Increased DNA binding activity of NF-κB, STAT-3, SMAD3 and AP-1 in acutely damaged liver

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Abstract

AIM: To investigate the role of genes and kinetics of specific transcription factors in liver regeneration, and to analyze the gene expression and the activity of some molecules crucially involved in hepatic regeneration.

METHODS: USING gel-shift assay and RT-PCR, transcription factors, such as NF-κB, STAT-3, SMAD3 and AP-1, and gene expression of inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF) and c-met were analyzed in an animal model of chemically induced hepatectomy.

RESULTS: Gene expression of HGF and its receptor c-met peaked at 3 h and 24 h after acute CCl4 intoxication. iNOS expression was only observed from 6 to 48 h. SMAD3 showed a high activity at all analyzed times.

CONCLUSION: TNF-α and IL-6 play a central role in hepatic regeneration. These two molecules are responsible for triggering the cascade of events and switch-on of genes involved in cell proliferation, such as growth factors, kinases and cyclins which are direct participants of cell proliferation.

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Key words: Hepatic regeneration; Transcription factor; Gene regulation

INTRODUCTION

The liver is a unique organ with with ability to regulate its growth. This capacity can be altered at various different conditions like tissue dismissal and cell loss caused by chemical or viruses[1]. Specific molecules are involved in the molecular events originated from these processes. One of these molecules is IL-6 which is an important factor for liver regeneration and repair after injury[2]. IL-6-deficient mice fail in regenerating its hepatic gland presenting liver necrosis, functional failure, blunted DNA response in hepatocytes, absence of STAT-3 and NF-κB activation, and selective dysfunction in AP-1, c-myc and cyclin D1 gene expression[3]. IL-6 signals via STAT-3, and STAT-3 activation increase in an IL-6-dependent manner post-hepatectomy (PH) and post acute CCl4 intoxication peaking at 2 h[4,5] and returning to basal levels at 12 h[6]. IL-6 is also an activator of AP-1 expression in liver regeneration[7]. AP-1 and STAT-3 act in a synergistic fashion enhancing transcription[8]. In our previous study IL-6 was strictly detected only at 24 h after acute CCl4 intoxication. We could not detect IL-6 mRNA in rats intoxicated with turpentine, indicating the need of the presence of acute phase response for hepatocyte proliferation[9]. On the other hand, TNF-α has also been shown as a major effector of signal pathways of liver regeneration[9]. Several lines of evidence invoke the role of TNF-α in the regulation of IL-6 secretion through a previous induction of NF-κB[10]. Our previous results showed that induction of TNF-α gene expression takes place as early as 6 h, peaking at 48 h post-acute CCl4 intoxication and this expression might induce IL-6 production[8]. In correlation with our results, others have shown that TNF-α activates NF-κB in many cells within 30 min after intra-peritoneal injection[11]. TNF-α and IL-6 also induce iNOS transcription through NF-κB activity[12] which occurs principally in hepatocytes due to NO production,
and is detected in these cells just after partial hepatectomy and before cell proliferation. HGF, the major growth factor involved in hepatocyte proliferation, signals through its receptor c-met, a transmembrane tyrosine kinase protein product of the proto-oncogene with the same name. HGF is produced by hepatic stellate cells (HSCs) and acts on cultured hepatocytes in a paracrine manner as a potent mitogen. HGF production is induced in animals by partial hepatectomy and hepato-toxin, detecting the mature form in significant levels in animals by partial hepatectomy and hepato-toxin as a potent mitogen.

In this study, we aimed to elucidate the kinetic of activation of several transcription factors and molecular mechanisms involved in hepatic regeneration in an animal model. Furthermore, we shed light on how these transcription factors are involved in the resolution of this process. The early activation of NF-κB, STAT-3 and AP-1 along with NF-κB, STAT-3 and AP-1, HGF and c-met observed in this study suggested that induction of events like production of TNF-α, IL-6, HGF and some proteins are involved in cell proliferation. Knowledge obtained regarding activation of these transcription factors might enable us to propose new pharmacological strategies of treatment for induction of hepatic regeneration in some cases of cirrhosis.

**MATERIALS AND METHODS**

**Animals**

Forty male Wistar rats (Charles Rivers Inc., Boston, MA), weighting 200 g, were used in this study and housed according to the principles and procedures outlined by the National Institute of Health's Guide for the Care and Use of Laboratory Animals. For acute intoxication experiments, five rats for each time point were intragastrically administered a single dose of a mixture 1:1 (v/v) of CCl₄ (Sigma Chemical Company, St Louis MO, USA) at 5 mL/kg of body weight. Control animals were administrated a single dose of mineral oil (Merck Company, Darmstadt, Germany) and mineral oil administered a single dose of a mixture 1:1 (v/v) of CCl₄ (Sigma Chemical Company, St Louis MO, USA) at 5 mL/kg of body weight. Control animals were administrated a single dose of a mixture 1:1 (v/v) of CCl₄ (Sigma Chemical Company, St Louis MO, USA) at 5 mL/kg of body weight.

**Extraction and quantification of RNA**

Isolation of total RNA from rat livers was carried out according to the modified method described by Chomczynski et al. Briefly, liver tissue was taken from three different lobes to obtain a representative sample and homogenized using a Polytron System (Brinkmann, Switzerland) in the presence of Trizol (Invitrogen). Chloroform was added, the aqueous phase was obtained and RNA was precipitated from the aqueous phase by isopropanol at 4°C overnight. Quantity and intactness of RNA were routinely tested by determining absorbance at 260/280 and ethidium bromide fluorescence of RNA electrophoresis on 10 g/L formaldehyde-containing agarose gels.

**Analysis of iNOS, c-met and HGF gene expression**

Expressions of iNOS, c-met and HGF gene were detected using RT-PCR as previously described. We amplified the target genes iNOS, c-met, HGF and the constitutive gene HPRT in different reaction tubes. RNA from liver samples was isolated with Trizol and 2 g of total RNA was reverse transcribed into complementary DNA (cDNA) using 0.05 mol/L Tris-HCl (pH 8.3), 0.04 mol/L KCl, 0.007 mol/L MgCl₂ buffer containing 0.05 g/L random hexamers (Invitrogen), 0.001 mol/L dNTPs mix (Invitrogen), 50 U/L RNase inhibitor and 400 U of Moloney murine leukemia virus reverse transcriptase (M-MLV) (Invitrogen). Samples were incubated for 10 min at 25°C and then for 60 min at 37°C. Reverse transcriptase was further inactivated by heating the sample tubes at 95°C for 10 min. The CDNs were used to perform PCR reaction according to the optimal amplification conditions for each gene. Amplification was performed in a PCR buffer of 0.05 mol/L Tris-HCl (pH 9.0) and 0.05 mol/L NaCl containing a mixture of 1 × 10⁻⁴ mol/L dNTPs and 1 unit of Taq DNA polymerase (Invitrogen). Amplification reactions were overlaid with light mineral oil and held at 94°C for “hot-start” PCR for 3 min and then run in an automated thermal cycler for different number of cycles and incubation temperatures according to each gene. Each PCR reaction was repeated at least in triplicate. Annealing temperature, number of cycles and primer sequence for each gene are shown in Table 1.

**Nuclear extract isolation**

Isolation of nuclear proteins was carried out according to the methods described by Andrews et al., with a few modifications. Briefly, 1 g of liver from CCl₄-treated and controls rats was homogenized in 5 × 10⁻⁴ L of buffer A (0.01 mol/L Heps-KOH (pH 7.9), 250 g/L glycerol, 0.420 mol/L NaCl, 0.0015 mol/L MgCl₂, 2 × 10⁻⁴ mol/L EDTA, 5 × 10⁻⁴ DTT, 2 × 10⁻⁴ PMSF) to disrupt extracellular matrix and cellular membranes. Homogenates were centrifuged at 1200 r/min for 10 s at 4°C. The pellet was resuspended in 2.5 × 10⁻⁴ L of buffer C (0.02 mol/L Heps-KOH (pH 7.9), 250 g/L glycerol, 0.42 mol/L NaCl, 15 × 10⁻⁴ mol/L MgCl₂, 2 × 10⁻⁴ mol/L EDTA, 5 × 10⁻⁴ mol/L DTT, 2 × 10⁻⁴ mol/L PMSF), homogenized and incubated at 4°C for 20 min. Cellular debris was removed by centrifugation at 4°C for 2 min. Supernatant fraction containing DNA binding proteins was recollected and quantified as described by Bradford. Supernatant was stored at -70°C in aliquots until use.

**Gel mobility shift assays**

Electrophoretic mobility shift assay (EMSA) was performed as described elsewhere. Binding reactions were prepared using 2 g of nuclear extracts from either acutely damaged rat livers or from control animals. Additionally, 100 000 cpm of radioactive probe and 1.2 g of poly (DIDC) as a non-specific DNA competitor were included in the binding reactions. Mixtures were incubated for 30 min at room temperature in binding buffer containing 0.01 mol/L Heps (pH 7.5), 0.05 mol/L NaCl, 0.001 mol/L EDTA and 100 g/L glycerol. For competition assays, a 100-fold excess of unlabeled probe was added to the reactions concomitantly with the hot probe. For supershift experiments, 1 mL of antibody against NF-κB,
Table 1  Oligonucleotide sequences used for PCR amplification

| Gene    | Sequence                      | Annealing temperature | Cycles | Size (bp) |
|---------|-------------------------------|-----------------------|--------|-----------|
| HPRT    | 5’TCC CAG CGT CGT GAT TAG TG 3’ | 60°C                  | 30     | 618       |
|         | 5’GCC TTT TTC ACT TTC GCT GA 3’ |                       |        |           |
| iNOS    | 5’TAG AGG AAC ATC TGG CCA GG 3’ | 58°C                  | 25     | 255       |
|         | 5’TGG CCG ACC TGA TGT TGG CA 3’ |                       |        |           |
| c-MET   | 5’TGG TCA GAA CCG GGC TGG CAA CAG GAT 3’ | 60°C                  | 28     | 725       |
| HGF     | 5’TAT CCA ATG ATG CAA TTT CTA ATA TAG TCT 3’ | 60°C                  | 27     | 618       |

Table 2  Oligonucleotide sequences used for EMSA

| Transcription factor | Sequence                      |
|----------------------|-------------------------------|
| NF-κB                | 5’AGT TGA GCC GAC TTT CCC AGG C 3’ |
|                      | 3’TCA ACT CCC GTG AAA GGG TCC G 5’ |
| STAT-3               | 5’GAT CCT TCT TGG GAT TCC 3’ |
|                      | 3’CTA GGA AGA TCC TTA AGG 5’ |
| SMAD-3               | 5’TGG AGA GC CAGA CAA AAA GC CAGA CAT TTA GC CAGA CAC 3’ |
|                      | 3’AGC TTC CG GTCT GTT TTG CG GTCT GTA AAT CG GTCT GTG 5’ |
| AP-1                 | 5’GAT GGA TGA TCT AGA GGA AAA 3’ |
|                      | 3’CTA GCT ACT GAG TCT CCT TTT 5’ |

Bold and underlined letters denote specific consensus DNA-binding sequences.

Figure 1  Semiquantitative RT-PCR analysis for iNOS expression. A: PCR products analyzed by agarose electrophoresis; B: normalized values of iNOS expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

STAT-3, AP-1 or SMAD3 (Santa Cruz Biotechnology) was incubated with the reaction mixture for 1 h at room temperature before regular incubation. The reactions were analyzed on 5% acrylamide non-denaturing gels in 0.5 × Tris-borate-EDTA buffer, dried and exposed. Intensity of each band, as the measure of DNA binding activity, was assessed by densitometric scanning Kodak ID 3.6 program. For gel retardation experiments, single-stranded oligonucleotides were obtained from Sigma and annealed in water. For annealing of complementary oligonucleotide pairs, 5 µg of each single-strand oligonucleotide was adjusted to a final volume of 5 × 10⁻⁶ L and placed on a heating block at 95°C for 5 min. Then the blocker was turned off and left to reach room temperature. Double-strand probe end labeling was performed using T4 polynucleotide kinase (Gibco) in the presence of (γ-32P) ATP. Each gel-shift experiment was performed in triplicate. Probe sequences for NF-κB, STAT-3, SMAD3 and AP-1 are shown in Table 2.

Statistical analysis
Results were expressed as mean ± SD. Student’s t test was used to analyze the data. P < 0.05 was considered statistically significant.

RESULTS
After normalization against the housekeeping gene HPRT, iNOS expression was detected at 6 h after acute liver damage and continued up to 48 h, being statistically different at these times (P < 0.05). However, iNOS expression was not detected before 6 h (Figure 1).

The hepatocyte growth factor (HGF) and its cognate receptor c-met mRNAs after CCl₄ acute intoxication are presented in Figure 2. Two peaks of gene expression were observed in both genes: one at 3 h and another at 24 h where significant difference was seen only at 24 h (P < 0.05).

It has been shown that transcription factors are
activated during hepatic regeneration in different hepatectomy models.\[9,22,23\] To determine the role and the kinetic of activation of transcription factors critically involved in hepatic regeneration after acute CCl4 injury, we performed gel-shift assays to evaluate the binding activity of NF-κB, STAT-3, SMAD-3 and AP-1 on DNA probes containing consensus sequences. We performed a chronological analysis between 0.5 h and 48 h after liver damage, since it has been reported by others and us, that the inflammatory process has declined and cell proliferation has concluded by this time\[23\]. Transcriptional activity of NF-κB, STAT-3, SMAD-3 and AP-1 on DNA were performed gel-shift assays to evaluate the binding activity of NF-κB, STAT-3, SMAD-3 and AP-1 on DNA probes containing consensus sequences. 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We performed a chronological analysis between 0.5 h and 48 h after liver damage, since it has been reported by others and us, that the inflammatory process has declined and cell proliferation has concluded by this time\[23\]. The transcription factor AP-1 showed a basal activity in normal animals. This activity increased as early as 30 min after CCl4-induced injury and increased progressively showing the maximum peak in the last analyzed time in
The role of AP-1 in the expression of molecule participants in cell proliferation, such as c-myc, D1 cyclin and cell growth factors, have been reported. The results obtained with AP-1 support, in fact, the role of AP-1 in hepatic regeneration. In this study, we observed a higher activity of AP-1 and also the maximum hepatocyte proliferation between 24 h and 48 h after CCl4-induced liver damage, which are in agreement with previous studies. The initiation step called “priming step” appears to be mediated by TNF-α and IL-6 and their downstream pathways involving activation of NF-κB, STAT-3 and AP-1. Activation of these transcription factors leads the progression to G1 phase of the cell cycle. “Priming” of hepatocytes induces them to respond to extra- and intra-hepatic growth factors, such as epidermal growth factor (EGF), transforming growth factor-β (TGFB) and HGF. In this study, we found HGF gene expression and its receptor c-met peaking at 1 and 24 h after the liver damage. These results allow to us think that HGF expression could be induced by two
different pathways involving two different molecules, first TNF-α and then IL-6. Since HGF strongly stimulates DNA synthesis in damaged hepatocytes, an increase in HGF and its receptor mRNA expression suggests that liver regeneration is taking place.\[8,9,10\]

TNF-α signals through two distinct receptors: TNFR-1 and TNFR-2.\[38\] Mice lacking functional TNFR-2 show completely normal DNA replication after hepatectomy and CCl₄ treatment. In contrast, lack of signaling through TNFR-1 greatly inhibits DNA replication after partial hepatectomy and cause significant mortality 24-40 h after the operation.\[19\] In TNFR-1 knockout mice, activation of NF-κB and STAT-3 is inhibited and AP-1 activation is decreased. The signal transduction pathway starting from TNF-α required for liver regeneration involves TNFR-1 with NF-κB activation. The sequence of events proposed for liver regeneration after CCl₄-intoxicated acute liver damage is similar to that observed with different animal models like partial hepatectomy. However, the time of activation seems to be more delayed. The sequence of these events is as follows: TNF-α binds to TNFR-1 and induces activation of NF-κB, NF-κB binds to IL-6 promoter and the protein is produced. IL-6 activates STAT-3, which in turn activates AP-1. AP-1 participates in expression of the genes involved in hepatocyte proliferation, such as D1 cyclin, c-myc and kinases.\[7,9\]

In conclusion, TNF-α and IL-6 are responsible for triggering the cascade of events and switch-on of genes involved in cell proliferation, such as growth factors, kinases and cyclins which are direct participants of cell proliferation.

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