Kupffer Cells Mediate Leptin-Induced Liver Fibrosis

JIANHUA WANG,* ISABELLE LECLERCQ,‡ JOANNE M. BRYMORA,* NING XU,* MEHDI RAMEZANI–MOGHADAM,* ROSLYN M. LONDON,* DAVID BRIGSTOCK,§ and JACOB GEORGE*

*Storr Liver Unit, Westmead Millennium Institute, University of Sydney and Westmead Hospital, Westmead, Australia; ‡Laboratory of Gastroenterology, Faculty of Medicine, Université Catholique de Louvain, Brussels, Belgium; and §Center for Cell and Vascular Biology, Children's Research Institute, Columbus, Ohio

BACKGROUND & AIMS: Leptin has profibrogenic effects in liver, although the mechanisms of this process are unclear. We sought to elucidate the direct and indirect effects of leptin on hepatic stellate cells (HSCs). **METHODS:** HSCs from Sprague–Dawley rats were exposed to leptin and expression of collagen-I, tissue inhibitor of matrix metalloproteinases-1 (TIMP1), transforming growth factor β1 (TGF-β1), and connective tissue growth factor (CTGF/CCN2) was assessed. The effects of medium from Kupffer cells (KCs) and sinusoidal endothelial cells (SECs) following leptin were evaluated in HSCs; α-smooth muscle actin (αSMA) production and KC signaling were analyzed. **RESULTS:** HSCs were not activated by incubation with leptin. However, HSCs cultured with medium taken from KCs that were incubated with leptin had increased expression of collagen I, TIMP1, TGF-β1, and CTGF/CCN2, as well as αSMA protein levels and proliferation. These effects were leptin receptor dependent because conditioned medium from KCs isolated from leptin receptor-deficient Zucker (fa/fa) rats did not activate HSCs. In KCs incubated with leptin, messenger RNA and protein expression of TGF-β1 and CTGF/CCN2 increased. Leptin potentiated signal transducer and activator of transcription 3, AKT, and extracellular signal-related kinase 1/2 phosphorylation in KCs and increased AP-1 and nuclear factor-κB DNA binding. Finally, addition of anti-TGF-β to KC-conditioned medium inhibited HSC expression of collagen I, TIMP1, and CTGF/CCN2, whereas signal transducer and activator of transcription 3 inhibitor attenuated TGF-β1 production by KC. **CONCLUSIONS: Leptin mediates HSC activation and liver fibrosis through indirect effects on KC; these effects are partly mediated by TGF-β1.**

Leptin, an adipocyte-derived hormone, has important effects in regulating body weight, metabolism, and reproductive function. Circulating levels of leptin are known to be increased in overweight and obese persons, in individuals with nonalcoholic steatohepatitis,[1,2] and in those with alcoholic liver disease and chronic viral hepatitis.[3–5] More recently, leptin has been shown to possess direct profibrogenic activity in the liver,[6–8] and the absence of leptin is associated with a marked attenuation of the hepatic response to a diverse range of fibrotic stimuli.[6,7] We previously demonstrated that leptin-deficient ob/ob mice failed to develop hepatic fibrosis in a rodent nutritional model of steatohepatitis and in response to chronic CCl_4-induced liver injury.[6] Restitution of physiologic levels of circulating leptin restored the liver's "fibrogenic" capacity.[6] Similar results have been obtained in models of fibrosis associated with bile duct ligation[9] and following the administration of thioacetamide.[10] The cellular and molecular mechanisms for this effect, however, have not been fully elucidated.

Because hepatic stellate cells (HSCs) are the main source of extracellular matrix (ECM) during the evolution of fibrosis, the effects of leptin on HSC behavior have been examined, but results are conflicting. One view holds that leptin acts directly on HSCs to trigger downstream response pathways that ultimately lead to ECM deposition.[7,11] Others suggest that Kupffer cells (KCs) and/or sinusoidal endothelial cells (SECs) contain a functional leptin receptor, which can stimulate the release of profibrogenic mediators such as transforming growth factor (TGF)-β1 that in turn drives HSC activation.[12] To date, there is no evidence to indicate that leptin-primed KCs or SECs exert direct stimulatory effects on HSCs. However, it is well-known that KCs and SECs play important roles in modulating stellate cell behavior by releasing proinflammatory and profibrogenic factors such as TGF-β1 and reactive oxygen species (ROS) upon stimulation by various noxious stimuli. In addition, recent data indicate that KC dysfunction as evidenced by decreased TNF-α production and down-regulation of TGF-β1 gene expression occurs in leptin receptor-deficient Zucker rats and may account for the attenuation of liver fibrosis in these rodents following the administration of pig serum.[13]

Abbreviations used in this paper: αSMA, α-smooth muscle actin; collagen I, collagen 1α1; CTGF/CCN2, connective tissue growth factor; ERK1/2, extracellular signal-related kinase 1 and 2; KCs, Kupffer cells; HSCs, hepatic stellate cells; SECs, sinusoidal endothelial cells; TGF-β1, transforming growth factor β-1; TIMP1, tissue inhibitor of matrix metalloproteinases-1; STAT3, signal transducer and activator of transcription-3.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.04.011

In this study, we undertook detailed in vitro experiments to clarify the cellular and molecular mechanisms whereby leptin exerts profibrogenic effects on the liver. Using primary cell culture models of hepatic nonparenchymal cells alone and in coculture, we demonstrate that the principal profibrogenic effects of leptin are mediated via direct effects on KCs leading to the release of soluble mediators including TGF-β1 and connective tissue growth factor (CTGF)/CCN2.

Materials and Methods

Animals

Male Sprague-Dawley rats were obtained from the Animal Resources Centre (Perth, WA, Australia). Zucker rats (fa/fa) and their lean littermates (Fa/Fa) were obtained from Professor Greg J. Barritt (Flinders University, Adelaide, Australia). All animals were maintained under 12-hour light/dark cycles with food and water ad libitum. Experimental protocols were approved by the Sydney West Area Health Service Animal Research Ethics Committee.

Materials

Rat recombinant leptin was purchased from Sigma–Aldrich (St. Louis, MO). Phospho-STAT3, pp38, pERK1/2(44/42), pAKT, and pJNK mouse monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant TGF-β1, monoclonal TGF-β antibody, platelet-derived growth factor (PDGF) BB, and PDGF ELISA Kit were purchased from R&D Systems (Minneapolis, MN). AP-1 and nuclear factor (NF)-κB consensus double-stranded oligonucleotides were purchased from Promega (Madison, WI). Signal transducer and activator of transcription (STAT) 3 inhibitor peptide (5730956), the MAP kinase kinase (MEK) inhibitor PD98059, and the phosphoinositide-3 kinase (PI3K) inhibitor LY294002 were obtained from Calbiochem (San Diego, CA). Soluble TGF-β receptor (sTGFβR) fusion protein was a gift from Biogen Inc.

Nonparenchymal Cell Isolation and Culture

HSCs were isolated by 2-step (collagenase B and pronase E) perfusion.[14] KCs and SECs were further obtained and purified by elutriation.[15] HSCs were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin and plated on 6-well plates at a density of 0.8×10^6 cells/well. Viability was routinely over 95% for all experiments. Purity was 95% as determined by morphology, vitamin A autofluorescence, and desmin positivity.

KCs were identified by their ability to phagocytose latex beads; viability was $>$ 96% and purity $>$ 98%. The viability of SECs was $>$ 98% and purity at least 94% as determined by morphology (cobblestone appearance) and absence of latex bead phagocytosis. KCs were cultured in 10% FCS/DMEM/1% penicillin-streptomycin in 6-well plates. SECs were cultured in M199 with 20% FCS, 1% penicillin-streptomycin, insulin (20 mU/mL), heparin (10 U/mL), VEGF (5 ng/mL), and dexamethasone (10 μmol/L). SEC culture wells were precoated with type I collagen (Nalge Nunc International, Rochester, NY).

Immunoblot Assays for Protein Expression

Culture media was removed, and cells were washed with phosphate-buffered saline (PBS) and then lysed on ice in a buffer containing 20 mmol/L Tris, 0.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 3 mmol/L NaN_3, with protease and phosphatase inhibitors. Cell lysates were disrupted with a sonicator on ice. Immunoblots were performed as previously described.[16] Immunoblotting was performed for TGF-β1 protein in culture medium after concentrating the media using Microcon YM-10 Centrifugal Filters (Millipore, Billerica, MA).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total cellular RNA was prepared from HSCs, KCs, and SECs using TRI REAGENT (Molecular Research Center). Complementary DNA (cDNA) was synthesized from 1 μg RNA using SuperScript III reverse transcriptase and 0.5 nmol of random primers (Invitrogen, Mount Waverly, VIC, Australia). Real-time quantitative reverse-transcription polymerase chain reaction (qPCR) was performed using SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich). The relative amount of messenger RNA (mRNA) was calculated by reference to a calibration curve. Each sample was normalized to the respective 18S value.

Cell Proliferation

HSC proliferation was assessed by using the Cell Proliferation Reagent WST-1 according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Immunocytochemistry for CTGF/CCN2

CTGF/CCN2 protein expression in KCs was assessed as previously described.[17]

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay

KC nuclear protein preparation and electrophoretic mobility shift assay (EMSA) were performed as previously described.[18]

Sirius Red Staining and Quantification of Collagen in HSCs

These experiments were performed as previously described.[19]