

Full Length Research Paper

Effects of leptin on colorectal cancer cell line HT-29

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The purpose of this study was to investigate the effect of leptin on the growth of HT-29 colorectal cancer cell line. HT-29 cells were treated with leptin at several concentrations of 0, 5, 50, 100, 200 ng/ml, respectively. Epidermal growth factor (EGF) was taken as the positive control. The effect of leptin on the growth, proliferation and apoptosis of HT-29 colorectal cancer cell line was assayed through drawing growth curve and by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] method and flow cytometry (FCM). According to the growth curve, the cell counts of HT-29 cell line increased in proportion to leptin concentration, varying from 5 to 200 ng/ml at a dose-dependent manner. However, the cell proliferation amounts of 200 ng/ml leptin group were still less than those of the positive control group (EGF). The MTT results were similar to those from cell counting method. Leptin at different concentration levels promoted the growth of HT-29 cell lines in a time- and dose-dependent manner, and the proliferation rate varied from 5.1 to 67.9%. FCM analysis showed that leptin can significantly affect cell cycle of HT-29 cells. With increasing concentrations, G0/G1 phase cells decreased gradually, and S and G2/M phase cells rose gradually, but no significant effect of leptin on apoptosis of HT-29 cells was observed. *In vitro*, leptin could promote growth and proliferation of HT-29 cell line according to the concentration and experimental time, but there was no significant correlation in apoptosis.

Key words: Leptin, colorectal neoplasms, cell proliferation, apoptosis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, flow cytometry.

INTRODUCTION

Colorectal cancer is a very common gastrointestinal malignancy. In western countries, it is the third most common malignancy in both males and females, and the second leading cause of cancer-related death (Song et al., 2012). In China, it is the fourth common malignant tumor and is the fifth key cause of cancer-related death (Hu et al., 2012). The prognosis is closely related with tumor TNM stage. It is generally believed that the development of colorectal cancer is closely related to dietary habits, genetic factors and colorectal adenoma (Zandonai et al., 2012; Parkin and Boyd, 2011; Di Gregorio et al., 2012; Mates et al., 2012). However, the pathogenesis of colorectal cancer is not clear yet. Leptin as the obese gene product, produced by adipocytes, is an important

regulatory molecule in energy regulation and food intake (Kishali, 2011; Türkmen, 2011; Gisou et al., 2009). The present study found that leptin was a growth factor for a wide variety of tumor. It promoted cell proliferation, angiogenesis, tumor invasion and meta-stasis, and inhibited cell apoptosis (Chen et al., 2007; Guo and Gonzalez-Perez, 2011; Zhou et al., 2011). In this study, the role of leptin on the growth and apoptosis of colorectal cancer cell line HT-29 was investigated, so as to provide a new theoretical basis for the pathogenesis of colorectal cancer.

MATERIALS AND METHODS

Cell line

The human colorectal cancer cell line HT-29 was obtained from the Cancer Research Institute, Xiangya School of Medicine, Central South University (Changsha, China). Cells were adherently cultured

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Table 1. Effects of leptin on the growth and proliferation of HT-29 by MTT assay (Optical densities).

Concentration (ng/ml)	Time (h)		
	24	48	72
0	0.20±0.03	0.18±0.05	0.26±0.07
5	0.27±0.03	0.33±0.05	0.37±0.07
50	0.36±0.02	0.40±0.02	0.51±0.04
100	0.54±0.05	0.60±0.02	0.67±0.04
200	0.56±0.05	0.75±0.03	0.82±0.04

P<0.05, compared between any two groups at different concentration and different time.

in RPMI1640 medium (Gibco) supplemented with 10% calf serum (Gibco), 50000 IU/L benzylpenicillin and 50 g/L streptomycin, and were incubated at 37°C in a humidified incubator containing 5% CO₂. Cells were digested and passaged with 0.25% trypsin (Sigma) every three days. Normally, HT-29 was epithelioid and grown adherently in a single layer. Before every experiment, cells were stained by trypan blue to ensure that the rates of living cells were greater than 98%. Cell Quest software was used for the analysis.

Detection of the effect of leptin on the growth of HT-29 by cytometry

Cells were dispersed with trypsin (Sigma). After cell counting, cells were seeded in a 96 well plate at 8×10⁴ cells per well. After cultured for 24 h, cells were treated by increasing concentrations of leptin (0, 5, 50, 100, 200 ng/ml) respectively. Adding just culture solution was the negative control group (0 ng/ml) and adding 100 ng/ml EGF was the positive control group. Then cells were cultured in the incubator for 24, 48, and 72 h. After incubation, cell counting was performed. Cellular growth curve was drawn according to incubation time and cell numbers.

Detection of cellular proliferation by MTT assay

Cells were plated in a 96 well plate at 1×10⁴ cells per well in triplicate for five treatment groups and cultured at 37°C in a humidified incubator containing 5% CO₂ for 24 h. Then cells were treated in the presence of increasing concentrations of leptin (0, 5, 50, 100, 200 ng/ml) for a sequence of time incubating for 24, 48, and 72 h, respectively. Twenty microlitre of 0.5% MTT reagent (Gibco) was added into each well and cells were cultured for additional 4 h. After that, the supernatant fluid was discarded, and the remaining formazan crystals were dissolved by adding 200 µl DMSO (Gibco) into each well. After concussion for 10 min, optical densities at 492 nm were measured using automatic microplate reader. The average results were reported. The growth rates of cells were calculated using the following formula: growth rate = (1 - average value of untreated control well/average value of experimental well) × 100%.

Cell cycle analysis by flow cytometry

Cells were seeded into several culture flasks of 75 ml. Five millilitre RPMI1640 culture solution was added to each culture flask. After incubation for 24 h, cells were treated in the presence of increasing concentrations of leptin (0, 5, 50, 100, 200 ng/ml) and then were

cultured in incubator. After 3 days, cells were harvested by trypsin (Sigma), and washed twice with PBS. Then cells were fixed in 70% ethanol on ice, and stained with 1ml PI staining solution (50 µg/ml PI, 20 µg/ml Rnase and 1.0% TritonX-100)(Sigma). The distribution of each cell cycle phase was analysed by FACSCalibur. The percentage of cells in each phase = cell number of specific phase /total cell number×100%. Meanwhile, proliferative index was also calculated following the formula: proliferative index = cell number of S and G₂/M phases/total cell number × 100%.

Statistical analysis

Measurement data was shown as and statistical analysis was performed using ANOVA for randomized block design. Enumeration data was shown as percentage and statistical analysis was performed using Chi-square test. p < 0.05 was considered to be statistically significant.

RESULTS

Detection of the effect of leptin on the growth of HT-29 by cytometry

After incubation with different concentrations of leptin, cellular growth curve was drawn as shown in Figure 1. The results show that after intervention, cells in the negative control group grew normally, and when the concentrations of leptin were more than 50 ng/ml, cellular growth curve obviously moved up. In the same period, cell number increased along with the growth of the concentrations of leptin from 5 to 200 ng/ml, showing that leptin dose-dependently promoted cell proliferation. However, even when the concentration of leptin increased to 200 ng/ml, cell number was still lower than that in the positive control group treated with EGF.

Detection of cellular proliferation by MTT assay

To further determine the effect of leptin on HT-29 cell growth, MTT assay was performed to further determine cell growth. The results (Tables 1 and 2) were similar to that of cell counting method, that was leptin dose-

Table 2. Effects of leptin on the growth rate of HT-29.

Concentration (ng/ml)	Time (h)		
	24	48	72
0	0	0	0
5	5.1	5.7	6.1
50	10.9	27.0	25.8
100	25.4	36.5	50.0
200	50.0	58.3	67.9

$P < 0.05$, compared between any two groups at different concentration; $P < 0.05$, compared between any two groups at different time except in the 0 and 5 ng/ml group.

Table 3. Effects of leptin on cell cycle and apoptosis of HT-29.

Concentration (ng/ml)	Apoptosis rate (%)	Phase (%)			Proliferative index (%)
		G ₀ /G ₁	S	G ₂ M	
0	2.31±0.56	68.23±2.01	13.52±0.59	18.11±2.93	31.63±2.92
5	2.33±0.40	67.39±1.77	13.94±1.07	18.66±0.78	32.60±1.12
50	2.41±0.35	61.87±0.22	16.83±0.82	21.29±0.15	38.12±0.33
100	2.36±0.45	52.33±2.04	19.45±1.55	28.22±2.05	47.67±2.03
200	2.42±0.31	42.52±1.72	25.40±0.55	32.08±0.62	57.48±0.33

Apoptosis rate: $P > 0.05$, compared between any two groups at different concentration; Phase: $P < 0.05$, compared between any two groups at different concentration; PI: $P < 0.05$, compared between any two groups at different time except in the 0 and 5 ng/ml group.

and time-dependently promoted HT-29 cell growth and proliferation in 72 h.

Cell cycle analysis by FCM

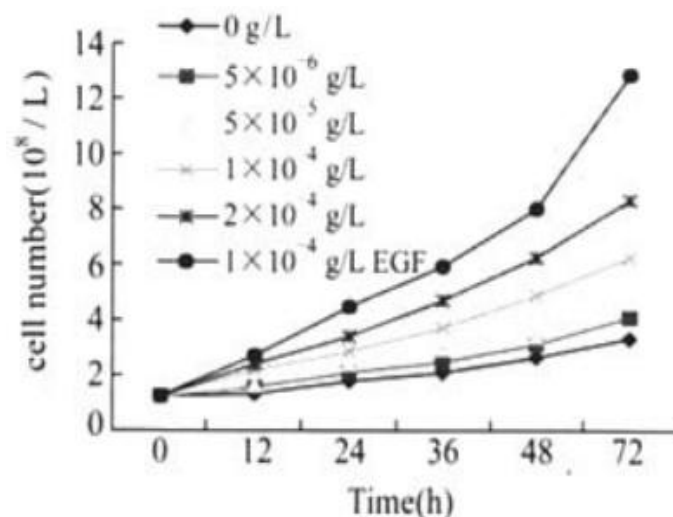
Cell cycle analysis was executed to determine the effect of leptin on cell proliferation and apoptosis of HT-29 (Table 3, Figure 2). The results show that leptin could significantly affect the cell cycle of HT-29. With the increase of the concentration of leptin, compared to the negative control group, the rates of G₀/G₁ phase cells gradually declined, and the rates of S and G₂/M phase cells gradually increased. But no obvious effect of leptin on the apoptosis of HT-29 was observed.

DISCUSSION

In the present study, leptin was observed to affect cell cycle, promote cell growth and proliferation, but had no obvious effect on apoptosis in colorectal cancer cell line HT-29.

The pathogenesis of colorectal cancer is a complex, multi-step and multi-stage process. In addition to genetic factors, certain promoting factors also play an important role (Morikawa et al., 2011; Liu et al., 2011). Several stu-

dies suggested that leptin participated in the developing of certain tumor (Chen et al., 2007; Guo and Gonzalez-Perez, 2011; Zhou et al., 2011; Liu et al., 2011), but the mechanism has not been clearly elucidated. In this study, the effect of leptin on HT-29 was observed through growth

**Figure 1.** The effect of leptin on proliferation of HT-29 by growth curve method.

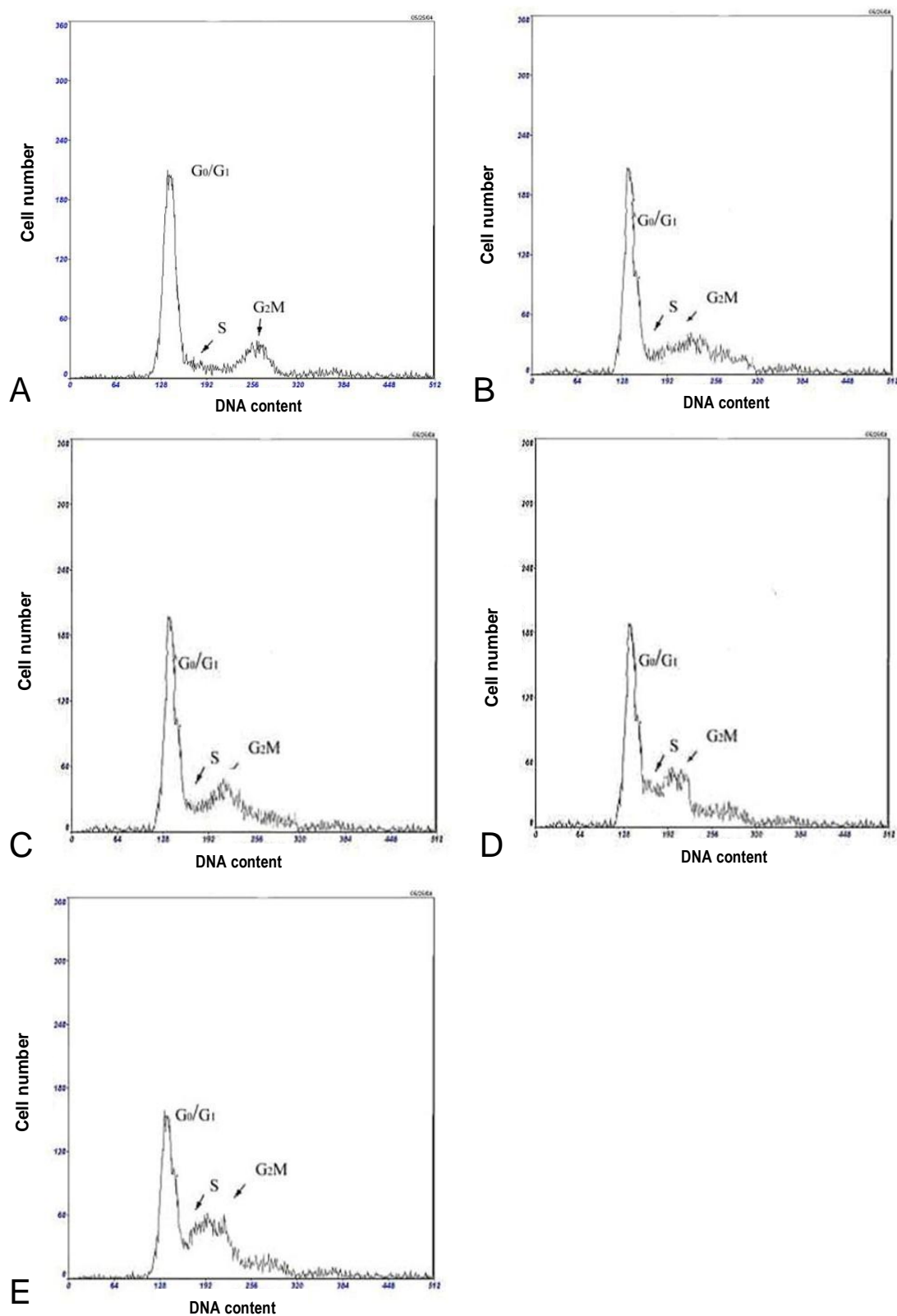


Figure 2. The effect of leptin on cell cycle of HT-29 by flow cytometry. (A) Cells were treated with 0 ng/ml leptin; (B) cells were treated with 5 ng/ml leptin; (C) cells were treated with 50 ng/ml leptin; (D) cells were treated with 100 ng/ml leptin; (E) cells were treated with 200 ng/ml leptin.

curve method, MTT assay and FCM. Growth curve method showed that with the increasing concentration of leptin ranging from 5 to 200 ng/ml, compared to the negative control group leptin promoted HT-29 cell proliferation in the dose- and time-dependent manner. Moreover, the proliferative effect was the highest as cells were treated with 200 ng/ml leptin.

The relationship between EGF and a variety of tumor development has been investigated. On one hand, EGF stimulates cellular growth in a single layer conversing to multi-layer, that is the growth characteristics of certain tumor cells. On the other hand, EGF receptor shares a common amino acid sequence segment with the product of oncogene c-erb-B. Our results show that 100 ng/ml EGF promoted HT-29 cell proliferation in positive control group more than 200 ng/ml leptin, and the difference was statistically significant. The proliferative effect of leptin on HT-29 was obvious, but was still lower than that of EGF.

The cell density in our MTT assay was $5 \times 10^7/L$, serum concentration in culture medium was 10%, and negative control was also set up. MTT method is mainly used to detect cell viability firstly. Later, it is widely applied in the detection of proliferation of cancer cells (Kang et al., 2002). It is simple, fast and accurate, needing less cell number, and without radiation pollution. The results show that leptin had a strong promoting effect on HT-29 cells in a dose- and time-dependent manner. The lowest growth rate of HT-29 cells was 5.1%, and the highest rate was 67.9%. FCM is applied in the detection of cell cycle and apoptosis, with the advantages of quick, comprehensive analysis, accuracy and reliability. Cell cycle can be divided into interphase and mitosis (M phase). Interphase includes G1, S, and G2 phase, among which S phase can reflect the activity of cell proliferation, as in this period DNA synthesis exists strongly. Flow cytometry was used in our experiment to detect the effect of leptin on cell cycle of HT-29 cells.

The results were presented that with the increase of the concentration of leptin, the rates of G0/G1 phase cells gradually declined, and the rates of S and G2/M phase cells gradually increased, suggesting that leptin has obvious growth promoting effect in a dose-dependent manner. DNA fragmentation is one of the main biochemical changes in apoptotic cells, because restriction endonuclease reaction is an assigned procedure.

Detection the exits of sub-G1 phase could indirectly reflect the exist and the extent of apoptosis (Wu et al., 2010). FCM can commendably reflect the status of cell apoptosis according to DNA content. Our data showed that with the increase of concentration, leptin has no obvious promoting or inhibiting effect on HT-29 cells, which was similar with the previous results in esophageal cancer cell lines (Somasundar et al., 2003).

In conclusion, in the same period, with the increase of leptin concentration, the amount of HT-29 cells increased,

the growth rates increased, the rates of S and G2/M phase cells also increased, suggesting that leptin promotes the proliferation of colorectal cancer cells in a dose- and time-dependent manner in vitro, and there is no relationship between leptin and apoptosis of colorectal cancer cells.

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