Gene 537 (2014) 230–237

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Interleukin 18 accelerates the hepatic cell proliferation in rat liver regeneration after partial hepatectomy

Jihong Zhang [a,b], Chengkai Ma [a,b], Yunqing Liu [a,b], Gang Yang [a,b], Yun Jiang [b], Cunshuan Xu [a,b,*]

[a] College of Life Science, Henan Normal University, Xinxiang 453007, Henan Province, China
[b] Key Laboratory for Cell Differentiation Regulation, Xinxiang 453007, Henan Province, China

ARTICLE INFO

Article history:
Accepted 30 December 2013
Available online 9 January 2014

Keywords:
Liver regeneration
Gene expression profile
Interleukin-18
Hepatocyte proliferation

ABSTRACT

Interleukin 18 (IL-18) is a proinflammatory cytokine with an ability to accelerate cell proliferation through activating other factors. However, little is yet understood of the role of IL-18 in the regulation of liver regeneration (LR). To study the effect of IL-18 on LR, the gene expression profiles of hepatocytes isolated from rat regenerative liver were determined by Rat Genome 230 2.0 microarray. Next, the synergistic effects of genes associated to IL-18 pathway were analyzed by expression profile function *Et*. Then, the expression level of IL-18 was examined by RT-PCR and ELISA. Finally, the effect of IL-18 on hepatocyte proliferation was detected by injecting recombinant rat IL-18 (rrIL-18) into rats immediately after partial hepatectomy (PH) and the rate of hepatocyte proliferation was detected by BrdU labeling. The microarray result showed that the expressions of 13 genes of IL-18 pathway and 49 cell proliferation genes regulated by the pathway were significantly altered at transcriptional level. The *Et* values of three branches of IL-18 pathway, NF-κB, p38 and JNK, were markedly enhanced during the priming and progressing phases of rat LR. The mRNA level of IL-18 was significantly elevated at 2 and 36 h, and its level in plasma was also significantly increased at 2 h, and reached the peaks at 12 h and 48 h after PH ($p < 0.05$). The number of BrdU positive cells was dramatically increased in rats treated with IL-18 compared to PBS control group ($p < 0.01$). In conclusion, IL-18 promotes rat hepatocyte proliferation in the LR priming and progressing phases after PH.

1. Introduction

Hepatocyte is the major cells in the liver, accounting for 70–80% of hepatic mass and 65% of total hepatic cells (Sell, 2003). They have many physiological functions including substance metabolism, amino acid utilization, biotransformation, bile secretion, oxidation protection, and detoxification (Fausto et al., 1995). In normal liver, the majority of adult liver cells are in the quiescent state and only 0.0012–0.01% hepatocytes undergo mitosis (Chen et al., 2013; Khan and Mudan, 2007). However, the hepatocytes are rapidly activated and enter cell cycle to compensate for the lost liver mass to its original level after partial hepatectomy (PH), which is called liver regeneration (LR) (Taub, 2004). LR is a tightly orchestrated process of compensatory growth and rodent liver 2/3 PH is a well-established liver regeneration model. Generally, LR process can be defined as priming phase (0.5–6 h after PH), progressing phase (6–72 h after PH) and terminal phase (72–168 h after PH) (Ma et al., 2009). It has been shown that hepatocytes are activated to proliferate mainly during priming and progressing phases (Fausto et al., 2006; Michalopoulos, 2007).

Interleukin-18 (IL-18), formerly called interferon-γ-inducing factor (IGIF), was initially found as a potent proinflammatory cytokine and played a critical role in the host defense against infections by intracellular microbes (Sugihara, 2000). Prior studies also showed that IL-18 serum level is closely connected with the severity of several liver diseases (Tsutsui et al., 2003) and increased significantly after PH (Shiraki et al., 2007). Using IL-18 transgenic mouse, IL-18 was found with anti-apoptotic effect on *fas*-mediated liver injury. IL-18 can increase the activities of NF-κB and X-linked inhibitors of apoptosis (XIAP) but inhibit caspase-3 activity (Yamamoto et al., 2008). The pleiotropic activities of IL-18 are mediated by IL-18 receptor (IL-18R). Upon binding to IL-18R, IL-18 initiates a signaling cascade that results in the activation of NF-κB (Kojima et al., 1998). It has also been shown that IL-18 has a role in the regulation of cell proliferation. When primary cultured rat chondrocytes and dog osteoblasts were treated with exogenous IL-18, the number of cells labeled with H3 thymidine was increased at 24 h in a dose-dependent manner (Cornish et al., 2003).

Abbreviation: BrdU, bromodeoxyuridine; DAPI, 4′6′-diamidino-2-phenylindole dihydrochloride; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immuno-sorbent assay; IGIF, interferon-γ-inducing factor; IL-18, interleukin 18; IL-18R, interleukin 18 receptor; IPA, Ingenuity Pathway Analysis; LR, liver regeneration; PH, partial hepatectomy; rrIL-18, recombinant rat interleukin 18; RT-PCR, real-time polymerase chain reaction; SO, sham operation.

* Corresponding author at: College of Life Science, Henan Normal University, No. 46, East of Construction Road, Xinxiang 453007, Henan Province, China. Tel.: +86 373 3326001; fax: +86 373 3326524.
E-mail address: cellkeylab@126.com (C. Xu).

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http://dx.doi.org/10.1016/j.gene.2013.12.062

To study the effect of IL-18 on hepatocyte proliferation, hepatocytes were isolated from rat regenerative liver, mRNA was prepared for Rat Genome 230 2.0 microarray. The results revealed 13 genes related-IL-18 signaling pathway and 49 genes related to hepatocyte proliferation were significantly changed. The synergistic effects of these genes on hepatocyte proliferation were analyzed by expression profile function *Et*, a method established by Xu et al. (2010). The *Et* values of three signaling branches of IL-18 pathway, NF-κB, p38 and JNK, were significantly enhanced during the priming phase (2–6 h after PH) and progressing phase (12–72 h after PH) ($p < 0.05$) of rat LR. In support, the expression level of IL-18 mRNA was significantly raised at 2 and 36 h after PH and its plasma level was significantly increased at 2 h, and reached the peaks at 12 h and 48 h after PH, ($p < 0.05$). To further study the direct effect of IL-18 on hepatocyte proliferation, rats were injected with an activated form of recombinant IL-18 immediately after PH, the number of dividing cells was significantly increased in rats treated with IL-18 compared to PBS control group ($p < 0.01$). Collectively, IL-18 promotes rat hepatocyte proliferation via the branches NF-κB, p38 and JNK branches in the LR priming and progressing phases after PH.

2. Materials and methods

2.1. Animals and partial hepatectomy

Sprague–Dawley (SD) male rats (6-week old), weighted 200 ± 10 g, were obtained from animal center of Henan Normal University. Rats were kept at 21 ± 2 °C, relative humidity 60 ± 10%, illuminated 12 h/d (8:00–20:00) with free access to standard rat chow and water. They were subjected to two-third hepatectomy (PH) under ether anesthesia condition (Higgins and Anderson, 1931). Rats in the sham operation (SO) received the same operation procedures but no liver lobes were removed. The rats and all experimental procedures used here were handled in compliance with the Animal Protection Law of China.

2.2. Isolation of hepatocytes

Rats were sacrificed at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168 h after hepatectomy, and their hepatocytes were isolated by conventional two-step perfusion and Percoll density gradient centrifugation (Vondran et al., 2008). Briefly, rats were anesthetized by ether and sterilized with 75% alcohol. Abdominal cavity was opened. Vena cava below and above the liver were ligated following the catheterization of the liver portal vein. The liver was perfused with calcium-free perfusate preheated at 37 °C via the portal vein till the liver surface turned gray–white, and then with 15 ml of 0.05% collagenase IV solution. The dispersed liver cells were collected and washed three times with PBS at 37 °C. Cells were resuspended in PBS at 1×10^8 cells/ml. 6 ml of mixed cell suspension was loaded onto 4 ml 60% Percoll in a 10 ml tube (Pharmacia, Biotech AB, Uppsala, Sweden) and centrifuged at 200 *g*, 4 °C for 15 min. The sediment was enriched with hepatocytes. Hepatocytes were identified by immunocytochemistry by their marker proteins ALB and G6P (Wang et al., 2008), and their viability was measured by trypan blue staining. Purity and viability of the hepatocytes used in this study were over 95%.

2.3. Rat Genome 230 2.0 microarray and data analysis

Total RNA was extracted and purified by the protocol previously described (Twigger et al., 2006). The cDNA first chain was synthesized by SuperScript II RT reverse transcription system, and the second chain was synthesized according to the guideline of Affymetrix cDNA kit. Biotin-labeled cRNA was prepared using GeneChip IVT kit as instructed by the manufacturer. cRNA fragments of 35–200 bp were prepared by fragmentation reagent treatment. The Rat Genome 230 2.0 array was hybridized with the cRNA fragments, which were pretreated. Then, they were stained, washed automatically using GeneChip fluidics station 450 (Affymetrix Inc., Santa Clara, CA, USA), scanned using GeneChip scanner 3000 (Affymetrix Inc., Santa Clara, CA, USA), and the spots were converted into signal values using Affymetrix GCOS 2.0 software (Guo et al., 2008). The signal values were normalized according to manufacturer's instruction. The *p*-values were determined based on the probe signal. When the *p*-value of a gene is <0.05, this gene is defined as present (P), <0.065 is marginal (M), and >0.065 is absent (A). To minimize the experimental operation and microarray test differences, each sample was repeated three times, and the average value was used for statistical analysis.

2.4. Real-time polymerase chain reaction (RT-PCR)

mRNA was prepared from the purified hepatocytes as described above. Primers for *Il-18*, *mapk10*, *ccnd1*, *agt*, *hmox1* and *β-actin*, were designed using Primer Express 5.0 software. Their first chain of cDNA was synthesized by SuperScript II RT reverse transcription system (Promega, USA). The PCR was performed by the conditions with SYBR Green I: 2 min at 95 °C, followed with 40 cycles for 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Each sample was performed in triplicates. Then PCR products were analyzed by melting curve to confirm the specificity of amplification. *β-actin* was used as an internal control.

2.5. Confirmation of the significant-expressed genes and liver regeneration-related genes in rat liver regeneration

The genes with ratio values of PH/SO ≥2 or ≤0.33 and a *p*-value ≤0.01 were considered as significant expressed genes (Fukuhara et al., 2003; Nault et al., 2013). This ratio value cut-off was chosen in order to include genes with a low change in expression and, consequently, a low *p*-value was used in order to reduce the likelihood of false positives. In addition, the genes, at least at one of PH time points in LR, with *F*-test difference $0.01 \le P < 0.05$ or $P \le 0.01$ between PH and SO (de Menezes et al., 2004) were considered as LR-related genes.

2.6. Construction of the interaction network of IL-18 signaling pathways

The terms of "IL-18 signaling pathway" were entered to the websites NCBI (www.ncbi.nlm.nih.gov) and RGD (www.rgd.mcw.edu) to find out IL-18 signaling pathway-associated genes. Then, the genes were collated according to physiological pathway maps embodied by Pathway Maps (http://pathwaymaps.com/maps/) and Ingenuity Pathway Analysis 9.0 (IPA) software (Kong et al., 2011; Xu et al., 2012). Subsequently, the transcription factors of IL-18 signaling pathway were input into TRED (http://rulai.cshl.edu/cgi-bin/ TRED/tred.cgi?process = searchTF GeneForm) and Lymph TF DB (http://www.iupui.edu/~tfinterx/activity.php) to search for their downstream target genes (Childress et al., 2007; Jiang et al., 2007), which were then matched against proliferation-related genes by Gene Ontology (http://www.ncbi.nlm.nih.gov/gene) to select the target genes only related to cell proliferation. Literature review was performed to confirm the function of these genes identified by NCBI database.

2.7. Gene synergy analysis of IL-18 signaling pathway

According to the gene expression abundance of IL-18 signaling pathway in hepatocytes of regenerating liver detected by Rat Genome 230 2.0 Array, expression profile function (*Et*), a mathematical model, was used to measure gene synergy between the related genes with in time series analysis (Tong, 1978), correlation analysis (r_{ik}) (Eisen et al.,