

Full Length Research Paper

Leptin decreases the sensitivity of human colon cancer cell HT-29 to 5-fluorouracil

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To verify whether leptin attenuated the sensitivity of colon cancer cells to 5-fluorouracil (5-FU). HT-29 cells were treated with 5-FU in the absence or presence of various concentrations of leptin. The cytoprotective effect of leptin was detected by MTT and flow cytometer. The expression of caspase-9 was measured by RT-PCR and Western blot analysis. Leptin attenuated the cytotoxic response of HT-29 cells to 5-FU by facilitating cells survival and antagonizing apoptosis. In addition, leptin decreased the expression of caspase-9 in HT-29 cells induced by 5-FU. Leptin treatment reduces the susceptibility of colon cancer cells to 5-FU by promoting cell survival. Attenuation of caspase-9 expression is one of the mechanisms underlying the cytoprotective role of leptin in HT-29 cells.

Key words: Leptin, apoptosis, caspase-9, drug resistance, 5-fluorouracil.

INTRODUCTION

Colon cancer is one of the leading causes of cancer death worldwide and its incidence continue rising. Chemotherapy plays an important role in anti-cancer treatment. Although various novel therapeutic approaches have been introduced, 5-fluorouracil (5-FU) is still a widely used agent in the first-line drugs for the colon cancer treatment (Yoshikawa et al., 2001). However, the development of drug resistance is a common problem encountered during the chemotherapy. Therefore, much interest has recently been devoted to enhance the sensitivity of 5-FU to colon cancer cells (Hayashi et al., 2004). The response of tumor cells to chemotherapeutic agents is not only determined by intrinsic properties of the tumor cells themselves, but is regulated by a number of growth factors (Stoika et al., 2003; Song et al., 2000; Zhang et al., 2001; Stoika and Yakymovych et al., 2003; Hayashi and Hideshima et al., 2004; John et al., 2005; Kwak, 2005; Moroni et al., 2005). Leptin is a 16-kDa circulating hormone produced primarily by adipose tissue (Zhang et al., 1994). After being released into the blood stream, leptin reaches its specific receptors (Ob-R) and

exerts its action. In gut, leptin participates in several physiological functions such as mucosal growth and nutrient and intestinal absorption (Morton et al., 1998). In addition, leptin appears to exert its action on colon carcinogenesis (Liu et al., 2001). Recent studies have confirmed that leptin is a new growth factor, which significantly enhances the cell proliferation (Genini et al., 2000; Hardwick, 2001; Hu et al., 2002; Tseng et al., 2003; Kaushal et al., 2004; Noordhuis, 2004; Rouet-Benzineb et al., 2004; Russo et al., 2004; Somasundar et al., 2004). Furthermore, this effect is in concert with epidermal growth factor (EGF), a known growth factor in HT-29 cells (Hardwick, 2001). It has been proved that the activation of the mitochondrial pathway involves the release of cytochrome *c* which associates with Apaf1 and procaspase 9 in the presence of dATP, leading to activation of caspase 9.

Subsequently, activated caspase 9 cleaves downstream caspases and ultimately leads to apoptosis (Rouet-Benzineb et al., 2004; Russo et al., 2004). However, whether caspase-9 is involved in lowering the sensitivity of 5-FU to leptin treatment remains unknown. In the present study, we investigated the role of leptin in retarding the apoptotic response of HT-29 cells to 5-FU. Our data indicated that leptin promotes colon cancer cell survival via suppressing caspase-9 expression.

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METHODS

Cell culture

HT-29 cells were purchased from Shanghai Cellular Research Institute (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% calf serum (Gibco) at 37°C in 5% CO₂ incubation.

MTT assay

The cytotoxicity of 5-FU (the 50% cell inhibition concentration, IC₅₀) was determined by colorimetric MTT assay. Cell survival rate was determined by measuring the absorbance of the cell suspension at 570 nm. The IC₅₀ value of 5-FU was determined as 9.6 µg/ml by MTT assay as described earlier. To clarify the correlation between leptin and the sensitivity of the colon cancer cells to 5-FU, the IC₅₀ values of the cells treated with 5-FU alone were compared with the cells treated with 5-FU combined in the presence of leptin (recombinant human leptin, U.S. Biological) at various concentrations (5, 50 and 500 ng/ml).

Flow cytometer analysis

HT-29 cells were treated with 5-FU (9.6 µg/ml) in the absence or presence of leptin (at 5, 50 or 500 ng/ml) for 48 h. The distribution of cells in the cell cycle was measured by flow cytometer.

RT-PCR

HT-29 cells were seeded in 6-well plates (2~4 × 10⁵ cells/well), then exposed to 5-FU and leptin as described earlier. Total RNA was isolated from cells by Trizol reagent. cDNA was synthesized from 1 µg total RNA using oligo (dT) primer and murine leukemia virus reverse transcriptase (Promega), followed by PCR using the Taq polymerase. The primer sequences were as follows:

Caspase-9: 5'-TAACAGGCAAGCAGGAAA-3' (sense);

5'-TCTTGGCAGTCAGGTCGC-3' (antisense);

β-actin: 5'-GGAGTCCTGTGGCATCCACG-3' (sense);

5'-CTAGAAGCATTGCGGTGGA-3' (antisense).

A 353-bp fragment of the caspase-9 gene was generated from the PCR analysis. β-actin was also amplified as a 320-bp product.

Western blot

HT-29 cells were harvested and lysed with lysis buffer (50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 1 mM PMSF and 10 µg/ml leupeptin) at 4°C for 1 h. 50 µg protein was separated by 12% SDS/PAGE and transferred to nitrocellulose membranes. Membranes were blocked with non-fat milk in TBST (20 mM Tris-HCl buffer, pH 7.6, 140 mM NaCl and 0.5% Tween 20), and then incubated with cleaved caspase-9 antibody (New England Biolabs) for 1 h. The membranes were washed three times with TBST, incubated with peroxidase conjugated secondary antibody (goat anti-rabbit, Santa Cruz Biotechnology) for 1 h and sufficiently washed with TBST. Bands corresponding to cleaved caspase-9 were detected by enhanced chemiluminescence (ECL kit, Amersham Life Science).

Statistical analysis

Values were presented as means±SE, 3 different experiments were performed. Data were analyzed by one-way ANOVA assay.

Differences with *P*<0.05 were considered significant.

RESULTS

Leptin treatment reduced the susceptibility of colon cancer cells to 5-FU

The IC₅₀ value of 5-FU alone was 9.6±0.6 µg/ml. In the presence of leptin (at 5, 50 and 500 ng/ml), IC₅₀ values of 5-FU were determined as 9.8±0.6, 11.6±1.0 and 23.6±1.9 µg/ml, respectively. These data indicated that leptin treatment (500 ng/ml) induced 2.4 folds increase of the IC₅₀ (*P*<0.01 versus 5-FU).

Leptin treatment reverses the inhibitory effect of 5-FU on HT-29 cell growth

Our data indicated that leptin stimulation led to increase the viability of 5-FU treated HT-29 cells with dose-dependent manner, in which the cell survival was completely recovered with 500 ng/ml leptin. In addition, the time course study indicated that cell proliferation was increased markedly with leptin treatment for 48 h (Figure 1). Accordingly, 48 h was then selected as an optimal exposure time point for the subsequent studies, which was also consistent with the previous report (Russo et al., 2004).

Leptin suppresses 5-FU induced cell cycle arrest and apoptosis

Flow cytometer data (Table 1) indicated that HT-29 cells treated with 5-FU showed a higher population in S phase and lower in G2/M phase compared with control cells (44.57±2.49 vs. 35.53±2.75; 2.07±0.21 vs. 5.73±0.55). Of note, there is a significantly elevated sub-G1 peak in the cells treated with 5-FU compared with the control, indicating that 5-FU induces apoptosis in HT-29 cells. Interestingly, leptin blocked 5-FU-induced cell cycle arrest and improved population of the cells in G2/M phase in a dose-dependent manner. Additionally, 5-FU-induced apoptosis was significantly decreased by 56.9% in the present of leptin (500 ng/ml) (*P*<0.01 vs. 5-FU).

The cytoprotective role of leptin in preventing ht-29 cells from 5-fu-induced cell death is via suppressing caspase-9 expression

Cells were exposed to 5-FU in the absence or presence of leptin. After 48 h, cells were harvested and stained with propidium iodide. The data were expressed in percentage of the cell population in the different phase of cell cycle and apoptosis. Results were expressed as the means±SD of three independent experiments **P*<0.05, ***P*<0.01,

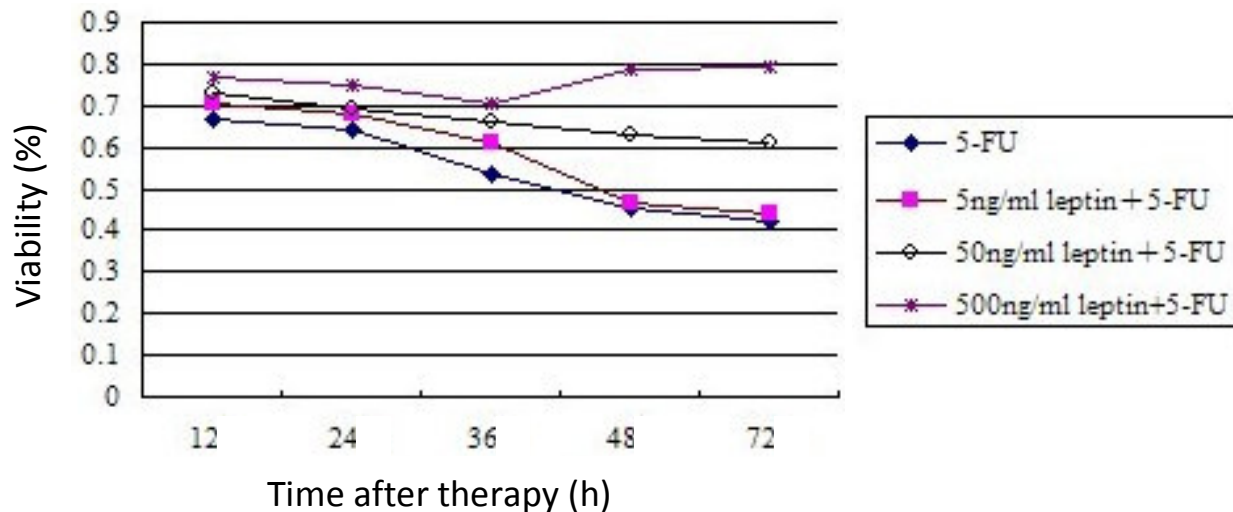


Figure 1. Cell growth curves determined by MTT.

Table 1. Flow cytometer analysis on cell cycle and apoptosis.

Group	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)	Apoptosis (%)
Control	58.73±2.80	35.53±2.75	5.73±0.55	1.47±0.15
5-FU	50.77±6.21	44.57±2.49	2.07±0.21	18.47±1.58
5-FU + 5 ng/ml leptin	55.40±2.65	42.00±3.00	2.60±0.26	17.20±1.76
5-FU + 50 ng/ml leptin	47.00±8.11	49.00±8.06	4.07±0.25*	9.43±0.83**
5-FU + 500 ng/ml leptin	51.73±4.96	40.03±6.37	8.23±1.46**	8.13±0.80**

versus 5-FU alone. RT-PCR analysis revealed that caspase-9 expression was increased 3 folds in the cells treated with 5-FU compared with the untreated control cells. Leptin treatment led to a significant decrease of caspase-9 expression induced by 5-FU ($P < 0.05$ vs 5-FU) (Figure 2). With a specific antibody for cleaved (activated) caspase-9, we detected the activation of caspase-9. Our data indicated that 5-FU treatment induced cleaved caspase-9 expression, which was partially blocked by the stimulation of leptin (Figure 3). The level of cleaved caspase-9 was reduced to 43.9% in the cells exposed to leptin (500 ng/ml) compared to the cells with 5-FU treatment alone. These results suggest that attenuation of caspase-9 activation is involved in leptin-induced cytoprotective effect on HT-29 cells.

DISCUSSION

5-FU is an important anti-metabolite cytotoxic drug that blocks DNA synthesis by inhibiting thymidylate synthetase and RNA incorporation (Noordhuis, 2004). In addition, 5-FU also has been reported to induce cell cycle arrest and apoptosis (Nita et al., 1998). However, cancer cells resistance to 5-FU is a major cause for the failure of chemotherapy (Paula and Angelis, 2004). Clinical study

has shown that a response rate of colorectal cancer patients with 5-FU treatment was only 20% (Schmoll HJ). This low efficacy of 5-FU is a big limitation for the colorectal cancer chemotherapy. Previous study has suggested that leptin is involved in the resistance to cisplatin of leukemic cells (Efferth et al., 2000). Herein, we investigated whether the cytotoxic response to 5-FU of colon cancer cells could be mediated by leptin. It has been shown that HT-29 cells express functional leptin receptors (Ob-Rb) on cell membranes (Rouet-Benzineb et al., 2004). Our present study indicated that leptin treatment results in HT-29 cells resistant to HT-29-induced cell death, suggesting that leptin blocks 5-FU toxicity in HT-29 cells via promoting cell survival. Recent studies have shown that leptin is a new positive factor for colon cancer via enhancing the proliferation of cells, promoting motility and invasiveness (Jenifer et al., 2008; Jaffe et al., 2008). However, the impact of leptin on chemotherapy sensitivity is still unclear. The present study has demonstrated that leptin enhanced cells viability, leading to prevent the colon cancer cells from 5-FU-induced cell death. Our report indicated for the first time that leptin may reverse the inhibitory action of 5-FU on colon cancer growth. In addition, our results also showed that 5-FU induced a cell-cycle delay in phase S, which is in agreement with other report (Tseng et al., 2003).

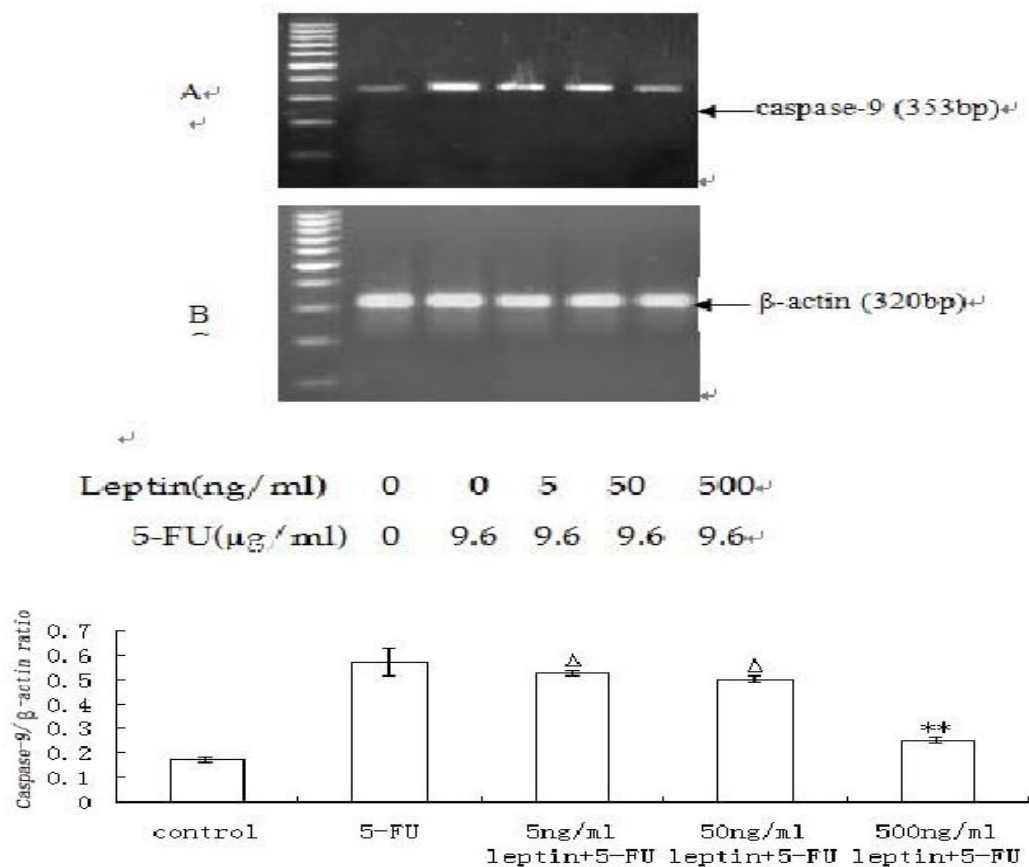


Figure 2. RT-PCR analysis for ratio of caspase-9 to β -actin $\Delta P < 0.05$, $**P < 0.01$ VS 5-FU.

Furthermore, leptin accelerated cell mitosis via shifting cell population into G_2/M phase, suggesting that leptin is a potent mitogen and anti-apoptotic cytokine for HT-29 cells. Collectively, enhancement of proliferation and inhibition of apoptosis mediated by leptin appear to be primary mechanisms accounting for the decreased sensitivity of colon cancer cells to 5-FU. Since most chemotherapeutic agents triggers tumor cell death via inducing apoptosis, it is likely that dysfunction of genes or signaling events that regulate apoptosis pathway can prevent cancer cells from drug-induced cell death (Inoue et al., 2001; Yu et al., 2005). PI3K/PKB (phosphatidylinositol-3-kinase/PKB) is a key anti-apoptotic and survival pathway that promotes cell survival in many cell types and play a central role in mediating resistance to chemotherapy (Inoue et al., 2001; Hofelder et al., 2005; Yu et al., 2005; JaffeSchwartz, 2008). Furthermore, PI3K/PKB appears to be a key element in the leptin-induced enhancement of cell number, a process potentially involving both enhanced cell proliferation and reduced apoptosis (Russo et al., 2004).

Caspase-9, one of substrates of PKB is considered to be an apoptosis initiator and served to sensitize tumor cells to cytotoxic therapy after activation (Herr et al.,

2003). Caspase-9 is an important member of the cysteine aspartic acid (caspase) family. Apoptotic stimulation leads to formation of the apoptosome (complex of Apaf-1, cytochrome c, dATP and procaspase-9) (Genini et al., 2000). This complex processes procaspase-9 into a large active subunit by self-cleavage. Cleaved and activated caspase-9 thereby triggers activation of downstream effector caspase and ultimately leads to apoptosis. Previous reports have shown that inhibition of caspase-9 leads to apoptosis resistance and drug resistance (Kaushal et al., 2004; Noordhuis, 2004). Activated PKB directly inhibits caspase-9 cleavage to impair drug induced apoptosis and cytotoxicity (Kim et al., 2004; Messina et al., 2004). A principal objective of this study was to determine whether caspase-9 was involved in leptin-mediated down-regulation of HT-29 cell sensitivity to 5-FU treatment. To this end, we detected the decrease of caspase-9 expression, including cleaved caspase-9 (the active form of caspase-9). This result suggests that caspase-9 dependent apoptosis signal pathway mediates 5-FU-triggered cell death in HT-29 cells. Leptin-induced resistant to HT-29 cell death is mainly via a mechanism by which leptin decreases caspase-9 expression and activation, inhibiting leptin-stimulated caspase-9 activation

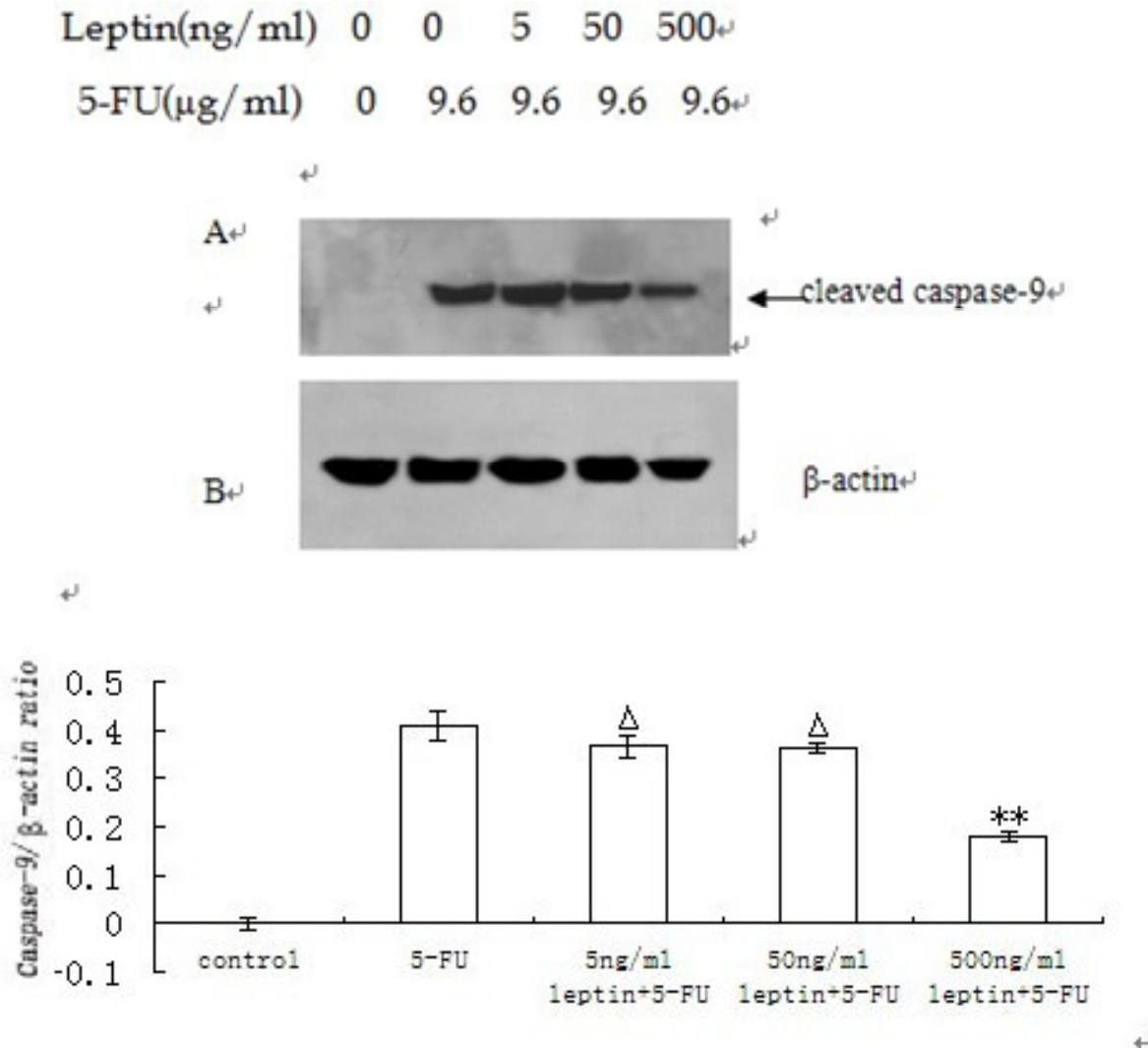


Figure 3. Western blot analysis for ratio of cleaved caspase-9 to β -actin; $\Delta P < 0.05$, $**P < 0.01$ VS 5-FU.

appears to be an approach for preventing leptin-induced cancer cell resistance to 5-FU treatment. In conclusion, this study demonstrates that leptin decreases the susceptibility of colon cancer cells to 5-FU by acting as a survival factor. Although a precise molecular target of leptin is incompletely understood, the results reported here reveal that attenuation of caspase-9 expression and activation is a mechanism underlying the inhibitory role of leptin in HT-29 cell death. Recent studies have demonstrated that hyperleptinemia or multiple signaling cascades associated with leptin-induced cell proliferation can be suppressed during colon carcinogenesis (Fenton et al., 2008; Miyamoto et al., 2008).

Further study should be done to investigate that whether blocking leptin-suppressed caspase-9 activation and expression can enhance the sensitivity of colon cancer cells to 5-FU and promote survival time for patients.

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