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Concomitant Activation of the JAK/STAT, PI3K/AKT, and ERK Signaling Is Involved in Leptin-Mediated Promotion of Invasion and Migration of Hepatocellular Carcinoma Cells

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Abstract

Various epidemiologic studies have shown that obesity is associated with hepatocellular carcinoma. Leptin, the key player in the regulation of energy balance and body weight control, also acts as a growth factor on certain organs in both normal and disease states. It is plausible that leptin acts to promote hepatocellular carcinogenesis directly affecting malignant properties of liver cancer cells. However, a direct role for leptin in hepatocellular carcinoma has not been shown. In this study, we analyzed the role of leptin and the mechanism(s) underlying its action in hepatocellular carcinoma cells, which express both short and long isoforms of leptin receptors. Treatment with leptin resulted in increased proliferation of both HepG2 and Huh7 cells and involves activation of signal transducers and activators of transcription 3 (STAT3), AKT, and extracellular signal-regulated kinase (ERK) signaling pathways. Leptin-induced phosphorylation of ERK and AKT was dependent on Janus-activated kinase (JAK)/STAT activation. Intriguingly, we also found that leptin potently induces invasion of hepatocellular carcinoma cells in Matrigel invasion and electric cell-substrate impedance-sensing assays. Leptin-stimulated invasion was effectively blocked by pharmacologic inhibitors of JAK/STAT and, to a lesser extent, by ERK and phosphatidylinositol 3-kinase (PI3K) inhibition. Importantly, leptin also induced the migration of both HepG2 and Huh7 cells on fibronectin matrix. Inhibition of JAK/STAT, ERK, and PI3K activation using pharmacologic inhibitors effectively blocked leptin-induced migration of HepG2 and Huh7 cells. Taken together, these data indicate that leptin promotes hepatocellular carcinoma growth, invasiveness, and migration and implicate the JAK/STAT pathway as a critical mediator of leptin action. Our findings have potential clinical implications for hepatocellular carcinoma progression in obese patients.

Introduction

Obesity is deemed as an important risk factor for many serious medical conditions. It greatly influences the risk and prognosis of many common forms of cancer, providing one of the few preventive interventions capable of making a significant effect on cancer (1,2). The management of normal body weight is regulated by adipocytokines that act on the brain to
regulate food intake (3). The adipocytokines are biologically active polypeptides that are produced exclusively or substantially by white adipose tissue, preadipocytes, and mature adipocytes and act by endocrine, paracrine, and autocrine mechanisms (3,4). Leptin, a product of the obese (ob) gene is a neuroendocrine hormone that has attracted attention since its identification in 1995 (5,6). It is a multifunctional peptide hormone with wide ranging biological activities including appetite regulation, bone formation, reproductive function, and angiogenesis (7–9). These biological activities suggest an important role in cancer proliferation, invasion, and metastasis (10). Leptin circulates as a 16-kDa protein partially bound to plasma proteins (11,12) and exerts its actions through its specific receptors present in a variety of tissues localized to the cell membrane (13).

Leptin receptor belongs to a family of class I cytokine receptors, which typically contain a cytokine receptor homologous domain in the extracellular region (14). All six isoforms have a similar extracellular ligand-binding domain at the NH\textsubscript{2} terminus but differ at the intracellular COOH-terminal domain. Although all five short isoforms have transmembrane domains, only the long form has the intracellular motifs necessary for activation of signaling pathways (14). As with other class I cytokine receptors, the leptin signaling is thought to be transmitted mainly by the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway (15,16). JAKs associate constitutively with conserved box 1 and 2 motifs in the intracellular domain of Ob-Rb (long isoform of leptin receptor). Binding of leptin to Ob-Rb results in autophosphorylation of JAK1 and JAK2 as well as phosphorylation of the cytoplasmic domain of Ob-Rb and the downstream transcription factors STATs (15). The leptin signal is terminated by induction of suppressor of cytokine signaling (SOCS-3), a member of a family of proteins that inhibits the JAK-STAT signaling cascade (17,18). SOCS proteins have a variable NH\textsubscript{2}-terminal domain, a central SH2 domain, and a COOH-terminal domain, termed SOCS-box motif. They are induced by cytokines and act in a negative feedback loop to inhibit the receptor. Thus, overexpression of SOCS-3 inhibits leptin-mediated tyrosine phosphorylation of JAK2 (17–19). Whether activation of the above pathways by leptin occurs in hepatocellular carcinoma cells remains unknown.

Large population prevalence studies have shown that hepatocellular carcinoma is clearly associated with obesity (20–24). A research group from Denmark assessed the relative risk of hepatocellular carcinoma in a study cohort of 43,965 obese patients and concluded that the risk of hepatocellular carcinoma for obese patients was increased to 1.9 compared with the general population (25). A more recent U.S. study reported a similar effect of obesity on hepatocellular carcinoma even after multivariate analysis. This study included 404,576 men and 495,477 women with body mass index (BMI) of 18.5 kg/m\textsuperscript{2} at enrollment who were observed for 16 years. Stratification of overall and site-specific cancer-related deaths according to BMI showed that hepatocellular carcinoma was 1.68 times higher among women with a baseline BMI of 35 kg/ m\textsuperscript{2} and was 4.52 times higher for men with a similarly increased BMI compared. Most notably, among the male group, hepatocellular carcinoma had the highest relative-risk increase as a consequence of obesity compared with all the cancers studied, including prostate, kidney, gallbladder, colon, rectum, esophagus, stomach, and pancreas (26). Another clinical study recently examined obesity as an independent risk factor for hepatocellular carcinoma in patients with cirrhosis who underwent transplantation, concluding that obesity is indeed a statistically significant independent risk factor after multivariate analysis (2,26,27). At present, a biological explanation for risk associated between obesity and hepatocellular carcinoma is not known. Therefore, the effects of obesity on human hepatocellular carcinoma represent a critical intersection between these two important health problems. However, whether there is a direct relationship between leptin and hepatocellular carcinoma cannot be conclusively stated as increased leptin and hepatocellular carcinoma may both be secondary
consequences of obesity. Considering the fundamental role of adipocytokines in cancer progression, the growth regulation of hepatocellular carcinoma cells by leptin might affect their malignant progression. Therefore, in the present study, we examined the expression of leptin receptors using the hepatocellular carcinoma cell lines. We further investigated the effects of leptin on the malignant properties of hepatocellular carcinoma, including proliferation, invasion, and migration. We also elucidated the signal transduction pathways regulating leptin-induced changes in the cancerous properties of hepatocellular carcinoma.

Materials and Methods

Antibodies

Antibodies for phosphorylated AKT (pAKT-Ser\(^{473}\)), anti-AKT, phosphorylated extracellular signal-regulated kinase (pERK-Thr\(^{202}/Tyr^{204}\)), anti-ERK, phosphorylated STAT (pSTAT-Tyr\(^{705}\)), and anti-STAT were purchased from Cell Signaling (Danvers, MA). Antibodies for short and long forms of leptin receptors Ob-R (C-20), Ob-R (B-3), and Ob-R (H-300) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture, reagents, and treatments

The following hepatocellular carcinoma cell lines were used: (a) HepG2 cells derived from a human hepatoblastoma (28) and (b) Huh7 cells derived from a hepatocellular carcinoma (29). HepG2 and Huh7 were grown in MEM and DMEM supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, West Sacramento, CA) and 2 \(\mu\)mol/L L-glutamine (Invitrogen, Carlsbad, CA), respectively. For treatment, cells were seeded at a density of 1 \(\times\) 10\(^6\) per 100-mm tissue culture dish. After 16 h of serum starvation, the culture media were changed to serum-free media containing leptin treatments as indicated. In other sets of experiments, cells were treated with the JAK/STAT inhibitor AG490 (Calbiochem, San Diego, CA) at 100 \(\mu\)mol/L, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Cell Signaling) at 10 \(\mu\)mol/L, and the mitogen-activated protein kinase (MAPK) inhibitor PD098059 (Sigma, St. Louis, MO) at 10 \(\mu\)mol/L for indicated durations. For electric cell-substrate impedance-sensing (ECIS) invasion assay, human umbilical vein endothelial cells [HUVEC; American Type Culture Collection (ATCC), Manassas, VA] were maintained in HAM’s F-12 medium (ATCC) containing 10% FBS, 0.1 mg/mL heparin (Sigma), and 0.05 mg/mL endothelial cell growth supplement (Sigma).

RNA isolation and reverse transcription-PCR

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) and quantified by UV absorption. RNA integrity was confirmed by using formaldehyde agarose gel electrophoresis and ethidium bromide staining. Complementary DNA (cDNA) was synthesized from 2.5 \(\mu\)g total RNA by reverse transcription at 42°C for 1 h using a first strand cDNA synthesis kit (Invitrogen). The synthesized cDNA was used as a template for PCR amplification. A semiquantitative PCR amplification was carried out using specific primers designed to amplify leptin receptors Ob-Rb (long isoform) and Ob-Rt (short isoform). The primers were as follows: Ob-Rb sense, 5’-TCACCCAGTGATTACAAGCT-3’; Ob-Rb antisense, 5’-CTGGAGAACTCTGATGTCCG-3’; Ob-Rt sense, 5’-CATTATTATCCCCATTGAGAAGTA-3’; Ob-Rt antisense, 5’-CTGAAAATTAAGTCCTTGTGCCCAG-3’. PCR generated 1,071- and 273-bp fragments of the Ob-Rb and Ob-Rt genes, respectively. To ensure that amplification of these genes was within the exponential range, different numbers of cycles (25–40) were run. Finally, 30 cycles of PCR amplification were chosen. PCR conditions were 95°C for 5 min (denaturation) and 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min followed by 72°C for 5 min. In addition, specific primers for the 18S RNA were used as control. The
primers were sense, 5′-GAGGGAGCCTGAGAAACGG-3′ and antisense, 5′-GTCGGGAGTGGGTAATTTGC-3′. PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Immunoprecipitation of Ob-Rb and Ob-Rt**

For immunoprecipitation of the long and short forms of leptin receptor (30), COLO 320DM cell lysate (Santa Cruz Biotechnology) and whole-cell lysates from HepG2 and Huh7 cells were incubated with either Ob-R (C-20; specific for the long form of leptin receptor) or Ob-R (H-300; for both long and short forms of leptin receptor), and the mixture was rotated slowly at 4°C for 16 h. IgG served as a negative control. A total of 20 µL packed protein A/G agarose beads was added, and mixture was incubated at 4°C for 1 h with rotation. The beads were collected by gentle centrifugation and washed twice with 1.5 mL ice-cold buffer [50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.25% Na-deoxycholate, 1 mmol/L phenyl-methylsulfonyl fluoride (PMSF), 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mmol/L Na3VO4, and 1 mmol/L NaF]. After the final wash, the precipitated protein-beads complexes were resuspended in SDS-sample loading buffer, fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. Immunodetection was done by blocking the membranes for 1 h in TBS buffer [20 mmol/L Tris-Cl (pH 7.5), 137 mmol/L NaCl, 0.05% Tween 20] containing 5% powdered nonfat milk followed by addition of the mouse monoclonal Ob-R (B-3) antibody (specific for both long and short forms of leptin receptor) in TBS for 2 h at room temperature. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibodies and developed by enhanced chemiluminescence (ECL system, Amersham Pharmacia Biotech, Arlington Heights, IL) according to manufacturer’s instructions.

**Analysis of ObRb expression in human liver tissue**

Normal liver tissue (two samples) was obtained during surgical liver resection for secondary liver cancer. The tissue was obtained at a minimum of 5-cm distance from the tumor, and normal histology was assessed by routine examination. Pathologic tissue was obtained from three patients undergoing surgical resection for trabecular hepatocellular carcinoma. Specimens from the tumor and from peritumoral noninvolved tissue were obtained immediately after resection and snap-frozen in liquid nitrogen. Liver cirrhosis was present in the peritumoral tissue of all three patients (two HCV related and one HBV related). All procedures were in accordance with the ethical standards of the Regional Committee on Human Experimentation (Florence, Italy). Tissue was homogenized with 20 strokes in a glass-Teflon homogenizer with a buffer of the following composition: 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 1 mmol/L Na3VO4, 2 mmol/L PMSF, 1 µg/mL trypsin inhibitor, 1 µg/mL leupeptin, and 1 µg/mL pepstatin A. Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration was measured in triplicate with a commercially available assay (Pierce, Rockford, IL). Equal amounts of proteins were analyzed by Western blotting as indicated below. Autoluminograms were scanned, and signals were quantified with Image J software. Background signal was subtracted in each sample. Ob-Rb levels are expressed as the ratio between specific signal and signal of a housekeeping gene (β-actin) analyzed on the same membranes.

**Western blot**

Whole-cell lysates were prepared by scraping cells in 250 µL of ice cold modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.25% Na-deoxycholate, 1 mmol/L PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mmol/L Na3VO4, and 1 mmol/L NaF; ref. 30]. The lysate was rotated 360 degrees for 1 h at 4°C followed by centrifugation at 12,000 × g for 10 min at 4°C to
clear the cellular debris. Proteins were quantified using the Bradford protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes, and Western blot analyses were done using the previously described antibodies. Immunodetection was done using enhanced chemiluminescence (ECL system, Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Quantification of DNA/cell proliferation assay by bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdUrd) incorporation analysis was done using an ELISA (Roche Diagnostics, Indianapolis, IN). Approximately $5 \times 10^3$ HepG2 or Huh7 cells were cultured in the presence of leptin at 25 to 200 ng/mL for 12, 24, and 48 h in 96-well tissue culture plates. Cells grown in serum-free media served as a negative control, whereas cells grown in the presence of serum (10% FBS) served as positive control for proliferation. BrdUrd incorporation assays were done as described previously (31). Subsequently, BrdUrd was added to the cultures for 4 h. Cells were fixed and DNA denatured. The anti–BrdUrd-POD monoclonal antibody from mouse-mouse hybrid cells conjugated with peroxidase was used at 1:100 and binds to the BrdUrd incorporated in newly synthesized cellular DNA. Resultant immunocomplexes were quantified by emitted light measurements using a microplate luminometer (Becton Dickinson, Franklin Lakes, NJ) with photomultiplier technology. Relative light units per seconds directly correlate to the amount of DNA synthesis and the number of proliferating cells.

Fluorescence-activated cell sorting analysis/cellular DNA flow cytometry using propidium iodide (S-phase analysis)

Fluorescence-activated cell sorting (FACS) analysis of HepG2 and Huh7 cells was carried out using Cellular DNA Flow Cytometry analysis (Roche, Indianapolis, IN), as per manufacturer’s instructions. Cells were grown and synchronized in serum-free for 16 h followed by treatment with leptin (100 ng/mL) for various time intervals (8, 12, and 24 h). Cells grown in serum-free media served as a negative control, whereas cells grown in the presence of serum (10% FBS) and platelet-derived growth factor served as positive control. Cells were harvested, adjusted to equal cell numbers, fixed in 70% ethanol for 30 min at −20°C, and suspended in 500 AL PBS containing RNase A (5 Prime 3 Prime, Inc., Boulder, CO; 250 mg/L) for 30 min at 4°C. Fixed cells were stained with propidium iodide (100 mg/L) before FACS analysis as described elsewhere (31). Briefly, after excitation of propidium iodide at 485 nm, red fluorescence from propidium iodide was collected through a 580-nm long-pass filter and recorded to measure cellular DNA content. After counting $3.5 \times 10^4$ cells, the number of cells in each phase of cell cycles (G₀-G₁, S, and G₂-M) was analyzed to assess the respective DNA content. The HepG2 and Huh7 cell cycle profile was determined using a Becton Dickinson FACScan, and data were analyzed using ModFit LT 3.1.

Tumor cell invasion assay

For an in vitro model system for metastasis, we did a Matrigel invasion assay by using a Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA; ref. 30). Cells were seeded at a density of $1 \times 10^5$ per insert and cultured overnight. After 16 h of serum starvation, the culture media were changed to serum-free media containing treatments as indicated. Triplicate wells were used for each treatment. Cells were treated with human recombinant leptin (Sigma) at 100 ng/mL. In other sets of experiments, cells were treated with the JAK/STAT inhibitor AG490 (Calbiochem) at 100 μmol/L, the MAPK inhibitor PD098059 (Sigma) at 10 μmol/L, and the PI3K inhibitor LY294002 (Cell Signaling) at 10 μmol/L along with leptin. After 24 h of incubation, cells remaining above the insert membrane were removed by gentle scraping with a sterile cotton swab. Cells that had invaded through the Matrigel to the bottom of the insert were fixed in methanol for 10 min.
After being washed in PBS, the cells were stained with H&E. The insert was subsequently washed in PBS and briefly air-dried and mounted. The slides were coded to prevent counting bias, and the number of invaded cells on a representative section of each membrane was counted with light microscope. The number of invaded cells for each experimental sample represents the average of triplicate wells.

**ECIS invasion assay**

Electrode arrays were obtained from Applied BioPhysics (Troy, NY), and ECIS invasion assays were done (32). ECIS array wells were precoated with a solution of 200 µg/mL gelatin in 0.15 mol/L NaCl. After 15 min of incubation to allow the gelatin to adsorb, the gelatin solution was aspirated, and the electrode containing wells were rinsed twice with PBS. They were partially filled with 200 µL HUVEC medium and allowed to equilibrate for 15 to 60 min in humidified CO\textsubscript{2} incubator. Approximately 1 × 10\textsuperscript{5} HUVECs were added in 200 µL HUVEC medium in each well. The attachment and spreading of cells into the ECIS wells was followed by impedance measurements using ECIS (model 1600R, Applied BioPhysics). The HUVECs were challenged with monodisperse cell suspensions of HepG2 and Huh7 cells (20 × 10\textsuperscript{5}/mL) in fresh HUVEC medium, and 50 µL was added to wells (one hepatocellular carcinoma cell for each endothelial cell). Triplicate wells were used for each treatment. Cells were treated with human recombinant leptin (Sigma) at 100 ng/mL. In other sets of experiments, cells were treated with the JAK/STAT inhibitor AG490 (Calbiochem) at 100 µmol/L, the MAPK inhibitor PD098059 (Sigma) at 10 µmol/L, and the PI3K inhibitor LY294002 (Cell Signaling) at 10 µmol/L along with leptin. The impedance of the challenged endothelial cell layer was monitored via ECIS for the next 12 to 20 h (32).

**Migration assay**

To perform migration assays (33), cells were plated into 24-well cell culture plate and precoated with human fibronectin (5 µg/cm\textsuperscript{2}; Sigma). Cells were allowed to grow in 10% FBS containing DMEM to confluence, washed with serum-free medium, and serum starved for 16 h. A 1-mm wide scratch was made across the cell layer using a sterile pipette tip. After washing with serum-free medium twice, DMEM containing 10 µg/mL human fibronectin was added to replace matrix depleted with the cells. Cells were treated with human recombinant leptin (Sigma) at 100 ng/mL. In other sets of experiments, cells were treated with the JAK/STAT inhibitor AG490 (Calbiochem) at 100 µmol/L, the MAPK inhibitor PD098059 (Sigma) at 10 µmol/L, and the PI3K inhibitor LY294002 (Cell Signaling) at 10 µmol/L along with leptin. Plates were photographed after 6, 12, and 24 h. All experiments were done at least six times.

**ECIS wound-healing assays**

Wound-healing assays were done with the ECIS (Applied BioPhysics) technology (34,35). For wound-healing assays, confluent HepG2 and Huh7 monolayers cultured on ECIS plates were submitted to an elevated voltage pulse of 40-kHz frequency, 3.5-V amplitude, and 30-s duration, which led to death and detachment of cells present on the small active electrode, resulting in a wound normally healed by cells surrounding the small active electrode that have not been submitted to the elevated voltage pulse. Wound healing was then assessed by continuous resistance measurements for 24 h.

**Statistical analysis**

All experiments were independently done thrice in triplicate. Data were analyzed using paired or unpaired Student’s t test, as appropriate. Data were considered to be statistically significant if \(P < 0.05\). Data are expressed as mean ± SE.
Results

Leptin augments proliferation and modulates cell cycle of epatocellular carcinoma cells

Leptin exerts its biological functions through binding to its receptors that mediate a downstream signal by activating multiple signaling pathways (15). We first examined the expression of leptin receptors in HepG2 and Huh7 cells. The expression of leptin receptor mRNA and protein was examined using reverse transcription-PCR and Western blot analysis. A predicted PCR product of Ob-Rb (long isoform) was obtained as 1,071 bp and Ob-Rt (short isoform) as 273 bp by specific primers (Fig. 1A) in both HepG2 and Huh7 cells. Immunoprecipitation was done using specific antibodies: Ob-R (C-20; recognizes only the long form of leptin receptor) and Ob-R (H-300; recognizes both long and short forms of leptin receptor) followed by Western blot analysis using mouse monoclonal Ob-R (B-3; recognizes both long and short forms of leptin receptor). Immunoprecipitates with specific antibodies show the presence of both long and short forms of leptin receptor in HepG2 and Huh7 cells, whereas IgG controls do not (Fig. 1B). We also investigated the expression levels of Ob-Rb in tumor, peritumoral, and normal liver tissue samples obtained from patients with hepatocellular carcinoma (Fig. 2C). Importantly, Ob-Rb was barely detectable in normal human liver, whereas all three hepatocellular carcinoma samples express high levels of Ob-Rb. Interestingly, Ob-Rb expression was higher in the peritumoral tissue in comparison with normal liver, whereas the tumor tissue showed the highest level of Ob-Rb expression.

We next examined the effect of leptin on hepatocellular carcinoma cell proliferation using BrdUrd incorporation analysis. For these experiments, HepG2 and Huh7 cells were serum-starved for 16 h followed by treatment with various concentrations (25–200 ng/mL) of recombinant human leptin for different time intervals (12, 24, and 48 h). Leptin treatment stimulated the growth of HepG2 and Huh7 cells in a time- and dose-dependent manner. Substantial stimulation was observed at 24- and 48-h time intervals after treatment of cells at 100 ng/mL leptin, whereas higher concentrations were equally stimulatory (Fig. 2A).

Cell cycle analysis revealed that the proportion of both HepG2 and Huh7 cells was increased in S-phase by leptin treatment at 24 h compared with lower treatment periods, and cells were subjected to serum-free conditions (Fig. 2B). D-type cyclins are active in the G1 phase of the cell cycle. They complex with cyclin-dependent kinases to catalyze the transition from G1 to S phase of the cell cycle (36). Leptin promotes proliferation of hepatocellular carcinoma cells, and one of the targets for leptin action may be cyclin D1. Under the treatment of leptin, the G1 arrest of cells was reduced and was accompanied with up-regulation of G1 phase–specific cyclin D1 (Fig. 2C) but down-regulation of cyclin-dependent kinase inhibitor p21\(^{WAF1/CIP1}\) (Fig. 2D). Kruppel-like factor (KLF5) is a cell growth mediator, and higher KLF5 increases cell growth rate and leads to transformed phenotypes (37). Tumor cell proliferation is tightly associated with tumor progression; therefore, we examined the effect of leptin on the expression of KLF5. Immunoblot analysis using specific antibodies against KLF5 showed an increase in the expression of KLF5 following leptin treatment in HepG2 and Huh7 cells (Fig. 2E). Collectively, the results showed that the proliferative effect of leptin on HepG2 and Huh7 cells was associated with the up-regulation of cyclin D1 and KLF5 and down-regulation of p21\(^{WAF1/CIP1}\).

Leptin activates the JAK/STAT-PI3K/AKT-ERK axis in growth stimulation of hepatocellular carcinoma cells

To gain insight into the mechanism underlying the proliferative effect of leptin on hepatocellular carcinoma cells, we next examined the changes in signal transduction pathways plausibly involved in mediating leptin action. Previous studies have shown that
leptin activates JAK, which in turn phosphorylates and activates STATs in other systems (15,16). Total cellular proteins were extracted from cells treated with 100 ng/mL leptin for various time periods, and lysates were immunoblotted with a specific phosphorylated tyrosine STAT3 antibody. STAT3 phosphorylation was stimulated by 100 ng/mL of leptin in a time-dependent manner, resulting in an increase in STAT3 phosphorylation within 30 min of treatment (Fig. 3A). Immunoblots were reprobed with antibodies against STAT3, showing that the increase in STAT3 phosphorylation was not due to the increased STAT3 protein expression (Fig. 3A). We further examined the phosphorylation of ERK and AKT after stimulating the cells with 100 ng of leptin for various intervals of time. An increased phosphorylation of ERK and AKT was observed within 1 h after leptin treatment followed by a decline (Fig. 3B and C). Leptin had no effect on total ERK and AKT protein expression levels.

Next, to investigate if activation of the JAK/STAT-PI3K/AKT-ERK axis is directly involved in leptin-induced proliferation of hepatocellular carcinoma cells, we studied the effect of pharmacologic inhibitors of JAK/STAT (AG490), ERK (PD098059), and PI3K (LY294002) on leptin-induced stimulation of proliferation. Treatment of cells with AG490 decreased the phosphorylation of STAT3 significantly without affecting the expression of total STAT3 protein (Fig. 4A), whereas PD098059 and LY294002 did not affect the phosphorylation of STAT3. As shown in Fig. 4B and C, both PD098059 and LY294002 specifically inhibited the phosphorylation of ERK and AKT, respectively, without affecting the expression of total ERK and AKT (Fig. 4B and C) or levels of phosphorylated STAT3 (Fig. 4A). Interestingly, treatment with the JAK/STAT inhibitor AG490 blocked leptin-induced hyperphosphorylation of both ERK and AKT (Fig. 4B and C). Importantly, simultaneous treatment with leptin and AG490 could not restore the level of phosphorylation of STAT3 (Fig. 4A) or ERK (Fig. 4B) or Akt (Fig. 4C), as achieved by treatment with leptin alone. These data suggest that activation of JAK/STAT is upstream of the activation of the ERK and AKT pathways, revealing the hierarchy of these events. Examination of cell proliferation in these treatment conditions clearly showed that blocking STAT3 phosphorylation significantly reduced the growth stimulation of HepG2 and Huh7 cells by leptin (Fig. 4D), indicating that the activation of STAT3 is essential for the cell-proliferative effect of leptin in hepatocellular carcinoma. In addition, blocking ERK and AKT phosphorylation significantly reduced the growth stimulation of HepG2 and Huh7 cells by leptin (Fig. 4D).

**Leptin promotes the invasive potential of hepatocellular carcinoma cells**

Invasion and metastasis are the key biological features of carcinoma cell behavior (38). As Ob-Rb receptors are connected with several signaling pathways involved in cell proliferation, apoptosis, and cancer progression (15), we addressed the question of whether leptin may participate in the regulation of invasion in hepatocellular carcinoma progression. For an *in vitro* model system for metastasis (30), we used a Matrigel invasion chamber (Becton Dickinson). In the absence of leptin, the invasion was very low. With 100 ng/mL leptin in the bottom chamber, significantly greater numbers of HepG2 and Huh7 cells invaded through Matrigel-coated inserts towards the bottom chamber (Fig. 5A). H&E staining of invaded HepG2 and Huh7 cells exhibited a remarkable invasion in response to 100 ng/mL leptin (Fig. 5A). Next, we examined the contribution of the JAK/STAT-PI3K/AKT-ERK kinases in leptin-induced increased invasiveness. Treatment with the JAK/STAT inhibitor AG490, the PI3K inhibitor LY294002, and the ERK inhibitor PD098059 significantly inhibited the invasiveness induced by 100 ng/mL leptin in hepatocellular carcinoma cells (Fig. 5A).

Next, we did a quantitative real-time impedance assay using an ECIS-based technique (32) to follow the invasive activities of HepG2 and Huh7 cells in culture. This assay is based on
the microscopic observations that metastatic cells attach and invade the established confluent layer of HUVECs on small gold electrodes. First, the initial attachment and spreading of this lot of HUVEC cells were analyzed via time course impedance changes (data not shown). Electrodes were followed from the time of inoculation (time 0) to 24 h after inoculation. The initial increase in the curve due to cell attachment and spreading increased the resistive portion of the impedance at 4 kHz six times more than that of the cell-free electrode. As evident in Fig. 5B to D, the spreading was completed in ~ 2.5 h, and the resistance fluctuations resulting from the movement or undulations of the established cell sheet constraining the current were evident. Next, the established HUVEC cell layers were challenged with HepG2 and Huh7 cells. The decrease in resistance of the wells challenged with the HepG2 and Huh7 cells showed direct interactions of the challenger cells resulting from retraction of the endothelial cell junctions and extravasation of the HepG2 and Huh7 cells on the substratum (Fig. 5B–D). Importantly, leptin-treated cells showed substantial steeper decrease in impedance than no treatment controls, clearly showing that leptin increases the invasive potential of both HepG2 and Huh7 cells (Fig. 5B–D). Next, we sought to determine the effect of inhibitors of JAK/STAT-PI3K/AKT-ERK on the leptin-induced increased invasiveness of HepG2 and Huh7 cells. Treatment with the JAK/STAT inhibitor AG490 (Fig. 5B), the ERK inhibitor PD098059 (Fig. 5C), and the PI3K inhibitor LY294002 (Fig. 5D) significantly inhibited the invasiveness induced by 100 ng/mL leptin in hepatocellular carcinoma cells.

**Leptin increases the migration capability of hepatocellular carcinoma cells**

Cancer progression is a multistep process that enables tumor cells to migrate to points far from a given primary tumor mass, leading to metastasis (39). We analyzed the effect of leptin on migration potential of HepG2 and Huh7 cells by using a migration assay. The movement of HepG2 and Huh7 cells across the scratched area of the cell monolayer indicates the migration of cells in a process independent of proliferation (40). As shown in Fig. 6A, both HepG2 and Huh7 cells cultured in the presence of leptin migrated rapidly and covered the wound in 12 h compared with the untreated controls. The ability of cells to migrate was significantly reduced when they were treated with the JAK/STAT inhibitor AG490 in the presence of leptin. Treatment of HepG2 and Huh7 cells with the ERK inhibitor PD098059 (Fig. 6A) and the PI3K inhibitor LY294002 (Fig. 6A) also impaired the migration potential but not to the extent of inhibition achieved by AG490.

Next, we did ECIS based wound-healing assays for a quantitative determination of effect of leptin on migration potential of hepatocellular carcinoma cells. HepG2 and Huh7 cells cultured on ECIS 8W1E plates were subjected to an elevated voltage pulse of 40-kHz frequency, 3.5-V amplitude for 30 s duration, and resistance was measured for 24 h. The application of the high-field pulse led to a drastic decrease of cell resistance (Fig. 6B). HepG2 and Huh7 cells treated with leptin showed increased resistance to reach the resistance values of the nonwounded cells at the start of the experiment, whereas untreated cells did not. Interestingly, HepG2 and Huh7 cells treated with the JAK/STAT inhibitor AG490, the ERK inhibitor PD098059, and the PI3K inhibitor LY294002 along with 100 ng leptin did not reach the resistance values of the nonwounded cells, indicating significant inhibition of leptin-induced migration in the presence of chemical inhibitors for the JAK/STAT-PI3K/AKT-ERK kinase pathway. Our demonstration that inhibition of the JAK/STAT-PI3K/AKT-ERK kinase pathway abrogates leptin-induced invasion of Matrigel and migration (scratch-wound healing) confirmed that the activity of these pathways is indeed a crucial component of the signaling machinery used by the leptin receptor in promoting malignant properties of hepatocellular carcinoma.
Discussion

Increasing epidemiologic data in humans and many in vitro investigative reports have linked obesity with various disease states and suggested a strong link between leptin and cancer growth. Several reports have described a mitogenic effect of leptin on gastric (40), breast (4), ovarian (41), prostate (42), and endometrial (30) cancer cells. However, it inhibits the growth of pancreatic carcinoma (43), suggesting a differential response of various cancer cells to leptin treatment. Hepatocellular carcinoma showed the highest relative risk increase in association with obesity compared with all the cancers studied, including prostate, kidney, gallbladder, colon, rectum, esophagus, stomach, and pancreas (22–26). A recent clinical study examining obesity as an independent risk factor for hepatocellular carcinoma in patients with cirrhosis who underwent transplantation concluded that obesity is indeed a statistically significant independent risk factor after multivariate analysis (27). Interestingly, leptin was reported to induce a significant suppression of human hepatocellular carcinoma via induction of natural killer cell proliferation and activation in a murine model. However, the use of athymic mouse model does not exclude additional leptin-mediated effects on regulatory T-cell population or effector cells (44). Recently, high leptin expression was associated with an increased intratumor microvessel density and hepatocellular carcinoma development (45). Leptin-mediated neovascularization coordinated with vascular endothelial growth factor playing an important role in the development of liver fibrosis and hepatocarcinogenesis in NASH (46). However, the direct role of leptin in hepatocellular carcinoma progression and the elucidation of signaling pathways involved have never been deciphered. Thus, in the present study, the expression of leptin receptor in HepG2 and Huh7 cells was investigated. Both short and long isoforms of leptin receptors were observed in hepatocellular carcinoma cells, suggesting that leptin may be involved in hepatocellular carcinoma. This hypothesis is supported by the observation that Ob-Rb is expressed in tissue obtained from patients with hepatocellular carcinoma, and that expression levels are higher than in the noninvolved counterpart. In the United States and Europe, hepatocellular carcinoma arises in more than 80% of cases on a cirrhotic liver. Accordingly, all three patients analyzed in this study had cirrhosis in the peritumoral liver tissue. Remarkably, Ob-Rb levels were also higher in tissue with cirrhosis than in normal liver tissue, supporting the proposed role of leptin signaling in the development of liver fibrosis.

As a first attempt to elucidate the signaling pathways involved in leptin-mediated induction of cancerous properties of hepatocellular carcinoma cells, we examined the effect of leptin on the activation of the JAK/STAT-AKT-ERK pathway. Our experiments clearly showed that leptin rapidly stimulates the JAK/STAT pathway and induced the phosphorylation of ERK and AKT, thus activating these key signal transduction pathways associated with cell growth. In addition, prevention of leptin-induced activation of JAK/STAT with chemical inhibitors in turn significantly reduced the activation of both the ERK and AKT pathways. Importantly, leptin induced the invasive and migration potential of both HepG2 and Huh7 cells. Inhibition of these pathways with specific chemical inhibitors not only decreases the invasive potential but also blocked hepatocellular carcinoma cell migration. Thus, we deciphered in this report that leptin is directly involved in the augmentation of invasion and migration potential of hepatocellular carcinoma cells. In addition, in the present study, it is clear that leptin can trigger invasion and migration of hepatocellular carcinoma cells via a pathway involving the JAK/STAT-AKT-ERK axis as pharmacologic inhibition of this pathway abolished leptin-induced invasiveness and migration significantly.

Our studies represent the first steps towards understanding the molecular mechanisms of leptin action in hepatocellular carcinoma. Recent studies have shown that the ERK pathway is an attractive target for therapeutic intervention due to its integral role in the regulation of proliferation, invasiveness, and survival of tumors (47). Multiple studies with small
interfering RNAs and pharmacologic inhibitors have shown the importance of ERK blockade, and several agents that target this pathway are already undergoing clinical testing, and some have already shown promise in clinical trials (48). AKT provides a survival signal protecting cells from apoptosis induced by various stresses by multiple mechanisms, such as the phosphorylation of Bad, glycogen synthase-3, forkhead transcription factor, and caspase-9 (49,50). Phosphorylation of these proteins results in inactivation of their apoptotic functions. As shown in our report, AKT phosphorylation was increased in leptin-treated human hepatocellular carcinoma cells, and inhibition of PI3K with LY294002 abolished leptin-induced proliferation. LY294002 has been tested in an ectopic skin and orthotopic brain tumor model and has been shown to inhibit glioma tumor growth (51). It has also shown efficacy against ovarian carcinoma (52). In addition, more potent AKT inhibitors, such as small-molecule inhibitor API-59CJ-OMe (9-methoxy-2-methylellipticinium acetate; ref. 53), are being developed.

Importantly, our studies not only implicate the AKT and ERK survival pathways in hepatocellular carcinoma cell proliferation and invasion, but we also report that inhibition of the JAK/STAT pathway significantly reduced the phosphorylation of AKT and ERK and proliferation mediated via these pathways, suggesting that JAK/STAT acts upstream of ERK and AKT. STAT3 is frequently found to be either constitutively activated or activated in response to specific stimuli. Multiple strategies have been used to block STAT activation, including indirect and direct approaches. Tyrphostin AG490 has been implicated in inhibition of proliferation of human acute lymphocytic leukemia (54) and human myeloma cells (55). A recent study reported that introduction of antisense STAT3 oligodeoxynucleotide specifically blocked expression of STAT3 mRNA in human head and neck squamous carcinoma cell lines inhibiting proliferation (56). In addition, a small-molecule inhibitor (STA21) discovered through virtual database screening was found to inhibit human breast cancer cells expressing constitutively active STAT3 (57).

In summary, our data for the first time deciphered the molecular mechanisms responsible for the leptin-mediated hepatocellular carcinoma cell proliferation, establishing direct association between obesity and hepatocellular carcinogenesis and presenting involvement of key molecules of multiple signaling pathways.

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References


Figure 1. Leptin receptors are expressed in hepatocellular carcinoma cells and hepatocellular carcinoma tumor tissue from human

A, total RNA was extracted from HepG2 and Huh7 cells and analyzed by reverse transcription-PCR using specific primers for long (Ob-Rb) and short (Ob-Rt) isoforms of leptin receptor. Primer set for 18S RNA was used as a control.

B, total protein was isolated from HepG2 and Huh7 cells, and equal amounts of proteins were subjected to immunoprecipitation using specific antibodies for long and short forms of leptin receptor. Immunoprecipitation with IgG was included as a negative control. Twenty-five micrograms of COLO 320DM cell lysate (Santa Cruz Biotechnology) were included as positive control. Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblot analysis.
using a mouse monoclonal antibody against both long and short forms of leptin receptor. Long (Ob-Rb; 120 kDa) and short (Ob-Rt; 90 kDa) forms of leptin receptors were found to be present in HepG2 and Huh7 cells. C, lysates from normal liver tissue (surrounding liver metastases), peritumoral tissue, and three cases of hepatocellular carcinoma (HCC) on liver with cirrhosis were prepared. Equal amounts of proteins were resolved by SDS-PAGE and subjected to immunoblot analysis using a mouse monoclonal antibody against the long form of leptin receptor. The long form (Ob-Rb) of leptin receptors was found to be present in all three cases of hepatocellular carcinoma. The expression was more in tumorous tissues (T) compared with peritumoral tissue (P). The normal liver tissue from two cases (NHL 1 and 2) were negative for the presence of Ob-Rb. Lysate from hepatic stellate cells (HSC) was used as positive control. The Western blot signals were quantified by densitometry (histogram) and normalized to β-actin.
Figure 2. Leptin is mitogenic for hepatocellular carcinoma cells
A, HepG2 and Huh7 cells were serum-starved for 16 h followed by treatment with 25 to 200 ng/mL leptin for 12, 24, and 48 h. BrdUrd incorporation assays were then done as described in Materials and Methods. Leptin treatment increased proliferation of HepG2 and Huh7 cells in a time-and dose-dependent manner. *, \( P < 0.01 \), for different time and dose compared with respective untreated cells. Cells grown in 10% FBS were included as positive controls. Columns, mean of three independent experiments done in triplicates; bars, SE. B, leptin increased the fraction of HepG2 and Huh7 cells in the S phase of the cell cycle. Cells were synchronized by serum starvation in a medium containing 0.1% serum for 16 h. Serum-starved cells were exposed to platelet-derived growth factor (PDGF; 30 ng/mL) or leptin.
(100 ng/mL) for various time intervals (8, 12, and 24 h) or control serum-free media (0.1% FBS). After each time interval, the distribution of both HepG2 and Huh7 cells in the cell cycle was determined by flow cytometry using propidium iodide–stained nuclei. The results indicate the distribution of hepatocellular carcinoma cells in various phases after serum starvation, leptin, or platelet-derived growth factor treatment. Columns, mean of three independent experiments done in triplicate; bars, SE. *, \( P < 0.01 \), compared with serum-starved conditions at respective treatment time interval. C to E, both HepG2 and Huh7 cells were grown to 80% confluence, serum-starved for 16 h, and incubated in the presence of 100 ng/mL leptin for 6 and 24 h. Cell lysates were prepared and normalized for protein content; 100 µg of protein was resolved on 10% SDS-PAGE followed by immunoblot analysis with antibodies for cyclin D1 (C), p21 (D), and KLF5 (E). The Western blot signals were quantified by densitometry and normalized to β-actin. Representative of multiple independent experiments. *, \( P < 0.05 \), compared with untreated controls.
Figure 3. Leptin stimulates the phosphorylation of STAT3, ERK, and AKT
HepG2 and Huh7 cells were cultured, serum-starved for 16 h, and treated with leptin (100 ng/mL) for various time intervals. Time 0 represents the absence of leptin or untreated cells. Cell lysates were prepared and quantified for protein content. A total of 100 µg protein was resolved on 10% SDS-PAGE followed by immunoblot analysis with specific antibodies against total or phosphorylated forms of STAT3 (A), ERK (B), and AKT (C). The representative histogram is the densitometric analysis of bands showing fold increase in levels of phosphorylated forms of STAT3, ERK, and AKT with respect to total STAT3, ERK, and AKT protein, at various intervals of leptin treatment. Representative of multiple independent experiments with a representative immunoblot for pSTAT3, STAT3, pERK,
ERK, pAKT, and AKT. *Columns, mean densitometric values of band intensities; bars, SE. *, $P < 0.05$, compared with untreated control cells. Immunoblot for $\beta$-actin was done as a loading control.
Leptin fails to stimulate proliferation of hepatocellular carcinoma cells in the presence of inhibitors of JAK/STAT, ERK, or AKT.

A to C. HepG2 and Huh7 cells were cultured, serum-starved for 16 h, and treated with leptin (100 ng/mL; L). For combined treatment, cells were pretreated with 100 µmol/L AG490 (A or A+L), 10 µmol/L PD98059 (P or P+L), or 10 µmol/L LY294002 (Ly or Ly+L) for 45 min followed by leptin treatment. Untreated controls (U). Cell lysates were prepared and quantified for protein content. A total of 100 µg protein was resolved on 10% SDS-PAGE followed by immunoblot analysis with specific antibodies against total or phosphorylated forms of STAT3 (A), ERK (B), and AKT (C). The representative histogram is the densitometric analysis of bands showing fold increase in levels of phosphorylated forms.
with respect to total protein. *, $P < 0.01$, compared with untreated control cells; #, $P < 0.01$, compared with leptin treatment; **, $P < 0.05$, leptin with inhibitor compared with respective inhibitor treatment alone. D, cells were treated as described earlier, and BrdUrd incorporation analysis was done as described in Materials and Methods. Columns, mean of experiments done in triplicates thrice; bars, SE. *, $P < 0.001$, compared with untreated control cells; #, $P < 0.005$, compared with leptin treatment. Immunoblot for β-actin was done as a loading control.
Figure 5. Leptin induces invasion potential of hepatocellular carcinoma cells
A, HepG2 and Huh7 cells were cultured in Matrigel invasion chambers in serum-free media containing 100 ng/mL leptin (L) for 24 h. For combined treatment, cells were pretreated with 100 µmol/L AG490 (L + AG), 10 µmol/L PD98059 (L + PD), or 10 µmol/L LY294002 (L + LY) for 45 min followed by leptin treatment. Untreated controls (U). The number of cells that invaded through the Matrigel was counted. Columns, mean of Matrigel invasion assay done in triplicate twice; bars, SE. *, P < 0.01, compared with untreated control cells; #, P < 0.01, compared with leptin treatment. H&E staining showing the invasion of HepG2 and Huh7 cells in a Matrigel invasion assay. B to D, resistance changes in the impedance at 4 kHz as confluent layers of HUVECs were challenged with HepG2 and Huh7 cell
suspensions. The control curve of HUVEC cells received media without HepG2 or Huh7 cells. HepG2 and Huh7 cells were treated with 100 µmol/L AG490 and 100 ng/mL leptin (AG + Leptin; B) or 10 µmol/L PD98059 and 100 ng/mL leptin (PD + Leptin; C) or 10 µmol/L LY294002 along with 100 ng/mL leptin (LY + Leptin; D). Changes in resistance were monitored for 24 h. All the experiments were done thrice in triplicates.
Figure 6. Leptin up-regulates the migration of hepatocellular carcinoma cells
A, both HepG2 and Huh7 cells were grown to confluence on fibronectin-coated surface, serum-starved for 16 h, and scratched with a pipette tip. The plates were photographed immediately following scratching. Culture media were replaced with control media (media containing 100 ng/mL leptin). For combined treatment, cells were pretreated with 100 µmol/L AG490 (Leptin + AG), 10 µmol/L PD98059 (Leptin + PD), or 10 µmol/L LY294002 (Leptin + LY) along with leptin treatment. The plates were photographed at the identical location of the initial image at 6 and 12 h. B, confluent HepG2 and Huh7 cells, seeded in ECIS plates and treated with leptin (100 ng/mL) alone or along with 100 µmol/L AG490 (AG + Leptin) or 10 µmol/L PD98059 (PD + Leptin) or 10 µmol/L LY294002 (LY +
Leptin), were subjected to an elevated voltage pulse of 40-kHz frequency at 3.5-V amplitude for 30 s. The wound was then allowed to heal from cells surrounding the small active electrode that did not undergo the elevated voltage pulse. Resistance was measured before and after the elevated voltage pulse application as described in Materials and Methods. The measurements were stopped 24 h after wound healing. All the experiments were done thrice in triplicates.