Liver Structural Injury in Leptin-Deficient (ob/ob) Mice: Lipogenesis, Fibrogenesis, Inflammation, and Apoptosis

Lesión Estructural del Hígado en Ratones con Deficiencia de Leptina (ob/ob): Lipogénesis, Fibrogénesis, Inflamación y Apoptosis

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SUMMARY: Nonalcoholic fatty liver disease (NAFLD) might progress the steatosis to nonalcoholic steatohepatitis (NASH), reaching a cirrhosis state and possibly hepatocellular carcinoma. The liver of three-month-old C57BL/6J mice (wild-type, WT group, n=10) and leptin-deficient obese mice (ob/ob group, n=10) were studied, focusing on the mechanisms associated with the activation of the hepatic stellate cells (HSCs) and pro-fibrogenesis. The obese ob/ob animals' liver showed steatosis, increased lipogenesis gene expressions, inflammation, increased pro-inflammatory gene expressions, inflammatory infiltrate, and potential apoptosis linked to a high Caspase 3 expression. In ob/ob mice, liver sections were labeled in the fibrotic zones by anti-alpha-smooth muscle actin (alpha-SMA) and anti-Reelin, but not in the WT mice. Moreover, the alpha-SMA gene expression was higher in the ob/ob group's liver than the WT group. The pro-fibrogenic gene expressions were parallel to anti-alpha-SMA and anti-Reelin immunofluorescence, suggesting HSCs activation. In the ob/ob animals, there were increased gene expressions involved with lipogenesis (Peroxisome proliferator-activated receptor-gamma, Cell death-inducing DFFA-like effector-c, Sterol regulatory element-binding protein-1c, and Fatty acid synthase), pro-fibrogenesis (Transforming growth factor beta1, Smad proteins-3, Yes-associated protein-1, Protein platelet-derived growth factor receptor beta), pro-inflammation (Tumor necrosis factor-alpha, and Interleukin-6), and apoptosis (Caspase 3). In conclusion, the results in obese ob/ob animals provide a clue to the events in humans. In a translational view, controlling these targets can help mitigate the hepatic effects of human obesity and NAFLD progression to NASH.

KEY WORDS: Steatosis; Stellate cell; Fibrosis; Confocal microscopy; Stereology.

INTRODUCTION

Leptin-deficient mouse (ob/ob) develops liver steatosis (comparable with the nonalcoholic fatty liver disease or NAFLD in humans). The pro-inflammatory state might progress the steatosis to nonalcoholic steatohepatitis (or NASH in humans), which, associated with fibrogenic processes in the long-term, may result in the deposition of excess matrix tissue and liver fibrosis (Bettermann et al., 2014).

The hepatic stellate cells (HSCs) are the dominant source of hepatic myofibroblasts (Lua et al., 2016). Improved fibrillar elements are produced by HSCs, modulated by fibroblastic factors, and accumulated in the extracellular matrix (ECM) in liver fibrosis (Sferra et al., 2017). The liver fibrosis pathogenesis starts with the HSCs activation to repair the damage, depositing collagen in the stroma, a process dependent on modulating cytokine induction and inflammation (Zhang et al., 2020). However, HSCs might remain activated depending on the stimuli, and liver fibrosis progresses, reaching a cirrhosis situation (Khomic et al., 2019) and possibly hepatocellular carcinoma (Martin et al., 2020).
The liver adaptation to nutrient excess, obesity, and HSCs activation are complex processes that involve biochemical, molecular, and immunological mechanisms (Fuchs et al., 2020). Activated HSCs change into myofibroblast-like cells and express alpha-SMA in response to liver injury (Carpino et al., 2005), and participate in hepatic inflammation by releasing a set of inflammatory cytokines and chemokines (Gupta et al., 2019).

Because of its central involvement in liver fibrosis' pathogenesis and its link with pro-fibrogenic pathways, new therapies for NAFLD/NASH might be expected regarding pathogenesis and its link with pro-fibrogenic pathways, new therapies for NAFLD/NASH might be expected regarding the HSCs. The anti-fibrotic drugs (e.g., pirfenidone and nintedanib), active via transforming growth factor (TGF) beta, still have related mechanisms not entirely known (Cho, 2018).

The study aimed to understand better the pathways linked to lipogenesis, HSCs activation, pro-fibrogenesis, pro-inflammation, and apoptosis in ob/ob mouse, which might help understand hepatic progression steatosis to a stage of steatohepatitis, with translation to the disease in humans.

**MATERIAL AND METHOD**

**Experimental protocol.** The current animal experiment complies with the ARRIVE guidelines and NIH Publication number 85-23 (revised 1996). The local Ethics Committee approved procedures (Protocol number CEUA 010/2016).

One-month-old male ob/ob and C57BL/6J mice (as the control group, wild-type, WT) stemmed from a colony derived from the Jackson Laboratory (B6.V-Lepob/J, stock no. 000632, Bar Harbor, ME, USA). The animals were kept in ventilated cages under controlled conditions (Nexgen system, Allentown Inc., PA, USA, 21 ± 2 °C, and 12 h/12 h dark/light cycle), with free access to food and water until three months of age (n= 10/group), feeding a balanced diet (Sigma-Aldrich, St Louis, MO, USA), and quantified (spectroscopy, Nanovue, GE Life Sciences), then one microgram was treated with DNAse I. Then, oligo (dT) primers for mRNA treated with DNase I. Then, oligo (dT) primers for mRNA and Superscript III reverse-transcriptase were applied to the synthesis of the first-strand cDNA (thermocycler CFX96 (Bio-Rad, Hercules, CA, USA), and SYBR Green mix). The endogenous beta-actin was used to normalize the expression of the selected genes. After the pre-denaturation and polymerase-activation program (4 min at 95 °C), 44 cycles of 95 °C for 10 s and 60 °C for 15 s were followed by a melting curve program (60–95 °C, the heating rate of 0.1 °C/s). The expression intensities of the genes were analyzed by qPCR, and the ratio of relative mRNA expression was calculated using 2-ΔΔCT - ΔCT as the difference between the number of cycles (CT).

Plasmatic measurements. After centrifugation (712 xg, 10 min), plasmatic hormone concentrations were measured (enzyme-linked immunosorbent assay kits, ELISA) to assess insulin (EZRMI-13K, Millipore, Merck, Temecula, CA, USA), leptin (EZML-82K), and adiponectin (EZRMDP-60K, Millipore, Merck, Temecula, CA, USA) concentrations (TP-Reader ELX800, BioTek Instruments Winooski, VT, USA).

**Liver**

a) **HSCs identification.** For antigen retrieval, sections were submitted to citrate buffer (pH 6.0, at 60 °C for 20 min), glycine 2 %, and blocking buffer (PBS/5 % BSA), then incubated overnight at 4 °C with anti-Reelin (1:100, AB78540, Abcam, Eugene, OR, USA), and anti-alpha-actin smooth muscle (SMA) (1:100, AB7817; Abcam). The sections were incubated with fluorochrome-conjugated secondary antibody IgG-Alexa 488 and IgG-Alexa 546 (Invitrogen, Molecular Probes, Carlsbad, CA, USA), diluted 1:50 in PBS/1 % BSA at room temperature for one hour. The slides were mounted (Slow Fade Antifade, Invitrogen) after rinsing in PBS. Digital images were taken with a Nikon Confocal Laser Scanning Microscopy (Model C2; Nikon Instruments, Inc., New York, USA).

b) **Steatosis assessment and liver fibrosis.** The volume density of fat in the hepatocyte (Vv [fat, hepatocyte] %) was estimated by stereology (point-counting method) in a sample of at least ten fields per animal obtained from nonconsecutive random sections of the liver (Mandarim-de-Lacerda & Del-Sol, 2017). Therefore, Vv [fat, hepatocyte] was estimated on HE-stained sections as described elsewhere (Catta-Preta et al., 2011). Other random sections of the liver were observed after Sirius red staining to study the collagen fiber distribution (Marinho et al., 2017).

c) **Real-time quantitative polymerase chain reaction (RT qPCR).** Total RNA of the liver (30 mg) was extracted (Trizol, Invitrogen, CA, USA), and quantified (spectroscopy, Nanovue, GE Life Sciences), then one microgram was treated with DNase I. Then, oligo (dT) primers for mRNA and Superscript III reverse-transcriptase were applied to the synthesis of the first-strand cDNA (thermocycler CFX96 (Bio-Rad, Hercules, CA, USA), and SYBR Green mix). The endogenous beta-actin was used to normalize the expression of the selected genes. After the pre-denaturation and polymerase-activation program (4 min at 95 °C), 44 cycles of 95 °C for 10 s and 60 °C for 15 s were followed by a melting curve program (60–95 °C, the heating rate of 0.1 °C/s). The expression intensities of the genes were analyzed by qPCR, and the ratio of relative mRNA expression was calculated using 2-ΔΔCT - ΔCT as the difference between the number of cycles (CT).
Statistical analysis. The data were shown as the means and standard deviations, and differences between the groups were tested with the unpaired t-test and Welch correction (GraphPad Prism v. 9.1 for Windows, La Jolla, CA, USA).

RESULTS

The three-month-old ob/ob mice showed an increased body mass by +110 % compared to the WT mice of the same age, as expected. Therefore, ob/ob animals showed higher plasma insulin (+70 %) and lower leptin (-60 %) concentrations than WT animals. Besides, adiponectin (an anti-inflammatory adipokine) was reduced in ob/ob mice compared to the WT mice (-50 %) (Table I).

Inflammation and apoptosis in the liver. The liver mRNA relative expressions of inflammation-related genes in the ob/ob group were higher than the WT group: Tnf-alpha (+230 %), Il 6 (+130 %). Moreover, the hepatocyte death rate in ob/ob mice was higher and linked with Caspase 3 increase than the WT mice (Table I).

Table I. Differences between the wild-type mouse (WT) and leptin-deficient obese (ob/ob) mouse (n = 10/group, Welch t-test).

| Data                  | WT        | ob/ob     | P       |
|-----------------------|-----------|-----------|---------|
| Body mass, g          | 26.46±1.09| 55.90±2.27| <0.000  |
| Adiponectin, 10^6     | 22.28±2.50| 11.16±1.04| 0.000   |
| Insulin, UI/L         | 14.66±0.17| 25.23±0.12| <0.000  |
| Leptin, 10^2 pg/mL    | 13.23±1.94| 5.01±0.82 | 0.000   |
| Liver qPCR            |           |           |         |
| Caspase 3, mRNA, a.u. | 1.17±0.09 | 3.41±0.16 | <0.000  |
| Il 6, mRNA, a.u.      | 1.07±0.07 | 2.49±0.28 | <0.000  |
| Tnf-alpha, mRNA, a.u. | 1.11±0.09 | 3.64±0.18 | <0.000  |

Abbreviations: a.u., arbitrary units; Il, interleukin; mRNA, messenger ribonucleic acid; P, probability; Tnf, tumor necrosis factor.

Fig. 1. Photomicrographs of the liver and steatosis assessment, interstitial collagen deposition, and immunofluorescence for activated hepatic stellate cell detection. (a) Wild-type (WT) mouse - liver tissue with an apparent typical structure, without sinusoidal dilatation or significative hepatocyte fat droplet accumulation. (b) Ob/ob mouse - liver tissue shows disarray and macro (arrows) and micro-steatosis with hepatocyte ballooning (a and b, same magnification, H-E stain). (c) The steatosis in the liver was estimated by stereology (mean ± SD, *P<0.001 comparing WT and ob/ob). (d) The ob/ob mouse - large interstitial collagen fibers network was identified by the Sirius red stain (red color, black arrows) and inflammatory infiltrates (open black arrows). (e) Possible activated stellate cell (like myofibroblasts) was identified with an anti-Reelin antibody (green color, open white arrow). (f) Possible activated stellate cells were likewise identified with anti-alpha-SMA antibody (red color, white arrows) (e and f, immunofluorescence, and confocal laser scanning microscopy). (n= 10/group).
Liver steatosis and HSCs activation. Liver steatosis (+650 %) and ballooned hepatocytes were more significant in the ob/ob mice than in the WT group (Figs. 1a-c). Besides, the ob/ob mice, but not the WT mice, showed a marked accumulation of collagen fibers in the liver stroma, interspersed with foci of inflammatory infiltrates (Fig. 1d). Accordingly, there was a positive labeling/signal for alpha-SMA and Reelin immunostaining, suggesting HSCs activation in the ob/ob mice, but not the WT mice (Figs. 1e-f).

Liver lipogenesis and profibrogenic markers. The genes linked with lipid metabolism and lipogenesis were more expressed in the ob/ob group than in the WT one: Ppar-gamma (+275 %, Fig. 2a), Cidec (+50 %, Fig. 2b), Srebp1c (+190 %, Fig. 2c), and Fas (+175 %, Fig. 2d). These data corroborate the pattern of hepatic steatosis developed in the ob/ob animals.

The relative mRNA expressions connected to profibrogenesis were higher in the ob/ob mice compared to the WT mice: Col1a1 (+780 %, Fig. 2e), Smad3 (+160 %, Fig. 2f), Yap1 (+140 %, Fig. 2g), Pdgfr-beta (+80 %, Fig. 2h), alpha-actin (+610 %, Fig. 2i), and Tgf-beta1 (+275 %, Fig. 2j). These findings are relevant, and the interaction between Smad3, Yap1, Pdgfr-beta, and Tgf-beta1 are original data for understanding liver fibrosis mechanisms in leptin-deficient obese mice.

Fig. 2. Gene expressions in the liver (relative mRNA expression in arbitrary units): MARKERS OF LIPOGENESIS. (a) Ppar-gamma (peroxisome proliferator-activated receptor-gamma). (b) Cidec (cell death-inducing DFFA-like effector c). (c) Srebp1c (sterol regulatory element-binding protein). (d) Fas (fatty acid synthase). MARKERS OF PRO-FIBROGENESIS. (e) Col1a1 (collagen type I). (f) Smad3 (smad proteins). (g) Yap1 (yes-associated protein). (h) Pdgfr-beta (protein platelet-derived growth factor receptor). (i) Alpha-actin; (j) Tgf-beta1 (transforming growth factor). Means ± SD, *P<0.001 comparing WT and ob/ob. (n= 10/group).

Fig. 3. Graphical abstract. The liver structure is standard in WT mice, presenting no steatosis or fibrosis and quiescent HSCs. Liver steatosis is marked in ob/ob mice, linked to higher expression of Ppar-gamma, Cidec, Srebp1c, and Fas, and inflammation. Therefore, activated HSCs mediated the pro-fibrogenic factors Tgf-beta1, Smad3, Yap1, and Pdgfr-beta, increasing collagen deposition fibers, generating fibrosis (figure created by FFM using the website https://app.biorender.com).
DISCUSSION

The inflammation and the increased flux of lipids in the liver might generate noteworthy changes in the ob/ob mouse hepatocytes. Also, interactions between pro- and anti-inflammatory cytokines such as TNF-alpha, adiponectin, and other cytokines are likely to play critical functions in liver disease development and progression (Mak et al., 2019).

Peroxisome proliferator-activated receptors (PPAR)-gamma is a ligand-activated transcription factor involved in the transcriptional regulation of lipid metabolism, glucose homeostasis, energy balance, and inflammation (Choudhary et al., 2019). The cell death-inducing DFFA-like effector c (CIDEc) is a direct target gene of Ppar-gamma. It generates high hepatic triacylglycerol levels, primarily by increasing the transcription of genes related to lipogenesis, and the suppression of Ppar-gamma-Cidec affects cell differentiation, maturation and reduces adipogenesis (Martins et al., 2020). Ppar-gamma enhanced by miR-942 might decrease the HSCs activation in hepatic fibrosis as an attempt to regulate fibrosis (Tao et al., 2020). In the study, both Ppar-gamma and Cidec were higher in the ob/ob mice.

Several factors might be enhanced, promoting HSCs survival, proliferation, and fibrosis, including transforming growth factor (TGF)-beta, connective tissue growth factor, ligands for Toll-like receptors 2 and 9, pro-inflammatory cytokines, platelet-derived growth factor (PDGF), and leptin (Choi et al., 2010). The PDGF receptor is a chemoattractant that drives HSCs proliferation and migration. Pdgfr-alpha showed a pro-fibrotic role in HSCs during a chronic liver injury in vivo via regulation of HSCs survival and migration, affecting the immune microenvironment, especially macrophages clearing dying hepatocytes (Kikuchi et al., 2020). Pdgfr-beta is absent in quiescent HSCs but upregulated in an early stage of liver injury (activating factors such as Tgf-beta1 stimulate the transcriptional induction of Pdgfr-beta in quiescent HSCs (Tsuchida & Friedman, 2017). Here, hepatic Pdgfr-beta was notably higher in ob/ob mice.

Sterol regulatory element-binding protein (SREBP)-1c is the dominant insulin-stimulated isoform responsible for inducing lipogenic gene expression and promoting hepatic fatty acid synthase (FAS) (Ferre & Foufelle, 2010). Srebp1c activation impacts the partition of triacylglycerol accumulation in the liver, resulting in adipose tissue remodeling by inflammation, fibrosis, and insulin resistance (Ohno et al., 2018). Srebp1c and Fas increased significantly in the ob/ob group, corroborating the report of dyslipidemia in ob/ob mice (Duong et al., 2018).

The expression of pro-fibrotic genes increased significantly in the ob/ob liver. Tgf-beta is usually the most potent fibrogenic cytokine released by numerous liver cells in a latent form (Hellerbrand et al., 1999). Tgf-beta binding and phosphorylation of the type I receptor induces phosphorylation of downstream SMAD proteins, mainly SMAD3. Activation of Smad3 during HSCs activation makes the transcription of type I and type III collagen (Breitkopf et al., 2006), and it was enhanced in the ob/ob animals. On the contrary, II-10 might mitigate hepatic fibrosis by inducing senescence of activated HSCs in vivo, linked to the p53 signaling pathway (Guo et al., 2020).

Tribbles homolog 2 (TRIB2) is an oncogene implicated in various cancers, including liver cancer, and colocalizes with alpha-SMA in fibrotic liver tissues (Xiang et al., 2021). Yes-associated protein (YAP, a mediator of the Hippo pathway) type 1 contributes to benign steatosis progression to fibrosis through interaction with Tgf-beta1 and Smad3. YAP/transcriptional co-activators with the PDZ-binding motif are mechano-regulators of Tgf-beta-Smad signaling that increase hepatic fibrosis (Mannaerts et al., 2015). TRIB2 promoted YAP stabilization, nuclear localization, and subsequent fibrotic gene expression. TRIB2 interacted with YAP to recruit phosphatase 1A and YAP dephosphorylation (Xiang et al., 2021). These genes were highly expressed in the liver of the ob/ob group.

Alpha-SMA labeling, a marker for a subset of activated fibrogenic cells, allows the detection of activated HSCs (Carpino et al., 2005). Reelin labeling was also used to underline HSCs’ identification (Kobold et al., 2002). In the study, liver sections in ob/ob mice were labeled by both anti-alpha-SMA and anti-Reelin in the fibrotic zones, but not in the WT mice. Moreover, the alpha-actin gene expression was higher in the ob/ob group’s liver than the WT group.

In conclusion, the study demonstrated original findings that contribute to a better understanding of the mechanisms related to liver changes in leptin-deficient obese animals. In the ob/ob animals, there were increased gene expressions involved with lipogenesis (Ppar-gamma, Cidec, Srebp1c, and Fas), pro-fibrogenesis (Tgf-beta1, Smad3, Yap1, Pdgfr-beta), pro-inflammation (Tnf-alpha, and Il 2), and apoptosis (Caspase 3). The results in obese ob/ob animals provide a clue to the events in humans. In a translational view, controlling these targets can help mitigate the hepatic effects of human obesity and NAFLD progression to NASH.

Author contributions

FFM, CAML – conceptualization. FFM - roles/writing - original draft. FFM, VSM, JJC, MDS, MBA, CAML - data
obesos con deficiencia de leptina (grupo ob/ob). El hígado del grupo ob/ob mostró esteatosis, aumento de la expresión génica proinflamatoria, infiltrado inflamatorio y posible apoptosis (caspasa 3). En conclusión, los resultados en animales obesos ob/ob proporcionan una pista de los eventos en humanos. Desde un punto de vista translacional, el control de estos objetivos puede ayudar a mitigar los efectos hepáticos de la obesidad humana y la progresión de HGNA a ENA.

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