

Original Research Article

Niacin downregulates chemokine (c-c motif) ligand 2 (CCL2) expression and inhibits fat synthesis in rat liver cells

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Abstract

Purpose: To elucidate the role of chemokine (c-c motif) ligand 2 (CCL2) in fat metabolism in hepatocytes.

Methods: Following partial hepatectomy, regenerated rat liver cells were isolated and cultured for 24 h were transfected with recombinant plasmid pEGFP-N1-CCL2 using liposomes. Niacin was added to the culture medium to inhibit fat synthesis. CCL2 expression was measured using western blot, while the expression of acyl-coa synthetase long chain family 4 (ACSL4) and apolipoprotein E (ApoE) were assessed using real-time PCR.

Results: At 12 h after transfection, GFP-positive rates in the pEGFP-N1 and pEGFP-N1-CCL2 transfection groups were 42.4 ± 5.6 % and 45.1 ± 3.5 %, respectively. Expression levels of CCL2 increased over time in pEGFP-N1 transfection group, pEGFP-N1-ccl2 transfection group, and niacin and pEGFP-N1-ccl2 transfection co-treatment group; however, CCL2 expression levels in the niacin and pEGFP-N1-ccl2 transfection co-treatment groups were similar to that of pEGFP-N1 transfection group, which were significantly lower than those of the pEGFP-N1-ccl2 transfection group. Expression level trends of fat-related genes ACSL4 and ApoE were similar to that of CCL2.

Conclusion: Niacin downregulates the expression of CCL2, thereby inhibiting lipid synthesis in liver cells.

Keywords: Chemokine 2, Niacin, Hepatectomy, Lipid synthesis, Transfection

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INTRODUCTION

Chemokines are small-molecule cytokines that mediate chemotactic cell-directed movement. The C-C motif subfamily is one of the largest chemokine families. One member, chemokine (c-c motif) ligand 2 (CCL2), is also known as mono-

chemotactic protein-1 (MCP-1) [1]. The N-terminal of the CCL2 molecule contains two adjacent Cys residues. Chemokines and their receptors mediate physiological functions such as cell growth, development, differentiation, apoptosis and distribution [2], and play important roles in inflammatory responses, pathogen

infections, wound repair, tumor formation and metastasis [3,4].

The liver plays an important role in the digestion, absorption, transport, decomposition and synthesis of lipids. Fatty liver is associated with disorders of fat metabolism [5]. Niacin, also known as nicotinic acid, is belong to the B vitamin group [6]. Nicotinic acid plays an important role in the prevention and treatment of nonalcoholic fatty liver disease [7].

A study found that CCL2 promotes fat accumulation during liver regeneration [8]; nevertheless, the mechanism is unclear. Therefore, in the present study, we investigated the molecular mechanisms of CCL2-mediated regulation of hepatocyte lipid synthesis by testing the ability of nicotinic acid to inhibit lipid synthesis in cultured hepatocytes.

EXPERIMENTAL

Laboratory animals

Sprague Dawley (SD) rats were purchased from the Laboratory Animal Center of Xinxiang Medical College. The rats were maintained in an animal room at constant temperature (21–22 °C). During the experiment, the rats were offered chow and water ad libitum. This study was conducted in accordance with the international guidelines for animal studies [9], and obtained approval from the ethics committee of Xinxiang Medical College (no. 20190315).

Establishment of partial hepatectomy model in rats

Rats were anaesthetized with 1% pentobarbital sodium (40 mg/kg), the hair of abdomen and chest was shaved and skin was disinfected with 75 % ethanol. We made an abdominal midline incision from the xiphoid extending 3 cm inferiorly. The abdominal cavity was exposed. The left and middle lobes of the liver (about 70 % of the total liver mass) withdrawn and the root of the liver was ligated using silk thread. One liver lobe was resected, the wound was sutured, and tetracycline powder was applied to prevent infection. The rats recovered for 24 h under routine feeding conditions.

Isolation and culture of regenerated hepatocytes

At 24 h after partial hepatectomy, collagenase was perfused to digest and disperse hepatocytes, filtered with 200 mesh screens, centrifuged at 50 g for 3 min, washed and

precipitated with PBS 3 times; isolated hepatocytes cultured in DMEM complete culture medium. A small number of cells were reserved for trypan blue measurement of survival rate.

Effect of niacin on proliferation of regenerated hepatocytes

The regenerated hepatocytes were inoculated into 96-well plates (0.2 mL medium, 3×10^3 holes). After 24 h of adherence, 10% FBS/DMEM culture was replaced and nicotinic acid gradient treatment was given. The final concentrations were 0, 0.25, 0.5, 1.0 and 2.0 mmol/L, and each gradient was provided with three pore complexes. After incubation for 24 hours, 10 ml MTT solution was added to the incubator and cultured for 4 h. The supernatants were carefully aspirated and discarded. The remaining crystals were dissolved and crystallized by adding 100 mL DMSO in each pore, such that the crystals were fully melted. The absorbance of each pore was measured using ELISA at 490 nm. The absorbance value A490 was measured.

Construction of eukaryotic expression vector of rat CCL2 gene

The TRIzol method was used to extract total RNA from rat liver, and then the RT-PCR was performed. The gene *cc12* was linked to pGEM-T vector by T4 DNA ligase for 18 h. The plasmid pGEM-T-CCL2 and expression vector pEGFP-N1 were digested, linked and transformed into JM 109 competent cells using Age I and Nhe I double enzymes. The positive clone was named pEGFP-N1-CCL2.

Liposomal transfection of rat regenerated hepatocytes

The regenerated hepatocytes were inoculated into 6-well plates at the concentration of 1×10^5 cells/wells. The recombinant plasmids pEGFP-N1-CCL2 and empty plasmid pEGFP-N1 were transfected with Lipofectamine 2000, and then the expression of green fluorescent protein (GFP) was measured 12 h later.

Determination of CCL2 expression using Western blot

Regenerated hepatocytes were treated with 1.0 mmol/L nicotinic acid 4 h after plasmid transfection. After 24, 48, and 72 h, total protein was extracted from cells, and proteins were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, then blocked with skim milk powder at 37 °C for 1 h. Membranes were incubated with CCL2 antibody

(1:500), then 1:2000 diluted horseradish peroxidase-labeled secondary antibody was added. The Beyo ECL Plus (P0018) chemiluminescence kit was used for measurement.

Assessment of fat-related gene expression using real-time polymerase chain reaction (PCR)

Regenerated hepatocytes were treated with 1.0 mmol/L nicotinic acid 4 h after plasmid transfection. At 24, 48, and 72 h, 1 µg RNA was extracted from the cells. Then, the target gene was amplified and tested by PCR on a PRISM 7900 Sequence Detector according to the operation guide of QuantiTect SYBR Green RT-PCR Kit (Qiagen). The relative content of target gene was calculated according to the copy number of beta-actin. The specific primers for each gene are shown in Table 1.

Statistical analysis

SPSS 13.0 statistical software was used to compare data for experimental and control groups. $P < 0.05$ and $p < 0.01$ were considered statistically significant as appropriate.

RESULTS

Rat regenerated hepatocytes

At 24 h after partial hepatectomy, hepatocytes were digested and dispersed by two-step perfusion method. Isolated regenerated hepatocytes were round and transparent, with over 85% viability. About 90% of the hepatocytes adhered to one another after 4 h of culture. The adherent hepatocytes grew in typical fashion: the cells changed from round to polygonal before adherence, and cell bodies became flat and thin, and increased in size significantly. The nuclei were round or oval (Figure 1).

Plasmid Mapping of Expression Vector pEGFP-N1-CCL2

The structure of pEGFP-N1-CCL2 vector confirmed using electrophoresis. Sequencing is shown in Figure 2.

Table 1: Primer sequences for real-time PCR

Gene	GenBank accession no.	Forward primer	Reverse primer	Length (bp)
acsl4	NM_053623	TCCGGGAGCTTCCTTCCCC G	CGGGTCCCCAGGCTGTCC TT	299
apoe	NM_138828.2	AACTGGGAAGATGAAGGCT C	CATCAGTACCGTCAGTTCC T	232

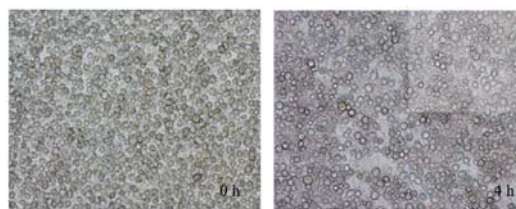


Figure 1: Isolated regenerated liver cells

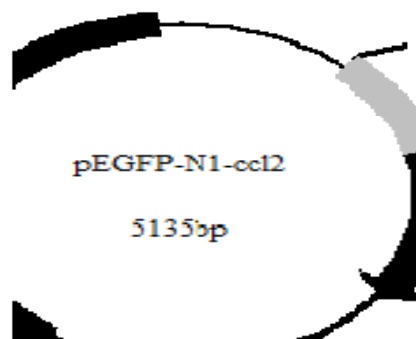


Figure 2: Plasmid map of pEGFP-N1-CCL2

Effect of niacin on proliferation of regenerated hepatocytes

When the concentration of nicotinic acid was more than 1.0 mmol/L at 24 h, there was a significant difference between the experimental and the control groups (Figure 3). Therefore, the concentration of 1.0 mmol/L was selected for subsequent experiments.

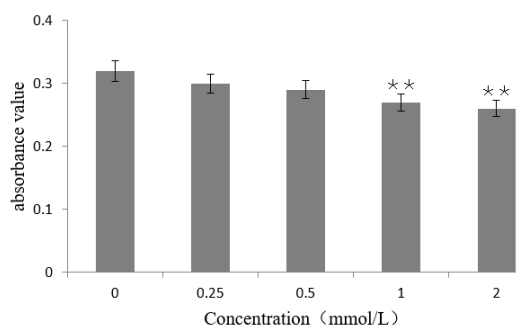


Figure 3: Influence of niacin on the proliferation of regenerated liver cells

Expression of green fluorescent protein (GFP) in cells

At 12 h after transfection with liposomes, the rates of GFP-positivity in the empty plasmid pEGFP-N1 and pEGFP-N1-CCL2 transfection groups were 42.4 ± 5.6 and 45.1 ± 3.5 %, respectively. There was no significant difference between the groups (Figure 4).

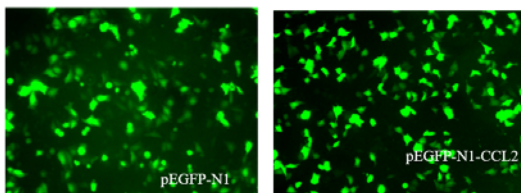


Figure 4: Expression of GFP in regenerated liver cells

Changes of CCL2 protein expression

Western blotting results showed that CCL2 expression in the pEGFP-N1 transfection, pEGFP-N1-CCL2 transfection group, and nicotinic acid and pEGFP-N1-CCL2 co-transfection groups increased over time; however, expression levels in the nicotinic acid and pEGFP-N1-CCL2 co-transfection group was similar to that of pEGFP-N1 transfection group, which was significantly lower than that of pEGFP-N1-CCL2 transfection group (Figure 5).

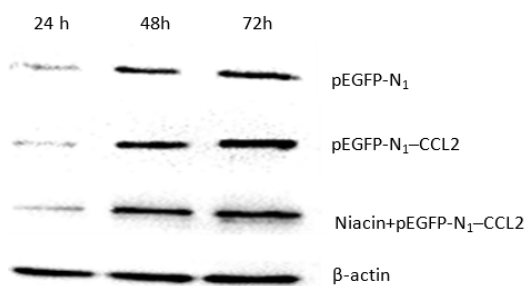


Figure 5: Expression of CCL2 at 24 h, 48 h and 72 h after transfection

Expression of fat-related genes

The expression of ACSL4 increased over time in the pEGFP-N1, pEGFP-N1-CCL2, nicotinic acid and pEGFP-N1-CCL2 groups. However, the expression of nicotinic acid and pEGFP-N1-CCL2 in the co-transfection group was significantly lower than that of the pEGFP-N1-CCL2 transfection group. Expression levels of apolipoprotein E (ApoE) increased over time in all three groups. Expressions levels of apolipoprotein E in the nicotinic acid and pEGFP-N1-CCL2 co-transfection group at 48 and 72 h

were significantly lower than those of the pEGFP-N1-CCL2 transfection group (Figure 6).

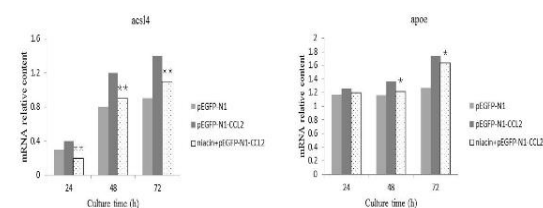


Figure 6: Expression of adipose related genes at 24 h, 48 h and 72 h after transfection with liposomes

DISCUSSION

Liver is the largest digestive organ in humans and higher mammals, with important physiological functions. Hepatocytes, or hepatic parenchymal cells, account for about 80% of all liver cells [10]. In normal physiological state, only about 0.0012–0.01 % of liver cells undergo mitosis; however, when the liver is traumatized or resected, it has a strong regenerative capacity [1]. The 70 % partial hepatectomy (PH) model is the main model for liver regeneration in rats. The remaining liver recovers its original weight and volume after about 1 week, maintains the optimal weight/volume ratio, and restores its original function [2]. After resection, hepatocytes first enter the cell cycle. After hepatectomy in rats, hepatocytes proliferate, and peak of DNA synthesis appeared 24 h after operation, while in mice, it appears 36 h [11]. Therefore, 24 h is the right time to study hepatocytes regeneration.

CCL2 participates in many liver diseases, including hepatitis [12,13], hepatic fibrosis [14], hepatocellular cancer, and carcinoma [15,16]. Clement *et al* found that CCL2 directly acted on hepatic parenchymal cells, causing liver parenchymal cells to express phosphoenol pyruvate carboxykinase, promote fatty acid esterification, and reduce the secretion of fat, resulting in the accumulation of fat in cells [17]. Thomas *et al* found that fat droplets appeared in liver tissue during liver regeneration [18,19]; nevertheless, the role of fat droplets in liver regeneration remained unclear.

The liver is an important digestive organ. Hepatocytes secrete bile that enters the duodenum via the biliary tract, where it aids digestion and absorption of fat and fat-soluble substances [20].

The role of fat in liver regeneration remains unclear. There may be two reasons: (1) Fat droplets provide a place to stay before protein degradation [17], preventing irregular

accumulation of proteins, and assisting in the degradation of proteasomes and lysosomes [21]. Protein inactivation of may expose hydrophobic groups, causing accumulation and cytotoxicity. After partial hepatectomy, many cells are damaged. To reduce inflammation, liver cells may produce fat drops for storing proteins to be degraded; (2) Fat droplets can also act as protein transporters to targeted organelles, including the endoplasmic reticulum, mitochondria and peroxisomes [22,23]. The process of liver regeneration produces large amounts of proteins. As a transport carrier, fat droplets may contribute to the rapid transport of proteins.

Nicotinic acid reduces the levels of serum total cholesterol, triglyceride, very low-density lipoprotein, lipoprotein A and apolipoprotein B in serum, and increase the level of high-density lipoprotein (LDL). Nicotinic acid is thought to be the most valuable drug for regulating lipid metabolism and has great significance in the prevention and treatment of NAFLD. Ganji *et al* found that nicotinic acid significantly inhibits the production of reactive oxygen species, the oxidation of LDL, the expression of vascular cell adhesion molecule-1, and CCL2 in human aortic endothelial cells [24]. In the present study, we also found that the expression of CCL2 in hepatocytes treated with nicotinic acid decreased.

CONCLUSION

CCL2 regulates fat formation, while niacin reduces the expression of CCL2 and inhibits fat synthesis. The signaling pathway mediating the action of niacin and CCL2 in fat synthesis as well as the specific role of fat droplets in liver regeneration need to be further studied.

DECLARATIONS

Acknowledgement

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Conflicts of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this

article will be borne by the authors. The first author conceived and designed the study, and wrote the manuscript. The second author collected and analyzed the data. Other authors carried out the experiment. All authors read and approved the manuscript for publication.

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