Leptin Enhances Cholangiocarcinoma Cell Growth

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Abstract

Cholangiocarcinoma is a strongly aggressive malignancy with a very poor prognosis. Effective therapeutic strategies are lacking because molecular mechanisms regulating cholangiocarcinoma cell growth are unknown. Furthermore, experimental in vivo animal models useful to study the pathophysiologic mechanisms of malignant cholangiocytes are lacking. Leptin, the hormone regulating caloric homeostasis, which is increased in obese patients, stimulates the growth of several cancers, such as hepatocellular carcinoma. The aim of this study was to define if leptin stimulates cholangiocarcinoma growth. We determined the expression of leptin receptors in normal and malignant human cholangiocytes. Effects on intrahepatic cholangiocarcinoma (HuH-28) cell proliferation, migration, and apoptosis of the in vitro exposure to leptin, together with the intracellular pathways, were then studied. Moreover, cholangiocarcinoma was experimentally induced in obese fa/fa Zucker rats, a genetically established animal species with faulty leptin receptors, and in their littermates by chronic feeding with thioacetamide, a potent carcinogen. After 24 weeks, the effect of leptin on cholangiocarcinoma development and growth was assessed. Normal and malignant human cholangiocytes express leptin receptors. Leptin increased the proliferation and the metastatic potential of cholangiocarcinoma cells in vitro through a signal transducers and activators of transcription 3-dependent activation of extracellular signal-regulated kinase 1/2. Leptin increased the growth and migration, and was antiapoptotic for cholangiocarcinoma cells. Moreover, the loss of leptin function reduced the development and the growth of cholangiocarcinoma. The experimental carcinogenesis model induced by thioacetamide administration is a valid and reproducible method to study cholangiocarcinoma pathobiology. Modulation of the leptin-mediated signal could be considered a valid tool for the prevention and treatment of cholangiocarcinoma. [Cancer Res 2008;68(16):6752–61]

Introduction

Cholangiocarcinoma, the malignant tumor arising from the epithelial cells lining the biliary tree (1), is a strongly aggressive malignancy with a bad prognosis. Cholangiocarcinoma represents ~10% to 15% of the total hepatobiliary tumors and its incidence and mortality are increasing worldwide (1). The lack of understanding of the molecular mechanisms regulating cholangiocarcinoma growth implies that this disease represents a challenge to establish effective tools for treatment (2). A consistent amount of data show that cholangiocytes acquire particular characteristics and transdifferentiate into neuroendocrine cells when involved in certain pathologic conditions (3). Moreover, many hormones, neuropeptides, and neurotransmitters regulate malignant and nonmalignant cholangiocyte pathobiology in the course of chronic biliary diseases (3, 4).

Leptin, a 16-kDa protein hormone produced by the Ob gene, is mostly secreted by the adipose tissue (5) and acts on the hypothalamus to regulate food assumption and caloric homeostasis (6). Thus, circulating serum leptin is increased in obesity, a risk factor for cholangiocarcinoma (7).

Leptin exerts its function through specific receptors, referred as long (OBRI) and short (OBRS) isoforms. Among leptin receptor isoforms, only OBRI contains an intact intracellular domain and has the ability to stimulate the intracellular Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway with the activation of STAT3 and extracellular signal-regulated kinase (ERK)1/2 (8). It has been shown that leptin plays an important role in the regulation of angiogenesis and growth of several cancers (9), and it has been proposed that the increased production of such a hormone by adipose tissue could explain the well-known correlation between obesity and increased incidence of various types of tumors (7, 9–12). It remains unknown, however, whether leptin directly regulate cholangiocarcinoma cell growth. Here, we show the effect of leptin on intracellular mechanisms, growth, migration, and apoptosis of human cholangiocarcinoma cells and the role of leptin in cholangiocarcinoma development and growth using an in vivo animal model.

Materials and Methods

Materials

Reagents were purchased from Sigma unless otherwise specified. Antibodies for immunohistochemistry and immunoblotting were purchased from Santa Cruz Biotechnology, unless differently indicated. Nitrocellulose membranes (0.2 μm/L) and the Pierce protein assay were purchased from Pierce Biotechnology. Cytoselect Cell Migration Assay kit was purchased by Cell Biolabs, Inc.

Cell Culture

We used three human cholangiocarcinoma cell lines (Mz-ChA-1, HuH-28, and TFK-1) with different origins. MZ-ChA-1 cells, from a human gallbladder (13), were a gift from Dr. J. Gregory Fitz (University of Texas A & M Health Science Center College of Medicine, Temple, Texas). Treatment with thioacetamide (0.2 g/kg per day) started at 8 weeks of age and was continued until 24 weeks. Control rats were fed with a regular diet (14)
Southwestern Medical Center, Dallas, TX). HuH-28 cells, from human intrahepatic bile duct (14), and TFK-1 cells, from human extrahepatic bile duct (15), were acquired from Cancer Cell Repository, Tohoku University, Japan. H69 cells were a gift from Dr. G. J. Gores (Mayo Clinic, Rochester, Minnesota). Cells were maintained at standard conditions as previously described (16, 17).

Expression of Leptin Receptors

Molecular analysis. We evaluated the expression of leptin receptor by reverse transcription PCR (RT-PCR) using total RNA from H-69, Mz-ChA-1, HuH-28, and TFK-1 cholangiocarcinoma cells. We extracted RNA by using Trizol Reagent (Invitrogen) purification kit. cDNA synthesis was performed as described (18). For PCR, we used primers specific for human common leptin receptor (sense, 5′-CTTCCACTTGTCTTCGCG-3′; antisense, 5′-CTGATTCTCCATAGTGAACC-3′) and primers specific for human leptin receptor long isoform (sense, 5′-CCATGATCATTTTATCCCCA-3′; antisense, 5′-GTCAATATACACAGAAGACC-3′) and human leptin receptor short isoform (sense, 5′-TTGGTGCACTTTATTTTTCTT-3′; antisense, 5′-AGT-GGCAACATTGGTTTCAT-3′). To check for RNA integrity, we used primers specific for human β actin (sense, 5′-GAAATGCTGCTGACATTAAG-3′; antisense, 5′-CTAGAAGCATTGGTGACAGTTGGAGGGGC-3′). PCR was performed in 50 μL of PCR-buffer [20 μmol/L Tris-HCl (pH 8.0), 300 μmol/L KCl, 1.5 μmol/L MgCl2, 0.2 μmol/L deoxynucleotide triphosphates, 0.2 μg each primer, 5 units Taq DNA-polymerase (Life Technologies Bethesda Research Laboratories)]. After a 3-min denaturation step at 94°C, the reaction proceeded in 30 cycles of 45 s at 94°C, 45 s at 54°C, 40 s at 72°C, followed by 10 min at 72°C (19). Ten microliters of each PCR product were separated on a 1% agarose gel and stained with ethidium bromide. The length of the amplicons was respectively 490 bp (common isoform), 481 bp (long isoform), and 200 bp (short isoform). The intensity of the bands was determined by scanning video densitometry using the Chemilager low-light imaging system (Alpha Innotech Corp.).

Immunofluorescence. Immunofluorescence to detect leptin and leptin receptor common isoform in H-69, Mz-ChA-1, HuH-28, and TFK-1 cells was performed as already described (18). The antibody used to detect leptin receptor recognizes the common sequence present in both short and long isoform.

Effect of leptin on HuH-28 cell proliferation and intracellular pathways. The changes in cholangiocarcinoma cell proliferation were assayed by the measurement of the BrdUrd incorporation (20) in the HuH-28 cell line. After trypsinization, cells were seeded into 96-well plates (10,000 per well) in a final volume of 200 μL medium. Cells were incubated until they reached 70% of confluence and then serum starved for an overnight period. Subsequently, the following day, medium was changed and cells were stimulated with increasing doses of leptin (0–80 ng/mL) in absence or presence of 30-minute preincubation with the JAK/STAT3 antagonist AG490 (100 μmol/L) or the mitogen-activated protein (MAP)/ERK kinase (MEK) antagonist PD98059 (25 μmol/L) up to 48 h. The BrdUrd incorporation was measured using the Cell Proliferation ELISA BrdUrd assay according to the instructions provided by the vendor (Roche), as previously described (20).

After trypsinization, HuH-28 cells (1 × 10^6) were incubated into 6-well plates until they reached 70% of confluence and then serum starved for an overnight period. Subsequently, the following day, medium was changed and cells were stimulated with increasing doses of leptin (0–80 ng/mL) up to 30 min, in absence or presence of 30-min incubation with the same intracellular inhibitors described above. After two washes with ice-cold PBS, cells were resuspended in lysis buffer and sonicated thrice (10-s bursts). We performed immunoblots as previously described (17, 18). The amount of protein loaded was evaluated by immunoblots for β-actin (18). Proteins were visualized using chemiluminescence (ECL Plus kit; Amersham). The intensity of the bands was determined by scanning video densitometry using the Chemi Doc imaging system (Bio-Rad).

Effect of leptin on the migration and apoptosis of HuH-28 cells. We evaluated the effect of leptin in HuH-28 cell migration, which is one of the malignant properties of the carcinoma of the biliary tract (1), by both the CytoSelect 24-well Cell Migration Assay and the “wound-healing” assay (18). We used the Cytoselect Cell Migration Assay kit according to the instructions given by the vendor. Briefly, after overnight starvation, HuH-28 cells were seeded at 30,000 cells per well in serum-free medium, in absence or presence of leptin (40 ng/mL) or fetal bovine serum (FBS) 10%, and were allowed to migrate toward FBS for 24 h. Migratory cells were subsequently lysed and detected by fluorescence measurement. In other set of experiments, HuH-28 cells were plated on 6-well culture dishes and grown using the Connaught Medical Research Laboratories medium 1066 supplemented with 10% FBS. After reaching the confluence, cell layers were disrupted by producing a linear wound with a sterile pipette tip (21). The cells were washed with PBS to remove debris and incubated in the absence or presence of leptin (40 ng/mL). Hydroxyurea (5 mmol/L) was added to the system to arrest cell proliferation (22). At time 0 and after 24 and 72 h, samples were examined by a phase contrast microscope (Olympus) and the wound size was measured as previously described (18). We also evaluated the effect of leptin in HuH-28 cell apoptosis. Briefly, after trypsinization, cells were seeded into 96-well plates (5,000 per well) in a final volume of 50 μL medium. Cell death by apoptosis was induced by incubating HuH-28 in FBS-free medium for 4 h with glycochenodeoxycholic acid (GCDC; 400 μmol/L). In addition, cells were incubated with GCDC as above indicated in the absence or presence of a 30-min preincubation with leptin (10-40 ng/mL). Changes in caspase-3 activation were then measured by the APO-ONE Homogeneous Caspase-3/7 Assay (Promega), according to the instruction provided by the vendor.

Effect of Leptin in Thioacetamide-Induced Neoplastic Changes in Biliary Epithelium

Experimental protocol. Four-week-old obese (fa/fa) and lean Zucker rats weighing ~220 g were purchased from Charles River Laboratories. Animals were housed for 2 additional wk in a temperature-controlled environment (20–22°C) with a 12-h light-dark cycle and with free access to drinking water and to standard rat diet. After this period, rats were divided into 4 groups: the first group (n = 4, control) was represented by lean Zucker rats fed with tap water, the second group (n = 8) was represented by lean Zucker rats fed with 0.03% thioacetamide (23) in tap water, the third group (n = 4, control) was represented by obese fa/fa Zucker rats fed with tap water, and the fourth group (n = 8) was represented by obese fa/fa Zucker rats fed with 0.03% thioacetamide in tap water. Food and water were available ad libitum. For all the treatment period, the weight of the animals was biweekly monitored. After 24 wk, animals were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and sacrificed according to institutional guidelines. At the time of sacrifice, from each animal, liver specimens obtained from thioacetamide-treated lean Zucker rats as given by the vendor.

Animals were housed for 2 additional wk in a temperature-controlled environment (20–22°C) with a 12-h light-dark cycle and with free access to drinking water and to standard rat diet. After this period, rats were divided into 4 groups: the first group (n = 4, control) was represented by lean Zucker rats fed with tap water, the second group (n = 8) was represented by lean Zucker rats fed with 0.03% thioacetamide (23) in tap water, the third group (n = 4, control) was represented by obese fa/fa Zucker rats fed with tap water, and the fourth group (n = 8) was represented by obese fa/fa Zucker rats fed with 0.03% thioacetamide in tap water. Food and water were available ad libitum. For all the treatment period, the weight of the animals was biweekly monitored. After 24 wk, animals were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and sacrificed according to institutional guidelines. At the time of sacrifice, from each animal, liver was isolated, fixed in formalin, embedded in paraffin, and processed for histopathology. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Morphologic Analysis of Liver Tissues

Animal liver tissues were fixed in formalin, embedded in paraffin, processed for histopathology, stained with H&E for routine examination or with CK19 for tumor visualization, and examined by light microscopy. c-Neu, a receptor protein preferentially expressed by malignant cholangio- cytes (24), was quantitatively evaluated by c-Neu immunohistochemical staining in frozen liver specimens obtained from thioacetamide-treated lean and fa/fa Zucker rats. Tumor sections (n = 4), obtained from each group of animals, were stained for c-Neu as previously described (17, 24, 25). After examination of the staining with a microscope, photographs of 4 different fields per section (selected in number of 10 from each animal in a blinded, random fashion) were taken. Then, by computerized analysis, the number of c-Neu-positive tumoral cells (24) was calculated. The quantitative analysis was performed using an Olympus microscope (Olympus Vanox AHBT3; Olympus Optical Co Ltd) equipped with a computerized image analysis system (CUE-3 Image Analyzer; Gala Production Ltd). Furthermore, leptin was detected by immunoblots in homogenized tumoral tissue obtained from the two groups of animals, as previously described (26). Immunohistochemical staining for Hep Par 1, also known as hepatocyte antigen (27), and biliary cell–specific cytokeratin CK7 were performed in liver specimens obtained from thioacetamide-treated lean Zucker rats as previously described (27, 28). Human cholangiocarcinoma tissues were
obtained from patients ($n = 3$), undergone to surgery, fixed in formalin, and embedded in paraffin. Immunohistochemistry for leptin and leptin receptor common isoform was performed in these samples, as previously described (26). After staining, sections were counterstained with hematoxylin and examined with a microscope (Olympus Vanox AHBT3; Olympus Optical Co Ltd). Negative controls, obtained by incubating the tumor sections only with the secondary antibody, were used to confirm the specificity of the staining.

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**Figure 1.** A, RT-PCR analysis for common short and long isoforms of leptin receptor and for leptin using total RNA from H69 normal and Mz-ChA-1, HuH-28, and TFK-1 cholangiocarcinoma cells. Leptin and leptin receptors are expressed by all these cell lines. B, immunofluorescence for common leptin receptor isoform and leptin in normal and malignant cholangiocytes. All the cell lines express leptin and leptin receptors. Bar, 50 μm. C and D, effect of leptin on HuH-28 cell proliferation. Leptin, at doses from 10 to 80 ng/mL, increases the proliferation of HuH-28 cells at 24 and 48 h of incubation. Leptin at 1 ng/mL did not affect HuH-28 cell proliferation. The stimulatory effect was assessed by BrdUrd incorporation. Columns, mean; bars, SE. *, $P < 0.02$; #, $P < 0.01$ versus corresponding basal value.
Changes in c-Neu and cyclooxygenase (COX)-2 protein expression were assayed by immunoblots, using specific primary antibodies. HuH-28 cells were incubated in FBS-free medium with and without leptin (40 ng/mL) for 48 h. Western blot on protein extracts was performed as previously described (17, 18). Proteins were visualized using chemiluminescence (ECL Plus kit; Amersham). The intensity of the bands was determined by scanning video densitometry using the Chemi Doc imaging system (Bio-Rad).

Animal Care
All animal procedures were previously approved by the Institutional Animal Care and Use Committee.

Statistical Analysis
All data are expressed as mean ± SE. Differences between groups were analyzed by the Student's unpaired t test when two groups were analyzed, and ANOVA when more than two groups were analyzed. A P value of <0.05 was used to indicate statistical significance.

Results
Expression of leptin receptors and leptin in cholangiocytes.
Immortalized normal human cholangiocyte H69 cells and Mz-ChA-1, HuH28, and TFK-1 human cholangiocarcinoma cell lines expressed bands corresponding to a common sequence present in both short and long OBR isoforms, and to short and long isoforms of leptin receptor by RT-PCR (Fig. 1A). These cells also expressed a band corresponding to leptin by RT-PCR (Fig. 1A). The common isoform of leptin receptor and leptin were also detected in the four cell lines by immunofluorescence (Fig. 1B).

Effect of leptin on cholangiocarcinoma cell growth and intracellular pathways.
The effect of leptin on cholangiocarcinoma cell growth was evaluated by BrdUrd incorporation, an index of cell proliferation. Leptin (10–80 ng/mL) significantly increased the proliferation index of HuH-28 cells after 24- and 48-hour incubation compared with basal values. Leptin at 1 ng/mL did not affect HuH-28 cell proliferation (Fig. 1C–D).

Figure 2. A to D, effect of leptin on the phosphorylation of JAK/STAT3/ERK1/2 pathway in the presence or absence of the selected inhibitors evaluated by immunoblots. A and B, leptin stimulation of cholangiocarcinoma growth is associated with an increase of p-STAT3 and p-ERK 1/2 protein expression, the effect higher after 15 min incubation. C and D, the phosphorylation of STAT3 increased proportionally to the concentration of leptin used, whereas phosphorylation of ERK1/2 reached a plateau after incubation with increasing doses of leptin (20–80 ng/mL). Columns, mean of four experiments; bars, SE. *, P < 0.05; #, P < 0.02; ψ, P < 0.01 versus basal values.
Leptin did not enhance AKT phosphorylation (data not shown). There was no variation in total STAT3, total ERK1/2, and total AKT protein expression in HuH-28 cells incubated up to 30 min with 0.2% bovine serum albumin and with leptin (20-80 ng/mL). Moreover, the increase of STAT3 phosphorylation after leptin stimulation was blocked by preincubation with the JAK inhibitor AG490 but not by preincubation with the MAP kinase (MAPK) inhibitor PD98059 (Fig. 3A). Moreover, the increase in leptin-induced ERK1/2 phosphorylation was blocked by the preincubation of tumoral cells with both AG490 and PD98059 (Fig. 3B). The JAK inhibitor and the MAPK inhibitor blocked stimulatory effect of leptin on cholangiocarcinoma growth (Fig. 3C). These results indicate that the increase of intracellular p-STAT3 levels and p-MAPK play an important role in leptin stimulation of cholangiocarcinoma growth.

Evaluation of the effect of leptin on the migration and apoptosis of HuH-28 cells. We showed that, after 24 hours of incubation, leptin (40 ng/mL) increased the migration of HuH-28 cells (Fig. 4A). Such a stimulatory effect was similar to that observed after incubation with 10% FBS (Fig. 4A). Moreover, we found that the addition of leptin (40 ng/mL) to HuH-28 cells in culture reduced the time necessary for wound closure with respect to the control. After 24 up to 72 hours of incubation, there was a significant difference in wound size between the two groups of cultured cells (Fig. 4B). These data support the hypothesis that leptin has a stimulatory effect on HuH-28 cell migration, which is one of the characteristics of malignant cholangiocytes (1).

Incubation with GCDC induced apoptosis in HuH-28 cholangiocarcinoma cells. GCDC markedly increased caspase-3 activity (Fig. 4C). We found that preincubation with leptin (10 up to 100 ng/mL) prevented the GCDC-induced increase in caspase-3 activity (Fig. 4C).

Effect of leptin in thioacetamide-induced neoplastic changes in biliary epithelium. Only a few papers from the literature describe in vivo animal models for the study of cholangiocarcinoma (23, 29–31). Some reports show the malignant transformation of biliary epithelium induced by chronic administration of thioacetamide (23, 29). In this study, thioacetamide dissolved in tap water at 0.03% was chronically administrated to the animals groups for a 24-week period. Specifically, to test the effect played by the leptin-mediated signaling in the development and growth of cholangiocarcinoma, we used fa/fa Zucker rats, possessing a mutated long form of leptin receptor (32). These animals, because of the faulty long isoform of leptin receptor due to this mutation, do not respond to the action of leptin in the hypothalamus and, therefore, become obese (5). In this study, there was no increase in mortality rate in thioacetamide-treated rats with respect to control groups during the 6-month treatment period (23). At the end of the observational period, the average body weight showed that controls gained significantly more weight (control lean, 468.5 ± 24.5 g; control fa/fa, 567.0 ± 40.0 g) than did thioacetamide-treated rats (treated lean, 299.6 ± 12.9 g; treated fa/fa, 417.1 ± 16.7 g; Fig. 4D). Significantly lower body weight gain was observed in the thioacetamide-fed lean and obese fa/fa Zucker rats beginning at 2 weeks posttreatment, compared with the correspondent lean and obese controls (Fig. 4D). At the time of sacrifice, the liver of the thioacetamide-treated animals had also decreased in weight after the ingestion of thioacetamide (control lean, 22.7 ± 3.2 g versus thioacetamide-treated lean 16.3 ± 0.6 g, \( P < 0.05 \); control fa/fa, 26.4 ± 0.5 g versus thioacetamide-treated fa/fa 21.2 ± 1.5 g, \( P < 0.05 \); Fig. 4D). In addition, 100% of the thioacetamide-treated lean Zucker rats developed multiple white, round, and firm nodules over the hepatic surface, including the left, middle, and right liver lobes (Fig. 5A).

Isolated white, round nodules were present on the liver surface in a reduced number of thioacetamide-treated fa/fa Zucker rats (Fig. 5A). No neoplastic changes were present in the liver of both groups of nontreated rats and no metastases were observed after the observational period in the principal organs of all the groups of

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**Figure 3.** A, the increase of p-STAT3 after leptin stimulation is blocked by preincubation with the JAK inhibitor AG490 (100 μM/L) but not by preincubation with the MAPK inhibitor PD98059 (25 μM/L). B, the increase of p-ERK1/2 by leptin stimulation is blocked by the preincubation with both AG490 and PD98059. C, effect of leptin on HuH-28 cell proliferation in the presence of the selected inhibitors. Both the JAK and the MAPK inhibitors block the stimulatory effect of leptin on cholangiocarcinoma growth. Columns, mean of four experiments; bars, SE. *, \( P < 0.05 \) versus basal value; &., \( P < 0.05 \) versus leptin.
animals. A diffuse positive staining was observed in the tumoral cells for CK-19 and CK-7 (Figs. 5A and 6A). In thioacetamide-treated lean Zucker rat liver sections, immunohistochemistry for Hep Par 1 showed a diffuse cytoplasmic granular staining pattern in normal hepatocytes, whereas cancer cells were generally negative for Hep Par 1 (Fig. 6A). However, as previously reported (33), scattered tumoral cells were distinctly decorated by Hep Par 1 (Fig. 6A). All together, these findings confirm the biliary ductal ontogeny of these neoplastic cells, thus distinguishing this tumor from hepatocellular carcinoma (27), the most frequent primary malignancy of the liver.

Liver sections of thioacetamide-treated Zucker lean rats showed higher number of c-Neu–positive malignant cholangiocytes compared with thioacetamide-treated fa/fa Zucker rats (Fig. 5A–B). Morphologic evaluation of liver tissue of control rats did not show any malignant transformation of biliary epithelium (data not shown). Immunoblotting analysis and immunohistochemical staining showed a higher expression of leptin protein in cholangiocarcinoma cells of thioacetamide-treated fa/fa obese Zucker compared with thioacetamide-treated lean Zucker rats (Fig. 5C). Moreover, immunohistochemical analysis showed a strong staining for both leptin and leptin receptor in human malignant cholangiocytes (Fig. 5D). Morphologic evaluation of liver tissue of thioacetamide-treated lean Zucker rats showed cholangiocarcinoma tissue with packed tubular glands lined by columnar and cuboidal epithelial cells, which show a clear or slightly eosinophilic cytoplasm (Fig. 6B; refs. 23, 28). Neoplastic cells displayed a middle size nucleus with a small nucleolus and dispersed chromatin. Also, the presence of mucin is evident in the cell cytoplasm and glandular lumen. All these characteristics were also present in cholangiocarcinoma tissue obtained from humans (Fig. 6B). To strengthen the hypothesis that leptin plays an important role in the oncogenic process, we stimulated HuH-28 cholangiocarcinoma cells with leptin 40 ng/mL for 48 hours and we...
showed that leptin enhances the expression of c-Neu and COX-2 (Fig. 6C). The fact that these factors are commonly involved in the cholangiocarcinogenesis process indicates that the increasing of the circulating leptin promotes malignant growth of biliary epithelium.

**Discussion**

In this study, we observed that normal and malignant cholangiocytes express leptin and both long and short isoform of leptin receptors. We showed that *in vitro*: (a) leptin stimulates growth, migration, and prevents apoptosis of HuH-28 cholangiocarcinoma cells; (b) the enhancement of cholangiocarcinoma cell growth by leptin is associated with an increase of STAT-3 and ERK1/2 phosphorylation, without changes in p-AKT; moreover, *in vivo* (c) genetic ablation of leptin-mediated signaling inhibits cancer development and growth in an animal model of cholangiocarcinoma.

Several studies show the involvement of neuroendocrine system in the regulation of cholangiocarcinoma cell growth. Estrogens and insulin-like growth factor I enhance cholangiocarcinoma cell proliferation (34), gastrin, somatostatin, and γ-aminobutyric acid inhibit cholangiocarcinoma cell proliferation and induce cell apoptosis (17, 18, 35). All these data suggest that a more accurate knowledge on which neuroendocrine factors affect cholangiocarcinoma cell pathobiology (4) would be very promising to design valid therapeutic strategies against bile malignancies. Strong epidemiologic evidences suggest the existence of a close link between obesity, a clinical condition characterized by high levels of circulating leptin (9) and a multitude of cancers such as mammary (11), endometrial (12), prostate (10), hepatocellular (36), colon (37), pancreatic (38), adenocarcinoma of esophagus (39), and cholangiocarcinoma (7). In this setting, consistent data show that leptin favors the genesis and progression of a multitude of tumors.
Moreover, leptin levels are elevated after common bile duct ligation and CCL4-induced liver fibrosis, both known risk factors for cholangiocarcinoma development (43). However, the link between leptin and cholangiocarcinoma has not been established yet. Thus, our study is the first exploring if leptin plays a role in modulating cholangiocarcinoma cell pathophysiology. There is only one evidence in literature of the presence of leptin receptor in bile duct cells of one of mammalian species (44). Here, we showed the presence of leptin and leptin receptors in cholangiocytes and showed that increasing doses of leptin stimulate HuH-28 (e.g., intrahepatic cholangiocarcinoma) cell proliferation in vitro in a dose-dependent manner up to 48 hours of incubation. Moreover, we found an increase of STAT3 and ERK1/2, but not of AKT, phosphorylation in HuH-28 cells incubated with leptin. Subsequently, we have shown that the stimulatory effect of leptin in cholangiocarcinoma cell growth was blocked by preincubation with AG490 and PD98059, respectively, JAK/STAT and MEK inhibitor, thus supporting the hypothesis that leptin, through stimulation of its own receptors, activates several intracellular mechanisms, among them JAK/STAT3 and ERK1/2, which lead to an increase of cholangiocarcinoma cell proliferation. Furthermore, because preincubation with AG-490, but not with PD98059, reduces p-STAT3 expression in cholangiocarcinoma cells after stimulation by leptin, and the increase of p-ERK1/2 consequent to leptin stimulation is blocked by both AG-490 and PD98059, it is evident that JAK/STAT3 is upstream of ERK1/2. The role played by STAT3 and ERK1/2 in regulating malignant cholangiocyte growth has been described (18, 45). We speculate that JAK/STAT3 and ERK1/2 are both activated in leptin-treated cholangiocarcinoma cells and that JAK/STAT3 represent an upstream of such a cascade, in a similar fashion to what has been found in gastric cancer cell lines (46). STAT family of transcription factors have been recognized as critical integrators of cytokine and growth factor receptor signaling required to perform several processes of cell metabolism such as proliferation, survival, differentiation, and motility (47). Emerging data show that a wide variety of human cancer cell lines, among them cholangiocarcinoma cells, and primary tumors, present a constitutively activated STATs (45, 47) and, particularly, several reports show that STAT3 participates in the development and progression of human cancers (47).

In our study, we also showed that leptin has a stimulatory action on the migratory capacities of HuH-28 cells; thus, this hormone might increase the metastatic potential of cholangiocarcinoma. To better reproduce the physiologic conditions, in the experiments aiming to characterize the intracellular pathways activated by leptin and to test the effect of such hormone in regulating malignant cholangiocyte migration, we used a dose of this protein of 40 ng/mL, which is generally correspondent to the quantity of leptin circulating in the obese patients (6). To strengthen the hypothesis that leptin enhances cholangiocarcinoma growth, we showed that this hormone prevented apoptosis of HuH-28 malignant cholangiocytes (Fig. 4C).

Only a few experimental carcinogenesis models of cholangiocarcinoma have been established in animals (23, 29–31). Thioacetamide, an agent originally used to preserve food, is a potent hepatotoxin and carcinogen (23, 29). In the present study, we induced cholangiocarcinoma in liver rats by administering thioacetamide dissolved at 0.03% in drinking water for 24 weeks. These results were in accordance with the data showed by Yeh and colleagues (23) in the only published study in literature, in which cholangiocarcinoma was induced in Sprague-Dawley rats by the
same dosage of thioacetamide we used. The negative staining of these tumor cells for the hepatocyte antigen Hep Par 1 (27), together with the positive staining for biliary cytokeratin CK7 and CK19, confirm the bile ductular ontogeny of these neoplastic cells, thus distinguishing this tumor from hepatocellular carcinoma (27), the most frequent primary malignancy of the liver. In addition, this animal model of carcinogenesis acquires relevancy because a high similarity between cholangiocarcinoma tissue developed in Zucker rats after chronic administration of thioacetamide and cholangiocarcinoma observed in human liver is observed (Fig. 6). Moreover, this is the first study in which cholangiocarcinoma was established in Zucker rats, an animal model previously never used to study the pathophysiology of biliary malignancies. Lean Zucker rats possess normal metabolic functions and represent ideal controls (48). We used fa/fa Zucker rats because we wanted to test the effect due to the lack of the functional long form of leptin receptor (32) on the process of cholangiocarcinogenesis. Zucker fa/fa rats, because of a congenital mutation at long form of leptin receptor (OBRI), possess such receptor nonresponding to the circulating leptin. This was the only way to eliminate the action of leptin through OBRI in all the body cells. We found that the inactivation of this receptor proper of the obese Zucker fa/fa rats induced a decrease in cholangiocarcinoma development and growth in these animals with respect to their correspondent lean controls. However, a difference was macroscopically evident in the liver of the sacrificed animals. In fact, whereas the liver of the thioacetamide-treated lean Zucker rats was completely subverted by the presence of neoplastic nodules, the liver of the thioacetamide-treated fa/fa Zucker rats showed a less-compromised structure, presenting isolated tumoral nodules on the surface (Fig. 5). We were unable to compare the neoplastic mass of the two groups of treated animals only by the macroscopic observation of the liver. Thus, we performed immunohistochemical analysis for c-Neu, the tyrosine kinase growth factor receptor overexpressed on the malignant cholangiocyte surface (24). By this technique, we found that thioacetamide-treated fa/fa Zucker rats showed less tumoral proliferation infiltrating their liver with respect to thioacetamide-treated lean Zucker rats, confirming that the signal mediated by leptin is important for cholangiocarcinoma growth.

To strengthen the hypothesis that leptin plays an important role in the oncogenic process, we showed that leptin enhances the expression of c-Neu and COX-2 in human cholangiocarcinoma cells. The fact that these factors are commonly involved in the cholangiocarcinogenesis process indicates that the increasing of the circulating leptin promotes malignant growth of biliary epithelium.

Moreover, the immunohistochemical analysis of the liver specimens showed a higher expression of leptin protein in cholangiocarcinoma cells of thioacetamide-treated fa/fa Zucker compared with thioacetamide-treated lean Zucker rats. This intriguing data suggests that malignant cholangiocytes could produce leptin, which contributes to modulate their own proliferation by an autocrine/paracrine mechanism. The increase of leptin content in cholangiocarcinoma cells in thioacetamide-treated fa/fa Zucker rats could be explained by the lacking of the negative feedback consequent to the loss of the functional long form of leptin receptor in these animals. To confirm the presence of leptin and leptin receptor in human cholangiocarcinoma tissues, we performed immunohistochemical analysis in samples obtained from patients that underwent surgery. A strong staining of both leptin and leptin receptor were expressed in human malignant cholangiocytes.

In summary, our study showed that leptin stimulates cholangiocarcinoma cell migration and proliferation in vitro through a STAT-3 dependent activation of ERK1/2. In addition, leptin-mediated signal enhances cholangiocarcinoma development and growth in an in vivo animal model. Together, these findings suggest that leptin is involved in the cholangiocarcinogenesis process and favors the growth of biliary malignancies. This implies that leptin could, at least in part, contribute to the increased incidence of cholangiocarcinoma in obese patients. In conclusion, these data advance the hypothesis that the modulation of the leptin-mediated signal is implicated in the growth of the biliary malignancies, thus being considered a novel potential target in cholangiocarcinoma prevention and treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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