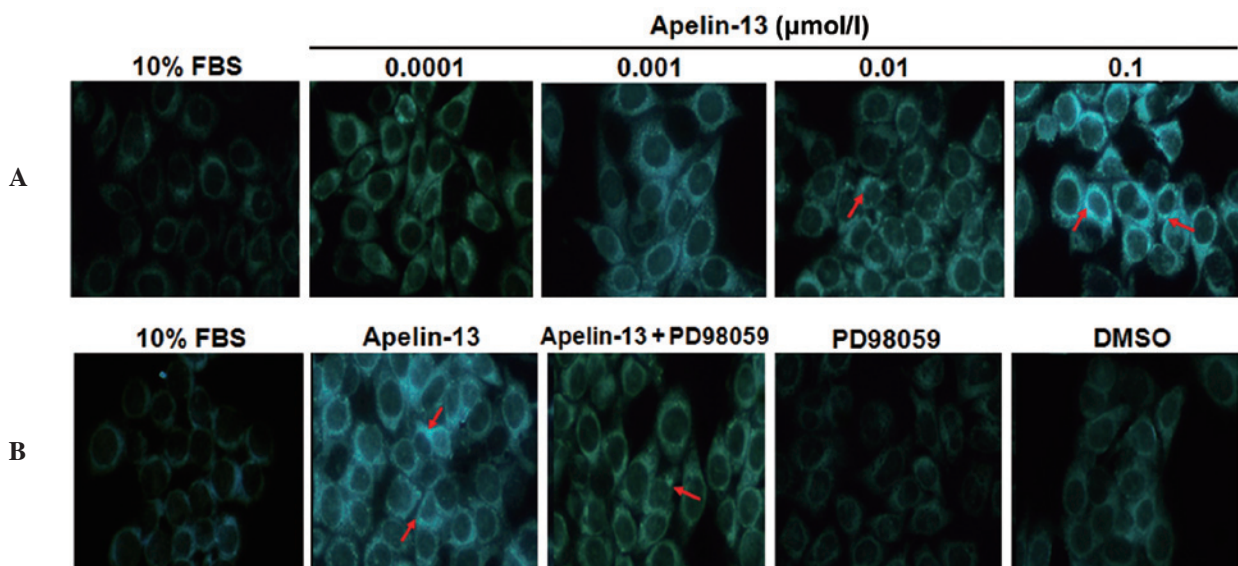


Figure 4. Analysis of autophagy in HepG2 cells subsequent to treatment with Apelin-13 and PD98059. Autophagy of HepG2 cells was observed under inverted fluorescence microscopy following MDC staining. Autophagosomes were stained with blue and green fluorescence. (A) The cells were incubated with 0.0001, 0.001, 0.01 and 0.1 $\mu\text{mol/l}$ Apelin-13 for 24 h. The cells treated with 10% FBS were used as a negative control. Representative MDC staining results are shown. Red arrows indicate autophagosomes. (B) Cells were treated with 0.1 $\mu\text{mol/l}$ Apelin-13 alone, 0.1 $\mu\text{mol/l}$ Apelin-13 combined with 10 $\mu\text{mol/l}$ PD98059 or 10 $\mu\text{mol/l}$ PD98059 alone for 24 h. The cells treated with 10% FBS and DMSO were used as negative controls. Representative MDC staining results are shown. Red arrows indicate autophagosomes. FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MDC, monodansylcadaverine.

treated with Apelin-13 and PD98059. In cells treated with PD98059 alone, the pERK1/2 relative level was 0.955 ± 0.023 . Compared with other treatments, the administration of Apelin-13 alone resulted in the highest level of pERK1/2, whereas the administration of PD98059 alone resulted in the lowest level of pERK1/2 ($P < 0.01$). Therefore, this result reveals that the Apelin-13 inhibitor PD98059 decreased the level of ERK1/2 phosphorylation induced by Apelin-13, further confirming the effect of Apelin-13 on ERK1/2 phosphorylation.

Subsequently, the level of Beclin1 protein and mRNA was detected following treatment with Apelin-13 and PD98059. Consistently, western blot analysis revealed elevated levels of Beclin1 protein in cells treated with Apelin-13 alone, with a relative level of 1.590 ± 0.010 (Fig. 3C and D). By contrast, the upregulation of Beclin1 expression was blocked in PD98059-treated cells, with a relative level of 0.889 ± 0.024 . The relative expression of the Beclin1 protein in cells treated with 10% FBS, Apelin-13 and PD98059, and DMSO were 1.009 ± 0.045 , 1.155 ± 0.039 and 1.141 ± 0.018 , respectively. Statistically, there was a significantly increased level of Beclin1 protein in Apelin-13-treated cells and a significantly decreased level of Beclin1 protein in PD98059-treated cells ($P < 0.01$). In addition, Beclin1 expression at the mRNA level revealed a similar pattern to the expression of the Beclin1 protein, as revealed by RT-PCR (Fig. 3E and F). The relative expression levels of Beclin1 mRNA in cells treated with 10% FBS, Apelin-13, Apelin-13 and PD98059, PD98059 and DMSO were 1.068 ± 0.056 , 1.582 ± 0.017 , 1.132 ± 0.028 , 0.917 ± 0.023 and 1.133 ± 0.008 , respectively. The differences between Apelin-13-treated, PD98059-treated and control cells were statistically significant ($P < 0.01$). Thus, these results indicate that the Apelin-13 inhibitor PD98059 inhibited the upregulation of Beclin1 protein and mRNA induced by Apelin-13, further verifying the effect of Apelin-13 on Beclin1 expression.

Apelin-13 promotes and PD98059 inhibits the formation of the autophagosome in HepG2 cells. The aforementioned results indicate that Apelin-13 promotes the formation of the autophagosome in HepG2 cells. In the present study, the autophagy of HepG2 cells following treatment with Apelin-13 and PD98059 was evaluated by MDC staining. Representative results of MDC staining are shown in Fig. 4. The autophagosome was stained with blue and green fluorescence. As shown in Fig. 4A, there was no evident blue or green luminescence in cells treated with 10% FBS, indicating that no autophagosome was formed. Following treatment with Apelin-13, blue and green fluorescence was evident in the HepG2 cells, indicating that autophagosomes were formed. With the increasing concentration of Apelin-13, more blue and green luminescence autophagosomes were detected in HepG2 cells under inverted fluorescence microscopy. By contrast, in PD98059-treated HepG2 cells, the formation of autophagosomes decreased significantly compared with Apelin-13-treated cells (Fig. 4B). Red arrows indicate representative clusters of fluorescent autophagosomes in the cytoplasm. These data suggest that Apelin-13 induces autophagy in HepG2 cells and that the Apelin-13 inhibitor PD98059 efficiently inhibits the formation of autophagosomes in HepG2 cells treated with Apelin-13.

Discussion

Hepatocellular carcinoma is a disease that leads to mortality worldwide and is one of the cancers with the highest level of cancer-associated morbidity. This disease is also the third most invasive cancer worldwide (10). In China, hepatocellular carcinoma is the second most invasive cancer (11). Therefore, studies have paid increasing attention to the development of more efficient treatments for this carcinoma. Autophagy is a process through which cells use the lysosome to degrade damaged

organelles and macromolecules. Autophagy is an important conservative cellular pathway and includes macroautophagy, microautophagy and chaperone-mediated autophagy (12,13). In the present study, the effect of Apelin-13 on HepG2 cells was observed. Through MDC staining, it was found that treatment with Apelin-13 induced autophagy in HepG2 cells in a dose-dependent manner. In addition, the increase in autophagy was inhibited by the Apelin-13 inhibitor PD98059. This data indicates that Apelin-13 may inhibit hepatocellular carcinoma cells through inducing autophagy and that Apelin-13 may be used to treat hepatocellular carcinoma.

Apelin-13 was also observed to upregulate the expression levels of pERK1/2 and Beclin1 in a dose-dependent manner. In 1999, Beclin1 was observed to be the key gene in the induction of autophagy, and it was the first gene that was identified to inhibit tumors through lysosome degradation (2). Studies have revealed that Beclin1 expression is decreased in cervical cancer (14), ovarian cancer (15) and cerebral tumors (16). Down-regulation of Beclin1 expression significantly reduces autophagy in cancer cells (17,18). Therefore, upregulating the expression of Beclin1 may induce autophagy and therefore promote tumor cell death. The present results indicate that activation of the ERK1/2 signaling pathway effectively upregulates the expression of Beclin1 in hepatocellular carcinoma, indicating that the ERK1/2 signaling pathway is one of the mechanisms that mediates the expression of Beclin1. Therefore, the present results indicate that Apelin-13 may promote autophagy through upregulating Beclin1 and that Apelin-13 may upregulate Beclin1 expression through the ERK1/2 signaling pathway. Subsequent to treatment with the Apelin-13 inhibitor PD98059, the level of autophagy and pERK1/2 and Beclin1 expression were decreased. This further verified the promoting effect of Apelin-13 on autophagy and Beclin1 expression. However, whether Apelin-13 regulates autophagy and Beclin1 expression through other pathways requires additional investigation.

In summary, the present study found that Apelin-13 induced autophagy in hepatoma cells through ERK1/2 signaling pathway-dependent upregulation of Beclin1. Fundamental low-level autophagy activity is vital to maintain a steady state in normal and tumor cells. Excessive autophagy may lead to the death of cells (19). Additionally, autophagy plays dual roles in tumor development (20). As a regulatory protein of autophagy, Beclin1 efficiently inhibits the development of tumors by inhibiting angiogenesis (21). Therefore, the present results provide experimental evidence for the use of Apelin-13 in the treatment of hepatocellular carcinoma.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (grant nos. 81102230 and 81270420).

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