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World J Gastroenterol 2008 May 14; 14(18): 2900-2904
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Primary study of leptin and human hepatocellular carcinoma *in vitro*

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Received: January 9, 2008 Revised: March 21, 2008

Abstract

AIM: To investigate the expression level and effects of leptin in human hepatocellular carcinoma cells *in vitro* and to explore the correlation between them.

METHODS: Human hepatocellular carcinoma cell line HepG2 was cultured *in vitro*, and (the expression level) mRNA of leptin and leptin receptors in HepG2 were assessed using reverse transcription polymerase chain reaction (RT-PCR). Effects of different concentrations of leptin (50 ng/mL, 100 ng/mL, 200 ng/mL) on HepG2 were detected with colorimetric assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after incubation periods of 24 h, 48 h, and 72 h. Flow cytometry was performed to assess cell cycle progression of different concentrations of leptin as stated above after each 24 h incubation period.

RESULTS: mRNA of leptin and leptin receptors (including short and long isoforms) were expressed in HepG2. The 72 h incubation of leptin at different concentrations (50 ng/mL, 100 ng/mL, 200 ng/mL) promoted proliferation of HepG2 in a concentration- and time-dependent manner. The experimental group shows significant statistical differences when compared to the controlled group which contained 0 ng/mL of leptin. As the concentration of leptin increases, significant fewer cells were detected in G_0-G_1 phase and more cells in S and G2-M phases.

CONCLUSION: Leptin and leptin receptor are simultaneously expressed in human hepatocellular carcinoma cell line HepG2. Addition of leptin (0 ng/mL-200 ng/mL) in 72 h periods indicated there is a concentration- and time-dependent correlation in the stimulation of HepG2 cell proliferation. The effect of proliferation by leptin is due to promotion of DNA synthesis and enhancement of mitotic activity. The relationship between leptin and human hepatocellular carcinoma cells might indicate that adipokine could be associated with the progression of human hepatocellular carcinoma.

Key words: Leptin; Hepatocellular carcinoma; Proliferation

Peer reviewers: Frank J Burczynski, Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba, R3T 2N2, Canada; Yukihiro Shimizu, Dr, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan; Byung Chul Yoo, Professor, Dr, Division of Gastroenterology, Samsung Medical Center, SungKyunKwan University School of Medicine, Seoul 135-710, Korea

Zhou J, Lei W, Shen L, Luo HS, Shen ZX. Primary study of leptin and human hepatocellular carcinoma *in vitro*. *World J Gastroenterol* 2008; 14(18): 2900-2904 Available from: URL: http://www.wjgnet.com/1007-9327/14/2900.asp DOI: http://dx.doi.org/10.3748/wjg.14.2900

INTRODUCTION

Leptin, a protein product encoded by the obese gene (ob gene)[1], is primarily derived from adipocytes which plays an important role in the regulation of food intake and the control of body weight[2]. It takes part in various physical conditions such as lipid metabolism, immune defense, neuroendocrine regulation, pituitary hormone secretion, and pubertal development[3,4]. In rodents and humans, leptin is related with many pathological syndromes including obesity, hyperphagia, hyperinsulinemia, reduced fertility, and cholelithiasis (including gallstone and hepatolithiasis)[5-8]. Studies have shown that individuals who are obese have increased risk for most cancers compared to individuals who are not obese[9]. Since leptin is closely associated with obesity, it may be the bridge conjoining obesity and cancer.

The biological functions of leptin on target cells and tissues are carried out through interaction with its specific receptors (ob-R) which belong to class I cytokine receptor

family[10]. In rodents and humans, two leptin receptor isoforms predominate: the short leptin receptor isoform (ob-Ra) and the long leptin receptor isoform (ob-Rb)[11,12]. They share the same extracellular domain, but they differ in the length of the transmembrane/cytoplasmic coding regions[13]. The physiologic significance of each isoform in relationship to obesity and cancer is still unknown.

Hepatocellular carcinoma (HCC) is one of the most malignant tumors in the world. It causes proximately one hundred and ten thousand deaths annually in China. HCC is extremely difficult to detect in prognosis due to its early metastasis and distant transmission. It has been reported that obesity was related to HCC complicated with cryptogenic cirrhosis and alcoholic liver disease[14,15]. Therefore, we presume that leptin might be associated with HCC. In order to investigate the relationship between leptin and HCC, (the expression level) mRNA of leptin and its receptors in human HCC cell line HepG2 were detected by reverse transcription polymerase chain reaction (RT-PCR). In addition, the effects of leptin on HepG2 *in vitro* were also discussed in this study.

MATERIALS AND METHODS

Materials

Human hepatocellular carcinoma cell line HepG2 was obtained from Shanghai Cell Biology Institute of Chinese Academy of Sciences. Human recombinant leptin was the product from Sigma (St. Louis, Missouri, USA). Roswell Park Memorial Institute-1640 (RPMI-1640), fetal bovine serum (FBS) and Trizol reagent were the products of Invitrogen (Carlsbad, California, USA). Trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethylene diamine tetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Amresco (Cleveland, Ohio, USA). Revert aid first strand cDNA synthesis kit and TaqDNA polymerase were the products of Fermentas (Burlington, Iowa, USA). All other reagents unless indicated were from Sigma (St. Louis, Missouri, USA).

Methods

Cell culture: HepG2 were grown as a monolayer in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 kU/L penicillin, and 0.1 g/L streptomycin. They were cultured in T-75 cm^2 culture flasks and maintained at 37℃ in 5% CO_2 humidified atmosphere. At the beginning of the experiment, cells in the exponential growth phase were detached from the flask with 0.25% trypsin and 0.02% EDTA solution.

RT-PCR: mRNA of leptin, ob-Ra and ob-Rb in cell line HepG2 were assessed using RT-PCR as described previously[16]. Total RNA was isolated from confluent cells using Trizol reagent according to the manufacture's instructions. The concentration of RNA was quantitated by absorbance change at 260 nm and 280 nm. Total RNA was suspended in DEPC-treated water and stored at -80℃.

Single-stranded cDNA was synthesized from 1 μg of RNA using revert aid first strand cDNA synthesis kit with random hexamer primer. The housekeeping gene β-actin was amplified to set a control for RNA loading and also to minimize variations in cDNA synthesis to ensure efficiency. Primer sequences for β-actin, leptin, ob-Ra and ob-Rb have been reported in the previous paper[17]. Following are the primers for: 1) β-actin: sense strand is 5'-ACCCACACTGTGCCCATCTA-3' and anti-sense strand is 5'-CGGAACCGCTCATTGCC-3' (encodes a 289-bp fragment); 2) leptin: sense strand is 5'-GTGCGGATTCTTGTGGCTTT-3' and anti-sense strand is 5'-GGAATGAAGTCCAAACCGGTG-3' (encodes a 174-bp fragment); 3) ob-Ra: sense strand is 5'-TTGTGCCAGTAATTATTTCCTCTT-3' and anti-sense strand is 5'-AGTTGGCACATTGGGTTCAT-3' (encodes a 200-bp fragment); 4) ob-Rb: the sense strand is 5'-TTGTGCCAGTAATTATTTCCTCTT-3' and antisense strand is 5'-CTGATCAGCGTGGCGTATTT-3' (encodes a 439-bp fragment).

Amplification of the resulting cDNA sequence was carried out using polymerase chain reaction (PCR). 3 μL cDNA was combined with 25 pmol oligonucleotide primers specific for β-actin, leptin, ob-Ra, ob-Rb and TaqDNA polymerase 1 U, PCR buffer (5 μL), 25 mmol/L $MgCl_2$ (4 μL), 10 mmol/L dNTP mixture (1 μL), and ddH_2O (34 μL in a 50 μL solution). PCR was performed in a thermal cycler (Gene Amp PCR system 2400, Perkin-Elmer Corp., Uberlingen, Germany). The condition for the reaction were the following: 1 min at 94℃ (predenaturation), followed by 30 cycles of 1 min (denaturation) at 52℃ (for β-actin), 55℃ (for leptin) or 50℃ (for ob-Ra and ob-Rb); 1 min at 72℃ (annealing), and then 5 min at 72℃ (extension). PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide, and the length were estimated to be 100-bp. Imaging was performed on JS-380 instrument (Peiqing Inc., Shanghai, China).

MTT for cell proliferation: MTT colorimetric assay was used to detect the effects of leptin on proliferation of cell line HepG2. Cells were washed extensively with filtered and sterilized phosphate-buffered saline (PBS) to remove the dead cells. HepG2 cells were suspended at a concentration of 1.0×10^4/mL, and then they were seeded into 96-well microplate at 150 μL/well and incubated to adhere overnight. RPMI-1640 medium containing different concentrations of human recombinant leptin were added in 0 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL to HepG2 cells with each concentration account for 6 wells. MTT assay was performed after incubation periods of 24 h, 48 h, and 72 h. MTT 20 μL (5 mg/mL) stock solution in PBS was added to each well. The microplate was incubated for 4 h and 100 μL DMSO was added (to each well). Optical density (*A*) value was measured by an ELISA plate reader (Digiscan Lab instruments, Austria) at wavelength of 492 nm. Each variant group was performed in triplicate wells for measurement accuracy.

Flow cytometric analysis: HepG2 cells in the exponential growth phase were detached by 0.25% trypsin and 0.02% EDTA. The cell suspension was then seeded uniformly

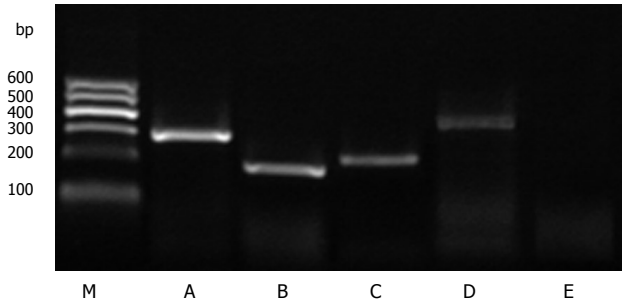

Figure 1 Expression of β-actin, leptin, ob-Ra and ob-Rb in HepG2. M: DNA marker; A: β-actin 289 bp; B: Leptin 174 bp; C: Ob-Ra 200 bp; D: Ob-Rb 439 bp; E: Negative control.

into four T-75 cm^2 culture flasks and incubated to adhere overnight. RPMI-1640 medium containing different concentrations of human recombinant leptin were added in 0 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL to HepG2 cells. Flow cytometric analysis was performed after incubated for 24 h. The cells were briefly washed twice with PBS, fixed in ice-cold ethanol (70% vol/vol in ddH_2O) and stained with propidium iodide (PI) solution (25 μg/mL PI, 180 U/ml RNase, 0.15% Triton X-100, and 30 mg/mL polyethylene glycol in 4 mmol/L critrate buffer w/pH = 7.8). DNA contents were detected using a FACScan flow cytometer (Becton Dickinson Co., San Jose, CA). The relative percentages of each phase of the cell cycle were analyzed using Cell Quest Software (Becton Dickinson Co., San Jose, CA). For the measurement accuracy, each variant group was tested in triplicate.

Statistical analysis

The data were presented as mean ± SD. ANOVA was used to analysis the results. $P < 0.05$ were considered to indicate statistically significant differences. Entire data were analyzed with the statistical software SPSS 11.5.

RESULTS

Leptin, ob-Ra and ob-Rb mRNA assay by RT-PCR

Total RNA extracted from HepG2 cells were reversely transcribed to check whether mRNA of leptin, ob-Ra and ob-Rb were expressed. RT-PCR detection with β-actin primers revealed a 289-bp fragment in HepG2. The 174-bp cDNA band indicated that the cells expressed leptin mRNA. PCR products for both the short isoform (ob-Ra, 200-bp) and the long isoform (ob-Rb, 439-bp) of leptin receptor were also detectable. No fragment was detected in the negative control that PCR product without the cDNA (Figure 1).

Effects of proliferation of leptin on HepG2

MTT assay indicated the cell proliferate conditions of HepG2. As shown in Table 1, leptin significantly stimulated HepG2 cells growth in a concentration- and time-dependent manner. Leptin caused significant growth potency on HepG2 within 72 h. There were significant statistical differences between concentration groups of 0 ng/mL *vs* 50 ng/mL ($P < 0.01$), 0 ng/mL *vs* 100 ng/mL ($P < 0.01$), 0 ng/mL *vs* 200 ng/mL ($P < 0.01$), 50 ng/mL *vs* 100 ng/mL ($P < 0.05$) and 50 ng/mL *vs* 200 ng/mL ($P < 0.01$). Meanwhile, significant statistical differences also existed between duration groups of 24 h *vs* 48 h ($P < 0.01$) and 24 h *vs* 72 h ($P < 0.01$).

Table 1 Effect of leptin on proliferation of HepG2

Concentration (ng/mL)	A		
	24 h	48 h[d]	72 h[d]
0	0.29 ± 0.01	0.34 ± 0.01	0.37 ± 0.01
50[b]	0.34 ± 0.02	0.42 ± 0.01	0.54 ± 0.02
100[b]	0.41 ± 0.03	0.53 ± 0.05	0.62 ± 0.02
200[b]	0.46 ± 0.04	0.63 ± 0.01	0.73 ± 0.03

[b]$P < 0.01$ *vs* group of 0 ng/mL leptin; [d]$P < 0.01$ *vs* group of incubation 24 h.

Table 2 Effect of leptin on cell cycle of HepG2

Concentration (ng/mL)	Cell phase (%)		
	G_0-G_1	S	G_2-M
0	61.8 ± 2.5	36.5 ± 1.0	1.6 ± 0.3
50	56.9 ± 2.2	38.5 ± 0.9	4.7 ± 1.4
100	50.2 ± 2.6[a]	40.3 ± 1.2	9.4 ± 2.1[a]
200	43.6 ± 2.7[b]	42.0 ± 0.3[a]	14.4 ± 2.5[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* group of 0 ng/mL leptin.

Effects of leptin on cell cycle progressions of HepG2

The effects of leptin on cell cycle progressions of HepG2 as determined by flow cytometry analysis are shown in Table 2. In the 24 h frame, the results indicate that as the concentration of leptin increases (e.g. 50 ng/mL, 100 ng/mL and 200 ng/mL), the proportion of the HepG2 cells in G_0-G_1 phase gradually reduces and the number of cells in S and G_2-M phases gradually increases. In comparison with control group, the data indicates a significant reduction of cells in G_0-G_1 phase ($P < 0.01$) and a significant increase of cells in S phase ($P < 0.01$) and G_2-M phase ($P < 0.01$) in response to treatment of leptin.

DISSUSION

The relationship between obesity and cancer has been excessively documented for cancers or adenocarcinoma in endometrium, breast, prostate, renal cells, pancreas, colon, and esophagus[18,19]. Epidemiological observations revealed that obesity was a risk factor of HCC complicated with cryptogenic cirrhosis and alcoholic liver disease[14,15], and in addition, clinical investigations identified that the leptin level of serum had increased significantly in alcoholic and post-hepatitis liver cirrhosis patients with or without HCC as compared to control subjects w/o the complication[20-24]. Still, there were few studies on the involvement of leptin in HCC.

It has been reported that leptin participates in an auto/paracrine manner in the pituitary gland, and it plays regulations roles in the pig[25]. One study reported that the proteins of leptin and ob-R had been detected in 72.22% and 30.56% of HCC, respectively[26]. Present study

indicated that the mRNA of leptin, and as well as the short and the long leptin receptor isoforms were all expressed in HepG2. These findings suggested that leptin might act as an auto/paracrine growth factor towards hepatocytes, and together with its receptors, they could play role in HCC initiation and progression.

Serum leptin level fluctuations are also detected in some non-physiological conditions. For example, leptin levels up to 400 ng/mL have been reported in children with chronic renal failure[27]. When obese, but otherwise healthy subjects treated with leptin (1 mg/kg per day), serum leptin levels rose up to 736 ng/mL[28]. However, serum leptin level in normal physiological situation is less than 11.4 ng/mL. Thus, a relatively higher concentration of leptin (200 ng/mL) was used in our study. There are some divaricate results among the studies of leptin and cancer. Leptin not only stimulated proliferation of some human cancer cell lines, including breast cancer cell lines (ZR75-1, MCF-7), esophageal cancer cell lines (KYSE 410) and prostate cancer cell lines (PC-3, DU 145), but also it inhibited the growth of other human cancer cell lines, such as pancreatic cancer cell lines (Mia-Paca, PANC-1)[18]. Data regarding leptin's effect on HCC cells are rarely reported, and they appear to be contradictory. In one *in vitro* study, leptin was found to have little effect on the proliferation of liver cancer cell line SMMC-7721[26], while in another study, leptin had shown anti-tumor activity[29]. In our *in vitro* study, exogenous leptin had effects on the proliferation of HCC cell line HepG2 in a concentration- and time-dependent manner. We might conclude that human cancer cell lines exhibit differential responses to the leptin treatments, depending upon the biological characteristics of the cancer cells and the organ of derivation of the cell lines. Because our study was to interfere HCC cell line with exogenous leptin *in vitro*, results may differ in concentration threshold and activation initiation time with *in vivo* HCC cells in tumor tissues with endogenous leptin. Therefore, it will be necessary to continue the research on leptin's effects on HCC with targeted animal models.

By adopting the techniques of flow cytometry, we were able to significantly expand the analysis of cell cycle progression. With respect to the cell cycle, we were able to accurately determine the relative proportion of cells in each phase of the cycle progression. From our data, it was evident that as the concentration of exogenous leptin increased from 0 to 200 ng/mL, it gradually reduced the relative proportion of the HepG2 cells in G_0-G_1 phase and gradually increased the number of the cells in S and G_2-M phases. Since the S phase of the cell cycle is the synthetic phase of DNA, and G_2-M phase represents a later synthetic phase of DNA and the splitting of the cells through mitotic phase, data from our study demonstrate that leptin increase cell proliferation of HepG2 by promoting of DNA synthesis and enhancing mitotic activity.

It has been reported that leptin replacement is a very promising therapeutic approach for managing the complications of lipodystrophy. In addition, leptin may have therapeutic potential in the treatment of epilepsy[30,31]. The primary finding of our study on leptin and HCC cell line indicates that adipokine could be associated with the progression of human hepatocellular carcinoma. Administration of the leptin antagonists or receptors in the targeted cells may open a new way in HCC prevention and treatment. Further studies are warranted and ongoing.

COMMENTS

Background

Leptin is the protein product encoded by obese gene, which plays an important role in the regulation of food intake, and the control of body weight. It is related with many pathological syndrome including obesity, hyperphagia, hyperinsulinemia, reduced fertility, and cholelithiasis. There are considerable researches about the effects of leptin.

Research frontiers

Recently, the researches of relationships between leptin and tumors are hotspots. But there are few studies on the involvement of leptin in human hepatocellular carcinoma.

Innovations and breakthroughs

In this study, we found mRNA of leptin and leptin receptors (including short and long isoforms) were expressed in human hepatocellular carcinoma cell line HepG2. Leptin (0 ng/mL-200 ng/mL) in 72 h could stimulate proliferation of HepG2 *in vitro*, and that effect was due to promotion of DNA synthesis and enhancement of mitotic activity.

Applications

The primary study of leptin and HepG2 may indicate that adipokine could be associated with the progression of human hepatocellular carcinoma, and thus may offer new aim for further understanding of the biological function of leptin and new therapeutic targets.

Peer review

The manuscript written by Zhou J *et al* describes a possible role of leptin in hepatocarcinogenesis and promotion of the disease. The data are interesting and potentially important. It's recommended the additional experiments using another HCC cell lines which could make this study's conclusions more confidential.

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