Competitive Inhibition of Leptin Signaling Results in Amelioration of Liver Fibrosis Through Modulation of Stellate Cell Function

Eran Elinav,[1] Mohammad Ali,[1] Rafi Bruck,[1] Eli Brazowski,[1] Adam Phillips,[1] Yami Shapira,[1] Meirav Katz,[1] Gila Solomon,[2] Zamir Halpern,[1] and Arieh Gertler[2]

Leptin signaling is involved in T-cell polarization and is required for profibrotic function of hepatic stellate cells (HSCs). Leptin-deficient *ob/ob* mice do not develop liver fibrosis despite the presence of severe long-standing steatohepatitis. Here, we blocked leptin signaling with our recently generated mouse leptin antagonist (MLA), and examined the effects on chronic liver fibrosis *in vivo* using the chronic thioacetamide (TAA) fibrosis model, and *in vitro* using freshly-isolated primary HSCs. In the chronic TAA fibrosis model, leptin administration was associated with significantly enhanced liver disease and a 100% 5-week to 8-week mortality rate, while administration or coadministration of MLA markedly improved survival, attenuated liver fibrosis, and reduced interferon γ (IFN-γ) levels. No significant changes in weight, serum cholesterol, or triglycerides were noted. *In vitro* administration of rat leptin antagonist (RLA), either alone or with leptin, to rat primary HSCs reduced leptin-stimulated effects such as increased expression of α-smooth muscle actin (α-SMA), and activation of α1 procollagen promoter. *Conclusion:* Inhibition of leptin-enhanced hepatic fibrosis may hold promise as a future antifibrotic therapeutic modality. (HEPATOLOGY 2009;49:278-286.)

Leptin, a 16 kDa hormone produced mainly by adipose tissue, is an important metabolic signal involved in body weight regulation, while its deficiency is associated with development of extreme obesity and the metabolic syndrome.[1] In addition, direct peripheral actions of leptin have been demonstrated in a variety of tissues and expression of the leptin receptor (LR) is now well documented on hemopoietic precursors, and immune cells, endothelium, liver, and adipose tissue, among others.[2]

Emerging evidence suggests a critical role for leptin in hepatic fibrogenesis. Leptin-deficient *ob/ob* mice and LR-deficient *db/db* mice develop significant steatosis, yet are resistant to the development of liver fibrosis.[3,4] Leptin enhances profibrogenic responses in the liver caused by hepatotoxic chemicals.[5] Leptin's profibrogenic activity is mediated mainly by increased production of collagen type I by hepatic stellate cells (HSCs), mediated via activation of the Janus kinase (JAK)–signal transducers and activator of transcription (STAT) mitogen-activated protein kinases, phosphatidylinositol 3-kinase/protein kinase B (AKT) signaling pathways, and reduced FAS-ligand-associated apoptosis.[6-9] In addition, leptin-induced HSC collagen accumulation is enhanced by decreased expression of matrix metalloproteinase 1 via the synergistic actions of the JAK/STAT pathway and the JAK-mediated H_2O_2-dependent extracellular signal-regulated kinase 1/2 and p38 pathways.[10,11] Similarly, reduced levels of leptin's major counterregulatory adipokine adiponectin are positively correlated with the progression of hepatic steatosis and fibrosis in animal models and in humans with nonalcoholic steatohepatitis (NASH).[12,13]

In addition, leptin has potent immunomodulatory properties. Structurally, leptin is similar to interleukins (ILs) 2, 6, and 15, making it a member of the cytokine superfamily.[14] LRs are structurally similar to hematopoietic cytokine receptors,[15] and they are found on CD4 and CD8 lymphocytes, monocytes,[16] natural-killer lymphocytes,[17] and HSCs.[18] Leptin enhances T-cell proliferation and pro-inflammatory cytokine secretion via activation of JAK/STAT signaling.[19] Leptin-deficient *ob/ob* mice are resistant to several T-helper 1 cell (Th1)-mediated immune disorders, including allergic experimental encephalomyelitis,[20] concanavalin A hepatitis, experimental arthritis, and autoimmune nephritis, but are extremely vulnerable to lipopolysaccharide induced hepatic damage.[21] Leptin replenishment reverses these disorders.[22] Leptin-induced pro-fibrogenic and pro-inflammatory responses may be linked, as it was recently demonstrated that LR activation in HSCs leads to increased expression of proinflammatory and proangiogenic cytokines, indicating a complex role for leptin in the regulation of the liver wound-healing response.[23]

One of the most effective methods of blocking leptin action *in vivo* is the use of leptin antagonists, capable of binding to, but not activating, LRs.[24] Using the sensitive bidimensional hydrophobic cluster analysis, LDFI in the loop that connects helices A and B of leptin were identified as a putative sequence contributing directly or indirectly to leptin's site III, responsible for the activation of the leptin receptor ObR.[25,26] While mutation of some or all of these amino acids to alanine in human, rat, mouse, and ovine leptin does not change their binding properties, it abolishes their biological activity and converts them into potent antagonists.[27,28]

Here, we present a first report on the use of mouse leptin antagonist (MLA) for the treatment of hepatic fibrosis in a murine model, and suggest that this effect is mediated by inhibition of HSC activation and function.

Materials and Methods

Antibodies and Reagents. Anti-rat α-smooth muscle actin (α-SMA) and anti-glyceraldehyde phosphodehydrogenase (GADPH) monoclonal antibodies (mAbs) were purchased from DAKO (Glostrup, Denmark). The anti-rat long isoform LR (OB-Rb) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Thioacetamide (TAA) and platelet-derived growth factor (PDGF) were obtained from Sigma-Aldrich (Rehovot, Israel). Mouse leptin, mouse leptin antagonist (MLA), and rat leptin antagonist (RLA) were prepared in-house as described.[27]

Mice. In all *in vivo* experiments, female 8-week-old to 10-week-old C57bl mice were used. For induction of liver cirrhosis, mice were injected intraperitoneally with 200 mg/kg TAA three times a week for 5 to 8 weeks. Experimental mouse groups (n = 8) were administered mouse leptin (1 or 0.5 μg/g per day in two injections), MLA (50 μg/g per day), or a combination of the two. For isolation of primary HSCs, 10-week-old female Wistar rats were used. All animals were maintained under 12-hour light-dark cycles, in accordance with regulations of the institutional animal and care authority of the Tel Aviv Sourasky Medical Center.

Serum Triglyceride and Cholesterol Levels. Sera for triglyceride and serum cholesterol measurements were obtained in glass tubes, centrifuged, and analyzed on the day of sampling using a Kone Progress Selective Chemistry Analyzer (Ruukintie, Finland). Serum triglyceride levels were measured in a spectrophotometer (Cobas DP-25) at a wavelength of 550 nm. All serum samples were processed in the same laboratory using the same methods and the same reference values.

Cytokine Measurement. Anti-mouse IL-10 and anti-mouse interferon γ (IFN-γ) enzyme-linked immunosorbent assay kits (ELISA MAX) were purchased from BioLegend (San Diego, CA). Serum cytokine levels were determined in accordance with the manufacturer's instructions.

Histological Examination. Histological specimens were taken at the end of the study period for all mouse groups, and prior to death in moribund leptin-treated mice in weeks 6 to 8 of the experiment. For each mouse, liver segments were fixed in 10% buffered formaldehyde and embedded in paraffin for histological analysis. Sections (5 μm) were stained with either hematoxylin/eosin or Sirius red. Additionally, α-SMA immunohistochemistry was analyzed using the DAKO kit according to the manufacturer's instructions. For Sirius red staining, sections of liver (3-5 μm) were stained in Sirius red solution, 1% (wt/vol) Direct Red (Sigma Aldrich, Rehovot, Israel) in picric acid for 1 hour and then washed in 0.5% (vol/vol) acetic acid solution. Stage of fibrosis was determined in both hematoxylin/eosin and Sirius red sections on a scale of 0 to 4 (0, normal; 1, portal fibrosis; 2, periportal fibrosis; 3, septal fibrosis; and 4, cirrhosis). Degree of severity of liver fibrosis were derived from blind analysis of each of the animals in each group. α-SMA staining was assessed by the product of the percentage area of positive staining by the intensity of staining (0, no staining; 1, weak staining; and 2, strong staining).

Isolation of Primary HSCs. HSCs were isolated from Wistar rats by sequential pronase/collagenase digestion followed by density-gradient centrifugation. After anesthesia and abdominal exploration, the liver was per-

fused via the portal vein with 50 mL Gey's balanced salt solution (GBSS; Gibco BRL, Rockville, MD). Perfusion was followed by 200 mL of GBSS containing 140 mg pronase (Roche Diagnostics, Basel, Switzerland) and 100 mg collagenase (Worthington Biochemedical Corporation, Lakewood, NJ). The digested liver was mashed *ex vivo* and incubated at 37°C for 25 minutes in 100 mL of GBSS solution containing 0.025% (wt/vol) pronase, 0.025% (wt/vol) collagenase, and 20 mg/mL deoxyribonuclease (DNase I; Sigma, Rehovot, Israel). The resulting suspension was filtered through a 150 mm steel mesh and centrifuged on an 8.2% Nycodenz cushion (Nycomed Pharma AS, Oslo, Norway) at 1,400*g* for 20 minutes at 25°C, which produced an HSC-enriched fraction in the upper whitish layer. Cells were washed by centrifugation (400*g*, 25°C, 10 minutes) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol,) fetal calf serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin for 7 days. Purity of the HSC culture, determined by Oil-Red-O staining, was routinely greater than 95%.

Proliferation Assay. Following 7 days of culture, primary HSCs were cultured for 48 hours in a 96-well plate, in triplicate, with various concentrations of leptin, RLA, or both, and PDGF. Proliferation was assessed by 2,3 bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide assay.

Western Blotting. HSC protein was extracted by incubating the cells for 30 minutes on ice in lysis buffer and a complete mini–ethylene diamine tetraacetic acid–free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Extracts were normalized to total protein content using Bradford reagent. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted onto a Hybond C-extra membrane, blocked overnight in 5% (wt/vol) dry nonfat milk, incubated with primary mouse anti-α-SMA mAb (diluted 1/20,000) for 1 hour at room temperature, and exposed to peroxidase-conjugated goat anti-mouse immunoglobulin G (diluted 1/10,000) for 1 hour at room temperature. Immunoreactivity was revealed using an enhanced chemiluminescence kit (Sigma Aldrich). Protein expression was normalized to that of GADPH.

Determination of Luciferase Activity Using Rat Pro-α1(I) Collagen Promoter and Rat α2(I) Collagen Promoter Upstream of the Luciferase Reporter. The plasmid ColCAT3.6 contains 3,520 bp of rat pro-α1(I) collagen promoter followed by 115 bp of the rat α1 (I) first exon cloned upstream of the chloramphenicol acetyltransferase gene within the *E. coli* phagemid vector pUC12; this plasmid was a gift from Dr. D. Rowe (University of Connecticut, CN). The promoter region was

excised and introduced into GL3-luciferase. The plasmid containing the 378-bp α2(I) collagen promoter upstream of the luciferase gene was obtained from Dr. F. Ramirez (Mount Sinai Medical Center, NY).

Primary HSCs were transiently transfected after 3 days of culture using the Gene Jammer kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, with a mix of 0.5 μg α2(I) collagen plasmid and 1.7 μg β-galactosidase plasmid. Transfected cells were cultured for 24 hours in 24-well plates with either 250 ng/mL leptin, 12.5 mg/mL leptin antagonist, or a combination of the two. Luciferase activity was determined using the Luciferase Assay kit (Applied Biosystems, Bedford, MA) using the Lumistar Galaxy Luminometer. The results were normalized to β-galactosidase activity. Each experiment was repeated three times.

Statistical Analysis. Data were expressed as mean $\pm$ standard error of the mean (SEM) of at least six mice. Statistical analysis was performed by *t* test and two-way analysis of variance. $P < 0.05$ was considered significant.

Results

Effect of Mouse Leptin and MLA on TAA-Induced Liver Fibrosis in Mice. Thrice-weekly injections of TAA resulted in progressively higher mortality rates, up to 40% after 6 to 8 weeks (Fig. 1A). This effect was drastically enhanced by simultaneously giving two daily injections of mouse leptin, each consisting of 0.5 μg/g body weight, resulting in 100% mortality after 5 weeks. Lowering the leptin dose to 0.25 μg/g twice daily had a milder effect, although 100% mortality was nevertheless achieved at week 8. Adding MLA to mice with TAA fibrosis resulted in a clear trend toward reduction of TAA-induced mortality, but the effect was not statistically significant (Fig. 1B). In contrast, coadministration of MLA totally abolished leptin's mortality-enhancing effect (Fig. 1C).

Histological analysis of liver sections from mice at the end of the experiment or at time of death (in the case of TAA + leptin treatments) revealed that TAA causes marked fibrosis (Fig. 2A) that is dramatically increased by simultaneous leptin treatment (Fig. 2 B) but almost completely abolished by MLA alone (Fig. 2C) or MLA coadministered with leptin (Fig. 2D). The fibrosis results were closely paralleled by Sirius red staining, which demonstrated significant enhancement of the extent and width of collagen septa in mice administered leptin + TAA, as compared to the significantly decreased fibrosis following MLA administration in mice treated with either TAA or TAA + leptin (Fig. 3). It should be noted that no collagen septa were observed in control, leptin-treated, or MLA-
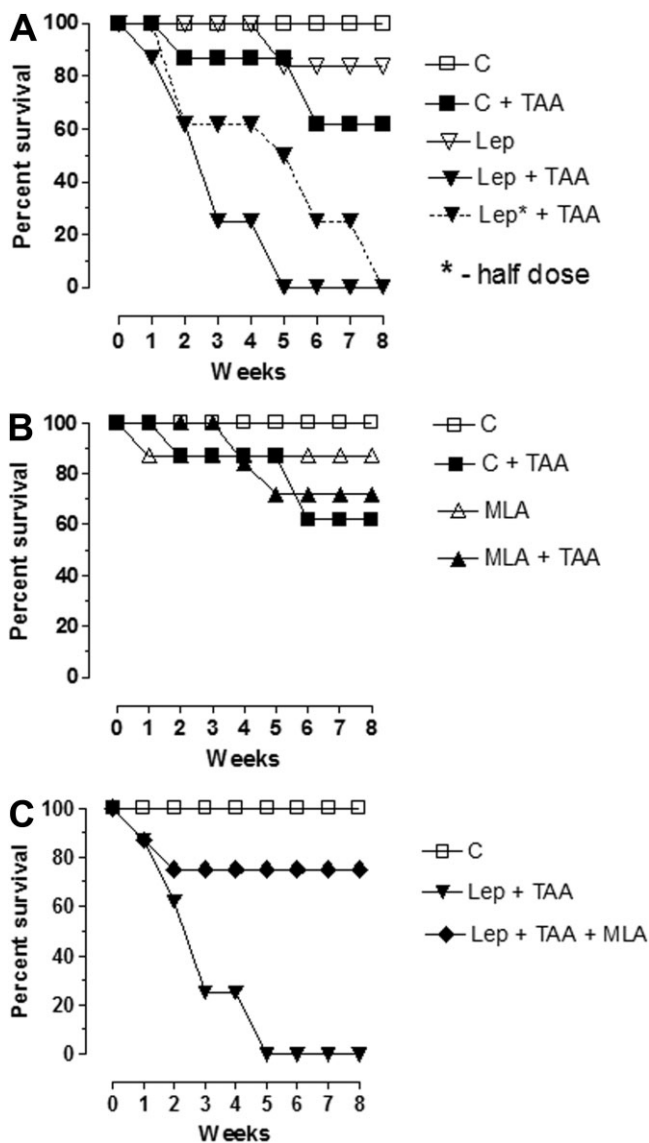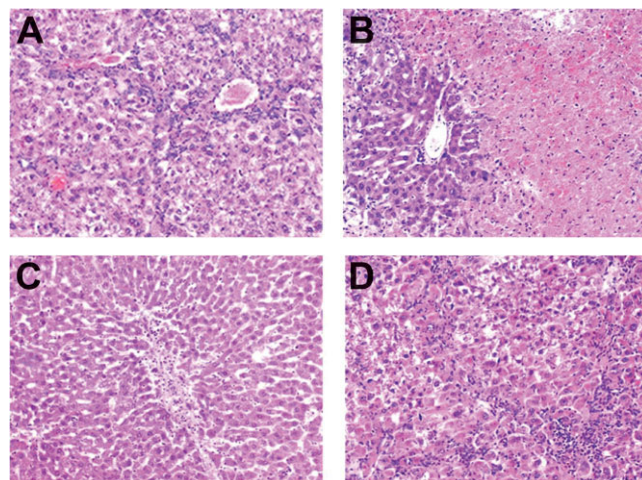
Fig. 2.   Representative hematoxylin-eosin staining of liver sections. For each mouse, a single liver segment was fixed in 10% buffered formaldehyde and embedded in paraffin for histological analysis. Hematoxylin-eosin staining was performed to ascertain tissue morphology and cell distribution ($\times$20 magnification). (A) TAA; (B) TAA + leptin; (C) TAA + leptin antagonist (MLA); and (D) TAA + leptin + MLA.

for TAA + leptin, 1.50 ± 0.29 for TAA + MLA, and 2.08 ± 0.42 for TAA + leptin + MLA. The effect of MLA addition was statistically significant ($P < 0.05$) in both comparisons. Histological analysis of fibrosis in control mice or in mice treated with leptin or MLA was graded as zero (data not shown). Moreover, immunostaining for α-SMA expression (Fig. 4) clearly showed enhanced hepatic α-SMA expression in TAA and leptin + TAA mice (calculated intensity of 65 ± 19), while

Fig. 1.   Survival in TAA fibrosis. TAA fibrosis was induced in wild-type mice by thrice-weekly injection of TAA (200 μg/g body weight) with or without 1.0 μg/g body weight or 0.5 μg/g body weight mouse leptin (L) and with or without 50 μg/g body weight of mouse leptin antagonist (A) (n = 8). (A) Mortality was significantly enhanced in leptin-administered (both doses) versus control TAA-induced mice ($P < 0.05$). (B) Mortality was reduced in leptin antagonist-administered versus wild-type TAA fibrosis-induced mice. (C) Mortality was reduced in leptin + leptin antagonist-administered versus leptin-administered TAA fibrosis-induced mice ($P < 0.05$).

treated mice (data not shown). No significant elevation or changes in hepatic aminotransferase levels between the different groups were noticed (data not shown). The stages of fibrosis (hematoxylin-eosin sections), as graded blindly by hepatic pathologists, were (mean $\pm$ SEM): 2.75 ± 1.1 for TAA, 4.00 ± 0 for TAA + leptin, 2.0 ± 0 for TAA + MLA, and 3.16 ± 1.16 for TAA + leptin + MLA. The stages of fibrosis in Sirius red–stained sections were (mean $\pm$ SEM): 2.40 ± 0.19 for TAA, 3.00 ± 0.07

Fig. 3.   Representative Sirius red staining of liver sections. For each mouse, a single liver segment was fixed in 10% buffered formaldehyde and embedded in paraffin for histological analysis. Sirius red staining was performed to ascertain the degree of hepatic fibrosis. Red-stained areas represent hepatic collagen deposits ($\times$20 magnification). (A) TAA; (B) TAA + leptin; (C) TAA + leptin antagonist (MLA); and (D) TAA + leptin + MLA.
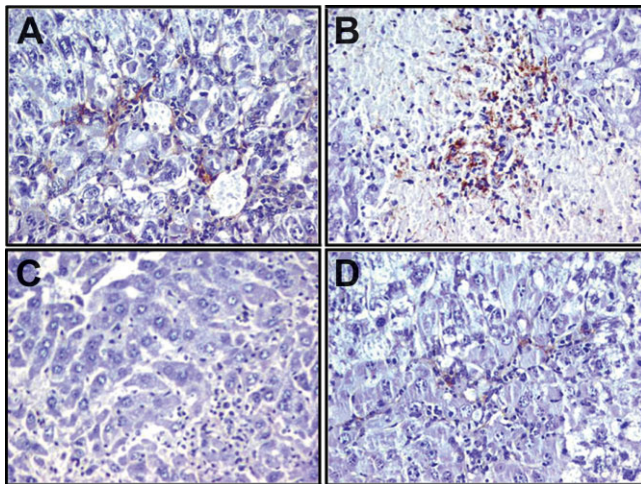
Fig. 4. Distribution of activated hepatic stellate cells (HSCs). Activated HSCs were identified by immunohistochemical staining of α-smooth muscle actin (α-SMA). Nuclei were counterstained with hematoxylin ($\times$20 magnification). (A) TAA; (B) TAA + leptin; (C) TAA + leptin antagonist (MLA); and (D) TAA + leptin + MLA.

Fig. 5. IFN-γ levels in mice with TAA-induced fibrosis. Eight mice were used for each treatment and serum samples were withdrawn after 0, 14, 22, 30, and 34 days of treatment. Because of the limited amount of sera aliquot obtained, from two to three mice in the same treatment group were randomly pooled to three pools. The results are presented as mean $\pm$ SEM (n = 3). Means significantly different from the respective controls are marked by *$P < 0.05$ or **$P < 0.01$.

this expression was significantly reduced following MLA administration (5 ± 5.5, 3.5 ± 4.3, and 6.5 ± 5 in TAA, TAA + MLA, and TAA + leptin + MLA, respectively; $P < 0.05$ for all comparisons).

The TAA-injection experiment was repeated under identical conditions but terminated after 34 days when the first mice in the TAA + leptin treatment (two out of seven) died. As peripheral antagonism of leptin activity may also result in immunomodulation, further contributing to a reduction in chronic hepatic fibrosis, we tested the blood profiles of INF-γ and IL10, hallmarks of T-cell Th1 and Th2 polarization, respectively. While serum levels of IL10 were generally very low and did not vary among mouse groups (NS = not significant, data not shown), serum IFN-γ was significantly elevated in TAA fibrosis-induced mice as early as 2 weeks after disease induction and continued to increase until day 34 (Fig. 5). IFN-γ was even more elevated in TAA + leptin–treated mice, but it was reduced to control levels in both TAA + MLA–treated and TAA + leptin + MLA–treated mice ($P < 0.05$ for both groups). These results support the strong immunomodulatory function of MLA.

Despite high doses of MLA, no weight gain was observed as compared to the controls (Table 1) after either 4 or 8 weeks of treatment. Both TAA and leptin treatments resulted in a mild (10%-15%) but nevertheless significant ($P < 0.05$) reduction in body weight, an effect that was fully or partially reversed by simultaneous MLA treatment. Serum levels of cholesterol and triglycerides, determined during the course of the experiment, varied between 74 and 120 mg/dL and between 55 and 130 mg/dL, respectively, but did not differ significantly
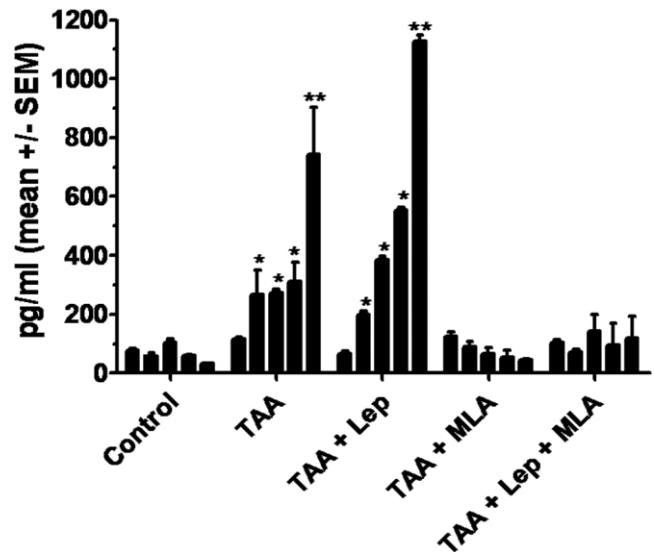
among the various treatments at any of the time points and did not reach pathological levels.

Effect of Leptin and Leptin Antagonist on Gene Expression and Proliferation in HSCs. The leptin responsiveness of HSCs has been well documented.[17] We therefore used primary rat HSCs to validate the observed leptin effects and to test whether those effects can be attenuated or abolished by RLA. Primary HSCs expressed the long isoform LR (data not shown). Culture with leptin dramatically elevated α-SMA expression while the addition of RLA attenuated this effect (Fig. 6A). To validate the effect of leptin on α1(I) and α2(I) collagen expression,

Table 1. Weight Changes in TAA-Induced Fibrosis After 4 and 8 Weeks of Treatment

Experimental treatment	Weight After 4 Weeks (g)	Weight After 8 Weeks (g)
Control	21.98 ± 0.57 (4)	22.78 ± 0.54 (4)
TAA	19.59 ± 0.42** (7)	19.72 ± 0.68** (5)
MLA	21.21 ± 0.38 (7)	22.58 ± 0.35 (7)
Mouse leptin	18.83 ± 0.25** (8)	20.03 ± 0.35** (7)
TAA + MLA	19.82 ± 0.49* (7)	21.28 ± 0.50 (6)
TAA + mouse leptin	17.16 ± 0.23** (3)	All dead
TAA + mouse leptin + MLA	19.62 ± 0.58* (6)	18.97 ± 0.44** (6)

Results are given as mean $\pm$ SEM and the number of mice at each time point follows, in parentheses. Means significantly different from the respective control are marked with asterisks (*$P < 0.05$; **$P < 0.01$). The initial number of mice per treatment was seven or eight, except for four in the control treatment.
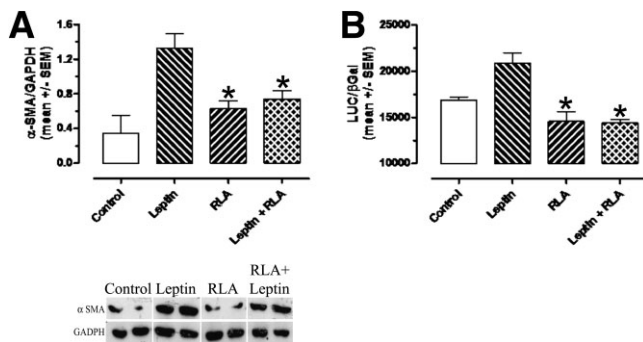
Fig. 6. Effect of leptin antagonist on HSC activity. Wild-type primary rat HSCs were cultured for 7 days in the presence of either 1 μg/mL leptin, 50 μg/mL leptin antagonist, or a combination of both. (A) α-SMA expression was measured after 7 days by western blotting. (B) Primary HSCs were transfected with a vector encoding a promoter reporter fused to a reporter gene (luciferase; LUC) and cultured for 48 hours in the presence of either 1 μg/mL leptin, 50 μg/mL rat leptin antagonist (RLA), or a combination of the two. Results are presented as the ratio of luminescence between reporter plasmid-transfected cells and control β-galactosidase vector-transfected cells.

we used its promoter fused to a reporter gene (luciferase). Using α2(I) collagen promoter, the significant effect of leptin was abolished by RLA (Fig. 6B). No leptin-mediated effect was detected using α1(I) collagen promoter (data not shown).

As PDGF is a known growth factor in HSC activation, we also tested whether its action is affected by leptin, and whether such an effect would be abolished by RLA. The results confirmed the growth-promoting activity of PDGF, and revealed that this activity is not affected by leptin or RLA (Table 2).

Discussion

This is the first report to demonstrate that leptin inhibition by a competitive leptin antagonist results in attenuation of chemically-induced hepatic fibrosis in a murine model, as manifested by improved survival, reduced hepatic α-SMA expression, and reduced serum IFN-γ levels. These effects are mediated, at least in part, by inhibition of HSC activation and function via suppression of α2(I) collagen gene expression. Throughout 8 weeks of treatment, neither significant changes in body weight, serum cholesterol or triglycerides, nor development of NASH were noted. The antagonist's antifibrogenic effect was most pronounced in hyperleptinogenic situations when fibrogenesis and related mortality were greatly enhanced, but was also evident in liver histology, liver α-SMA immunostaining, and IFN-γ levels of non-leptin-treated mice. In addition to the potential therapeutic implications, our approach provides direct evidence for the notion of a link between "metabolic" alterations, reflected by changes in leptin levels and alterations in liver

fibrosis and chronic hepatitis. A similar association has been recently suggested to exist between hepatitis B virus replication and survival and local hepatic metabolic factors, hence termed the "metabolovirus" theory.[29,30]

Chronic hepatitis and associated fibrosis are a worldwide medical problem, with chronic alcoholism and hepatitis C virus being the two major causes of the disease.[31] A direct link has been suggested between hyperleptinemic states and the progression and severity of several chronic liver diseases. Obesity, type 2 diabetes mellitus, and dyslipidemia, conditions closely associated with the development of NASH, are characterized by both insulin and leptin resistance.[32] In NASH patients, especially those with fibrosis, increased LR messenger RNA and protein expression have been noted, which are closely related to the anthropometric characteristics analyzed, and independent of NASH development.[33] While hepatitis C virus–associated insulin resistance is most likely an adipokine-independent phenomenon,[34] it has been suggested, in both animal models and humans, that chronic hepatitis due to hepatitis B and C is associated with higher leptin levels and that increased serum leptin levels represent a negative prognostic factor for response to therapy.[35,36] Moreover, it has been demonstrated in many animal models that leptin augments both inflammatory and profibrogenic responses, and is in fact an essential mediator of hepatic fibrosis in response to chronic liver injury, be it metabolic or toxic in etiology.[37,38]

Leptin's profibrogenic effect is mediated directly by activation of HSCs through their LR, as manifested by expression of α-SMA and activation of both collagen α1(I) and α2(I) genes, leading to secretion of extracellular matrix.[39,40] Leptin's activation of both collagen type I

Table 2. Effect of Platelet-Derived Growth Factor (PDGF), Rat Leptin, and Rat Leptin Antagonist (RLA) on Hepatic Stellate Cell (HSC) Proliferation

Treatment	Absorbance at 595 nm (mean ± SEM)
Control	1.33 ± 0.04
PDGF	2.17 ± 0.05*
PDGF + leptin (100 ng/mL)	2.31 ± 0.06*
PDGF + leptin (250 ng/mL)	2.22 ± 0.04*
PDGF + leptin (500 ng/mL)	2.17 ± 0.07*
PDGF + leptin (100 ng/mL) + RLA†	2.13 ± 0.08*
PDGF + RLA†	2.14 ± 0.02*
Leptin (100 ng/mL)	1.42 ± 0.04
Leptin (250 ng/mL)	1.41 ± 0.04
Leptin (500 ng/mL)	1.44 ± 0.03
Leptin (100 ng/mL) + RLA†	1.11 ± 0.03
RLA†	1.13 ± 0.11

*Significantly different from the control ($P < 0.001$). †5 μg/mL.

genes has been demonstrated to be direct and not dependent upon transforming growth factor (TGF)-β1–responsive elements.[41] In addition, leptin also plays a permissive role in HSC activation and function through induction of heightened sensitization to TGF-β1, secreted by Kupffer cells, sinusoidal endothelial cells, and HSCs themselves. The centrality of leptin's direct profibrogenic effect has also been demonstrated in wound healing, as well as in diabetic ophthalmopathy and glomerulosclerosis, in which leptin has been shown to augment collagen gene expression.[42,43]

Our data provide convincing evidence for a mechanism of liver fibrosis attenuation by leptin antagonist via direct inhibition of HSC activation and function. Our *in vitro* results demonstrate a significant reduction in HSC α-SMA expression, as well as direct inhibition of α2(I) collagen promoter activity. These results are further supported by our *in vivo* results, demonstrating a reduction in chemically-induced liver fibrosis accompanied by a significant reduction in α-SMA-positive cells in immunostained liver sections from mice treated with leptin antagonist alone or in combination with leptin. The most dramatic antifibrotic and prosurvival effect was achieved in hyperleptinogenic fibrosis states, created by exogenous coadministration of leptin. Nevertheless, significant attenuation of fibrosis and mildly improved survival were also noted in mice administered only the leptin antagonist, implying that under all conditions favoring hepatic fibrosis, inhibition of endogenous leptin activity may ultimately lead to inhibition of HSC function, resulting in a potent antifibrogenic effect.

Our data do not exclude other possible mechanisms contributing to the leptin antagonist's antifibrogenic activity, such as reduced expression of TGF-β1 or its receptor, or alteration of the expression of genes such as tissue inhibitors of metalloproteinases. Another possible contributor to leptin antagonist's antifibrogenic effect is its direct immunomodulatory function, which in turn may be linked to a reduction in profibrogenic signaling. Most, but not all, reports indicate that leptin-deficient *ob/ob* mice are resistant to inflammation at baseline.[44,45] In our study, a reduction in serum and hepatic IFN-γ levels also suggested that chronic inflammation is alleviated in leptin antagonist–treated mice, which may also contribute to the alleviation of fibrosis. In another currently ongoing project, we recently found further support for leptin antagonist's antiinflammatory activity in acute hepatic inflammation in several murine models, mediated in part by inhibition of several T-cell subsets (Elinav et al., unpublished results).

A major limitation to systemic clinical application of the current generation of leptin antagonists is the relatively high doses required to achieve significant suppressive activity. The antagonist's affinity to LR has been shown to be identical or highly similar to that of leptin,[24,27,28] necessitating a 10:1 to 100:1 antagonist-to-leptin molar ratio to block 90%-99% of the LRs, as calculated on a purely thermodynamic basis. Administration of such high doses of leptin antagonist has been demonstrated to be safe in this proof-of-concept stage. Nevertheless, we are in the advanced developmental stages of a higher-affinity leptin antagonist with a prolonged *in vivo* half-life that will enable the achievement of similar therapeutic effects with lower doses, facilitating future clinical application of this approach.

The major leptin-antagonist related metabolic side effect was abolishment of exogenous leptin-related weight loss, while leptin antagonism did not affect lipid levels, suggesting that its use, even in high doses, may be clinically safe. However, other metabolic parameters such as glucose tolerance, insulin and adipokine levels, and reproductive function, as well as effects on other physiological systems, need to be further studied to establish its clinical safety. An explanation for the lack of significant weight gain despite a robust antifibrogenic effect may lie in possible differences in leptin responsiveness between peripheral and central nervous system (CNS)-responsive cells or, alternatively, modified CNS penetration of leptin antagonist. Indeed, direct CNS administration of leptin has been shown to promote significantly more weight loss than systemically-administered leptin, suggesting that regulation of CNS leptin levels and activity may occur via altered penetration through the brain-blood barrier, or via modified sensitivity of hypothalamic sensory cells to leptin signaling.[46] Other possible mechanisms underlying the lack of weight and lipid alterations might be tight metabolic regulation mediated by compensatory alteration of counterregulatory hormones, such as adiponectin and resistin.

In conclusion, this is the first report of the *in vivo* application of a novel competitive leptin inhibitor as a therapeutic modality for experimental liver fibrosis. We demonstrate that antifibrotic activity of our competitive antagonist is mediated through inhibition of HSC activation and function, with a possible contribution of immunomodulatory function. This effect was achieved with no significant adverse metabolic effects. We believe that in the future, systemic leptin inhibition will play a role in a novel antifibrogenic and antiinflammatory therapeutic approach.

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