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Transdifferentiation of Rat Hepatic Stellate Cells Results in Leptin Expression

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Leptin is a peptide hormone that appears critical in regulating Fat metabolism. Recently, circulating leptin levels were reported higher in patients with alcoholic cirrhosis. In health, hepatic stellate cells store retinoids, but following liver injury they transdifferentiate into myofibroblast-like cells with loss of the retinoid stores. Leptin expression was demonstrated by detection of leptin mRNA by RT-PCR analysis and by immunohistochemistry viewed with confocal microscopy in transdifferentiated stellate cells after 14 days, or more, of culture. Leptin expression was not found in freshly isolated quiescent stellate cells. Leptin expression was not demonstrated in freshly isolated or cultured Kupffer cells. Treatment of activated stellate cells with either 1 μM retionic acid or 10 μM retinol acetate resulted in the inhibition of leptin mRNA expression. The observation that activated stellate cells in culture can express leptin has implications for understanding adipocyte biology in liver disease and treatment of malnutrition in cirrhotics. © 1998 Academic Press

Leptin is a 16-kilodalton (kDa) peptide hormone that was recently shown to reduce body fat in mice (1). Recombinant leptin decreases food intake and increases energy expenditure in wild-type mice. Its absence in the *obese (ob)* mouse strain leads to a massive increase in body fat(1–3). Leptin mRNA expression has been associated with adipocytes (1,4,5), although receptors for this hormone have been found in the hypothalmus, as well as in the choroid plexus, lungs, kidneys, and liver (6–8). Recently, leptin receptors were isolated in early granulocytes, and monocytes, and in mature peritoneal macrophages, where leptin appeared to enhance the production of cytokines, as well as to increase phagocytic activity (9). These studies suggest that leptin may result in the regulation of such processes as hemopoiesis.

Cytokines are thought to play a role in liver injury (10). Recently leptin levels were reported to be higher in patients with alcoholic cirrhosis regardless of body mass index (11). This group of investigators hypothesized that the cachexia of alcoholic cirrhosis may be due to elevated circulating leptin levels. Stellate cells, a group of hepatic non-parenchymal cells, store retinyl esters in health, but lose this capacity as they become activated once liver injury is sustained. Similarly, transdifferentiation of stellate cells occurs after freshly isolated cells are cultured on plastic dishes. We report here that activated hepatic stellate cells, *in vitro,* produce leptin, and that its production can be inhibited by the addition of retinoids.

METHODS

Rat hepatic stellate cells and hepatocytes were isolated by in situ liver perfusion from male Sprague-Dawley rats (300–400 g) obtained from Charles River Laboratories (Wilmington, MA) and cultured as described previously (12,13). The stellate and hepatocyte preparations were greater than 95% and 90% pure respectively. Kupffer cells were also isolated by liver perfusion with 0.2% pronase and 0.15% collagenase, and separated from other cell types by a metrizamide gradient (14). The Kupffer cell preparations were greater than 95% pure as demonstrated by confocal microscopy with ED2 antibody (Serotek, Oxford, England) immunofluorescence. Stellate and Kupffer cells were plated in DMEM containing 20% FBS in 150 mm^2 uncoated plastic tissue culture dishes (13).

Total RNA was extracted from freshly isolated stellate cells, hepatocytes, and Kupffer cells as well as from stellate cells and Kupffer cells maintained in culture as described previously. Total cellular RNA from various stellate cell populations was isolated by the method of Chomczynski and Sacchi (15). The concentration of isolated RNA was determined from the optical density at 260 nm and its purity from the 260/280 nm ratio. RNA message was determined by reverse transcriptase polymerase chain reaction (RT-PCR). Leptin primers were designed using the PCR Primer Selection Program, MacVector. The leptin primers were: 5′-GGCTTTGGTCCTATCTGTCCTATG-3′; 3′-TGCAGCACGTTTTGGGAAGG-5′.

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Abbreviations used: RT-PCR, reverse transcriptase-polymerase chain reaction; FBS, fetal bovine serum; RNA, ribonucleic acid; PBS, phosphate buffered saline; cDNA, copy deoxyribonucleic acid; RA, retinoic acid.

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Total RNA (1 μg) from freshly isolated stellate cells (Day 0), or activated cells in culture, was amplified using r*Tth* DNA polymerase (obtained from Perkin-Elmer, Norwalk, CT). Control reactions were performed with a 500-bp cDNA included with the RT-PCR reagents. Reverse transcription was performed at 60°C for 60 min. Thirty cycles of PCR were performed at 94°C for 30 sec, 60°C for 1 min, with subsequent elongation at 72°C for 10 min. PCR products were resolved by 1.2% agarose gel electrophoresis, and subsequently cloned employing the T/A cloning system (Invitrogen,). Identical PCR analysis was performed with RNA from freshly isolated hepatocytes and Kupffer cells. Also, activated stellate cells in culture for 14 or more days were treated for 48 hr with either, 10 μM retinol acetate, or 1 μM *cis*-retinoic acid (RA), to suppress leptin expression before RT-PCR analysis was performed. All PCR products were sequenced by the Department of Biological Chemistry at our Institution to confirm that the cDNA generated was leptin.

To perform immunofluorescent confocal microscopy, quiescent, freshly isolated stellate cells were centrifuged onto slides, washed with PBS, and fixed in cold (−20°C) acetone/methanol for 10 min. Stellate cells activated after 14 days in culture on Lab-Tek chamber slides were also washed with PBS and fixed in cold 50/50 acetone/methanol. Cells were blocked with 1% FBS for 1 h at 4°C and washed three times with PBS. For immunostaining, the cells were incubated with mouse anti-α-smooth muscle actin (Sigma, St. Louis, MO) or with rabbit anti-leptin mouse recombinant IgG (Calbiochem, La Jolla, CA) for 1 h at 37°C. Cells were incubated for 1 h at 37°C with either anti-mouse IgG CY3 conjugate (Sigma Chemical) or with goat anti-rabbit IgG FITC conjugate (Sigma Chemical). The immunostained slides were mounted in 50% glycerol in PBS containing 2 mg/ml p-phenylenediamine and examined with a laser confocal imaging system.

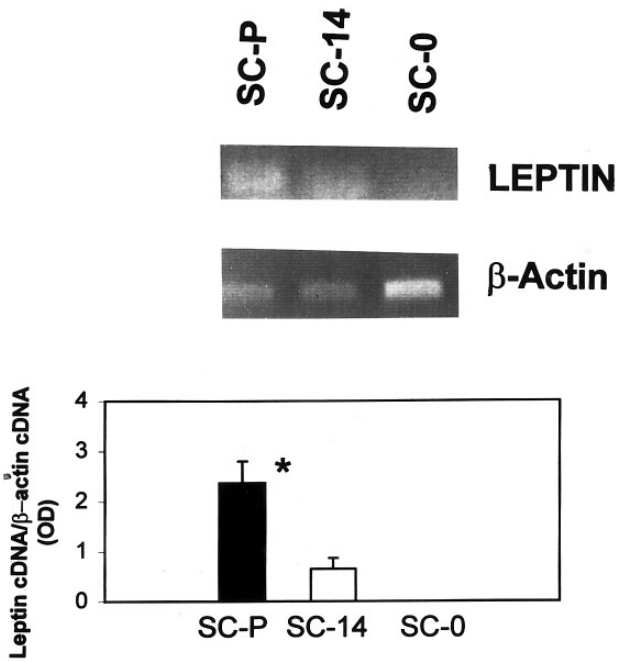

FIG. 1. RT-PCR analysis of leptin and β-actin mRNA in quiescent and activated stellate cells. One μg of total RNA subject to RT-PCR as described in text for leptin (TOP) and β-actin (BOTTOM). LANE 1: Passaged stellate cells (SC-P): LANE 2: Stellate cells, 14 days in culture after isolation (SC-14); LANE 3: Freshly isolated, quiescent, stellate cells (SC-0); bar graph represents the mean +/- S.E. ratio of leptin cDNA production to β-actin cDNA production as measured by scanning laser densitometry (OD). *$P < 0.03$ (two-tailed *t*-test); results shown are from three separate experiments.

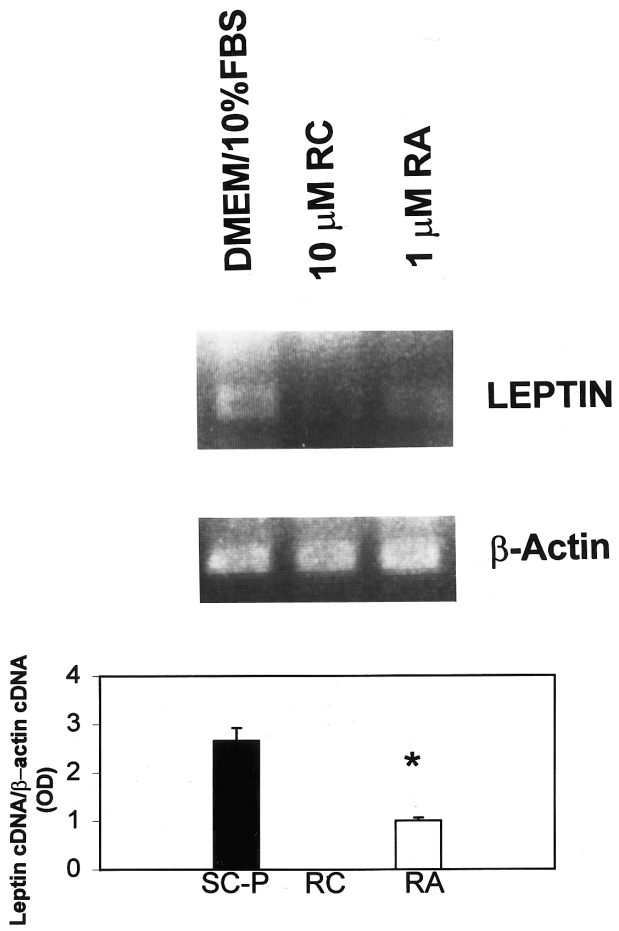

FIG. 2. RT-PCR analysis of leptin and β-actin mRNA from activated stellate cells treated with either *cis*-retinoic acid or retinol acetate. RT-PCR as in FIGURE 1. LANE 1: Passaged stellate cells in culture (>14 d) in DMEM with 10% FBS; LANE 2: As in lane 1, except 48 h pre-treatment with 10 μM retinol acetate (10 μM RC), or LANE 3, 1 μM *cis*-retinoic acid (1 μM RA). Corresponding bar graph, and statistical analysis as in FIG. 1, *$P < 0.01$.

RESULTS AND DISCUSSION

Leptin mRNA expression was not present in freshly isolated stellate cells (Fig. 1). By contrast, we consistently found a 256 bp PCR product, sequenced to be leptin, in 14 day-old cells in culture, and greater quantities in activated, passaged stellate cells in culture (Fig. 1). Furthermore, treatment of stellate cells with 10 μM retinol acetate for 48 h, abolished mRNA production in stellate cells, while 1 μM *cis*-retinoic acid resulted in a significant decrease in leptin mRNA compared to untreated cells (Fig. 2). All results were normalized to β-actin PCR products by scanning laser densitometry. We confirmed the presence of leptin by immunohistochemistry employing antibodies for leptin as demonstrated in Fig. 3A. α-smooth muscle actin was used as a marker of the activated stellate cell phenotype (Fig. 3B). Freshly isolated Kupffer cells and hepa-

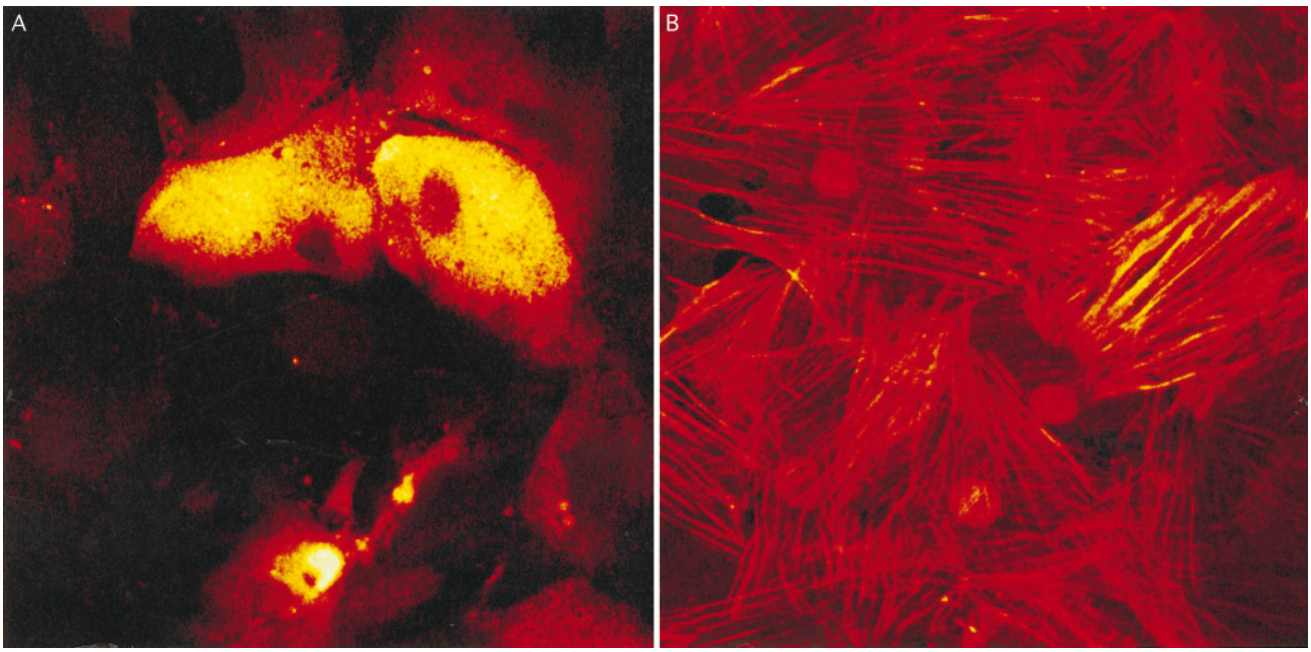

FIG. 3. Immunofluorescence of leptin and α-smooth muscle actin in activated hepatic stellate cells. Confocal microscopic examination of activated hepatic stellate cells in which antibodies to leptin are employed (A) and anti-α-smooth muscle actin (B). The stellate cells were activated by culture in DMEM containing 20% FBS in plastic dishes for >14 d.

tocytes, and Kupffer cells after 5 d in culture, did not express leptin mRNA. Also neither α-smooth muscle actin nor leptin were detected by immunohistochemistry with confocal microscopy in each of these cell populations.

The potential relationship of cachexia in cirrhosis, and the recent identification of elevated circulating leptin levels in patients with alcoholic liver disease is paradoxical. It is known that the leptin gene is down-regulated by fasting (16) and up-regulated by obesity (4) and thus serves as a molecular regulator of fat mass and appetite. It is well established that humans afflicted by cirrhosis from alcohol are malnourished (17). While leptin production is found predominantly in adipocytes, the product and its receptor are found in other tissues, including the liver (8). Our study shows that leptin production is increased in activated stellate cells, and that this occurs once retinyl ester stores are depleted; furthermore, the addition of retinoic acid suppresses leptin expression in the activated phenotype.

The murine leptin promoter has been cloned and sequenced (18) and, at least two adipocyte transcription factors appear critical in the regulation of leptin mRNA production. First, the consensus CCAAT/enhancer binding protein (C/EBPα) is required for leptin promoter activity (19–21). Second the peroxisome proliferator-activated receptor-γ (PPARγ) isoforms that typically mediate positive effects on gene expression by binding to a hexameric sequence with the retinoid X receptor (RXR) as a heterodimer, is inhibitory to leptin expression (22–28).

Quiescent hepatic stellate cells possess nuclear RA receptor-beta (RARβ), while in cultured (activated) cells, RARβ expression is decreased (29). In addition RARβ mRNA is suppressed in *in vivo* activated stellate cells; and, the mRNA level of RXR-α, a predominant subtype of RXR in hepatic stellate cells, is also depleted in activated cells from rats with cholestatic liver fibrosis induced by bile duct ligation (30). This information, taken together with the PPARγ/RXR inhibitory function of the leptin promoter, still cannot explain an increase of leptin production by activated hepatic stellate cells, or elevated leptin levels in patients with alcoholic cirrhosis. In fact, the major profibrogenic cytokine, transforming growth factor beta one (TGFβ1) stimulates transcription of both RAR and RXR in the presence of RA, probably by an AP-1 dependent mechanism (31).

AP-1 binding sites, of which the leptin promoter contains at least one *cis*-binding site, play a role in controlling the activation of various genes crucial to liver fibrosis. AP-1 binding sites influence the activation of matrix metalloproteinase gene promoters by growth factors and cytokines (32,33) and their inhibition by RA (33–35). While RARs and RXRs can induce transcriptional activation through specific DNA binding sites, they can also interact indirectly with AP-1 through transcriptional mediators to repress gene transcription (36–38). Furthermore, the presence of physiologic concentrations of RA inhibits the transactivation of the MMP, interstitial collagenase (39).

In cultured hepatic stellate cells the level of RARβ

mRNA has been shown to correlate with cellular retinoid responsiveness in vitro. The loss of RARβ most likely results from a decrease in stellate cell retinoid levels, because retinoid treatment, up regulates stellate cell RARβ expression, in cultured stellate cells (30). Depletion of retinoids in hepatic stellate cells allows for marked proliferation and collagen synthesis (29). Hence, even though TGFβ1 can induce RXR-α, and RAR-β gene activation, there appears to be a critical role of the diminished retinoid signaling in promoting AP-1 activity and TGFβ1 expression (30). These observations, taken together with our findings regarding leptin expression in stellate cells, and the recent report of elevated leptin levels in alcoholic cirrhotics, implies an important role of retinoids in repression of leptin expression, as it does with other gene products critical to hepatic fibrogenesis.

It is not known whether the lack of retinoid storage by activated stellate cells *in vivo* is responsible for the permissive effect of AP-1 transactivation of leptin in activated stellate cells, or whether this phenomenon occurs in adipocytes outside the liver in patients with chronic liver disease. Nevertheless, the absence of RA in cultured stellate cells, and the concomitant increase in leptin mRNA and protein expression which is ameliorated *in vitro* by retinoid rescue, strongly suggests that activated stellate cells synthesize leptin. The elevated circulating levels of leptin in cachectic patients with alcoholic cirrhosis may be explained, in part, by the presence of leptin message and protein in activated hepatic stellate cells. Further, even though leptin levels should be low in such patients, this work suggests the loss of RARβ permits AP-1 and perhaps additional factors, to constitutively express leptin message. These findings have potential implications for the treatment of malnutrition in cirrhotic patients and correlate with work done by others suggesting that chronic inflammatory conditions resulting in cytokine release also potentiate peripheral leptin production by adipocytes (40).

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