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World J Gastroenterol 2006 October 7; 12(37): 5995-6001
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

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

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Supported by CONACyT grant # 28827-M to Adriana Salazar-Montes and, in part, by COECyTJal grant # 08-2004 to Juan Armendariz-Borunda
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Received: 2005-10-20 Accepted: 2005-11-18

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 CCl_4 intoxication. iNOS expression was only observed from 6 to 48 h. Transcriptional factor NF-κB had an early activation at 30 min after acute liver damage. STAT-3 peaked 3 h post-intoxication, while AP-1 displayed a peak of activation at 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.

Key words: Hepatic regeneration; Transcription factor; Gene regulation

Salazar-Montes A, Ruiz-Corro L, Sandoval-Rodriguez A, Lopez-Reyes A, Armendariz-Borunda J. Increased DNA binding activity of NF-κB, STAT-3, SMAD3 and AP-1 in acutely damaged liver. *World J Gastroenterol* 2006; 12(37): 5995-6001

http://www.wjgnet.com/1007-9327/12/5995.asp

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 CCl_4 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[3]. AP-1 and STAT-3 act in a synergistic fashion enhancing transcription[7]. In our previous study IL-6 was strictly detected only at 24 h after acute CCl_4 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[8]. 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 CCl_4 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[13]. 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[14]. HGF is produced by hepatic stellate cells (HSCs) and acts on cultured hepatocytes in a paracrine manner as a potent mitogen[14,15]. HGF production is induced in animals by partial hepatectomy and hepato-toxin[16], detecting the mature form in significant levels[17]. The results in animal models correlate with the elevated levels of serum HGF in patients with hepatic disorders[18]. 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 the expression of iNOS, 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 CCl_4 (Merck Company, Darmstadt, Germany) and mineral oil (Sigma Chemical Company, St Louis MO, USA) at 5 mL/kg of body weight. Control animals were administrated a same volume of vehicle. Animals were sacrificed at 0.5, 1, 3, 6, 12, 24, and 48 h after CCl_4 intoxication. Livers were removed, immediately frozen in CO_2-acetone and stored at -70° until use.

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*[19]. 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 the RNA was precipitated from the aqueous phase by isopropanol at 4℃ overnight. Quantity and intactness of RNA were routinely tested by determining absorbance (*A*) 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[8]. 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 $MgCl_2$ 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℃ and then for 60 min at 37℃. Reverse transcriptase was further inactivated by heating the sample tubes at 95℃ for 10 min. The cDNAs 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℃ 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*[20], with a few modifications. Briefly, 1 g of liver from CCl_4-treated and controls rats was homogenized in 5×10^{-4} L of buffer A (0.01 mol/L Hepes-KOH (pH 7.9), 250 g/L glycerol, 0.420 mol/L NaCl, 0.0015 mol/L $MgCl_2$, 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℃. The pellet was resuspended in 2.5×10^{-4} L of buffer C (0.02 mol/L Hepes-KOH (pH 7.9), 250 g/L glycerol, 0.42 mol/L NaCl, 15×10^{-4} mol/L $MgCl_2$, 2×10^{-4} mol/L EDTA, 5×10^{-4} mol/L DTT, 2×10^{-4} mol/L PMSF), homogenized and incubated at 4℃ for 20 min. Cellular debris was removed by centrifugation at 4℃for 2 min. Supernatant fraction containing DNA binding proteins was recollected and quantified as described by Bradford[21]. Supernatant was stored at -70℃ in aliquots until use.

Gel mobility shift assays

Electrophoretic mobility shift assay (EMSA) was performed as described elsewhere[3]. 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 (DI-DC) 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 Hepes (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℃	30	618
	5′ GGC TTT TCC ACT TTC GCT GA 3′			
iNOS	5′ TAG AGG AAC ATC TGG CCA GG 3′	58℃	25	255
	5′ TGG CCG ACC TGA TGT TGC CA 3′			
c-MET	5′ CAG TGA TGA TCT CAA TGG GCA AT 3′	60℃	28	725
	5′ AAT GCC CTC TTC CTA TGA CTT C 3′			
HGF	5′ AGC TCA GAA CCG ACC GGC TTG CAA CAG GAT3′	60℃	27	618
	5′ TTA CCA ATG ATG CAA TTT CTA ATA TAG TCT3′			

Table 2 Oligonucleotide sequences used for EMSA

Transcription factor	Sequence
NF-κB	5′ AGT TGA **GGG GAC TTT CCC** AGG C 3′
	3′ TCA ACT **CCC CTG AAA GGG** TCC G 5′
STAT-3	5′ GAT CC**T TCT GGG AA**T TCC 3′
	3′ CTA GG**A AGA TCC TT**A AGG 5′
SMAD-3	5′ TCG AGA GC **CAGA** CAA AAA GC **CAGA** CAT TTA GC **CAGA** CAC 3′
	3′ AGC TCT CG **GTCT** GTT TTT CG **GTCT** GTA AAT CG **GTCT** GTG 5′
AP-1	5′ GAT CGA **TGA CTC A**GA GGA AAA 3′
	3′ CTA GCT **ACT GAG T**CT CCT TTT 5′

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

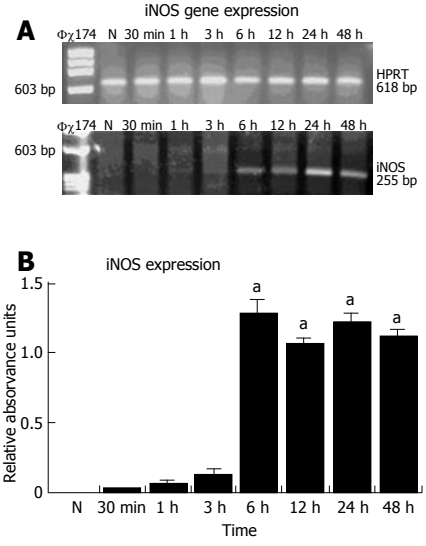

Figure 1 Semiquantitative RT-PCR analysis for iNOS expression. **A:** PCR products analyzed by agarose electrophoresis; **B**: normalized values of iNOS expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

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

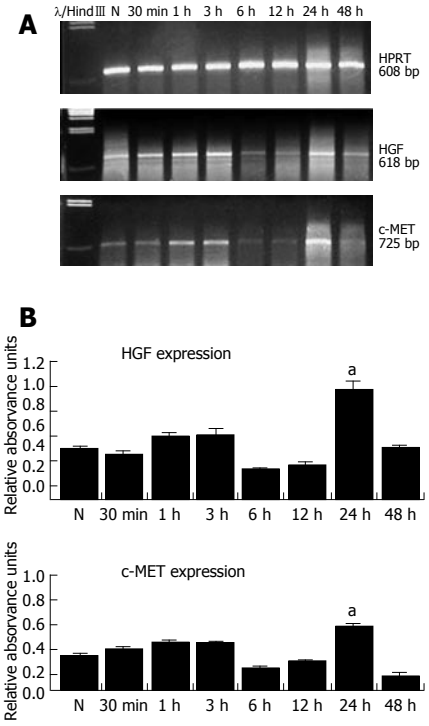

Figure 2 Semiquantitative RT-PCR analysis for HGF and c-met expressions. **A**: PCR products analyzed by agarose electrophoresis; **B**: Normalized values of HGF and c-met expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

in water. For annealing of complementary oligonucleotide pairs, 5 μg of each single-strand oligonucleotide was adjusted to a final volume of 5×10^{-5} L and placed on a heating blocker at 95℃ 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 CCl_4 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

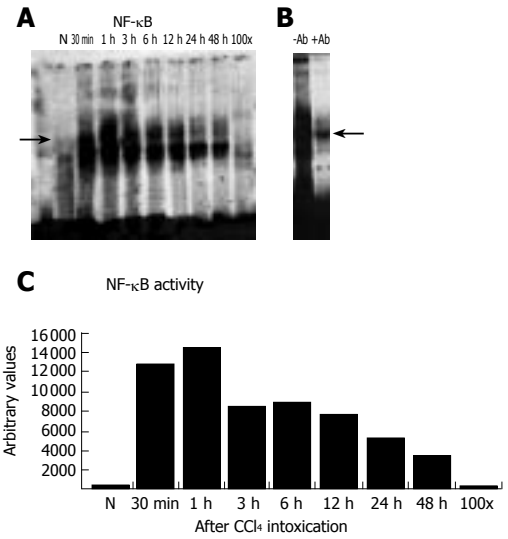

Figure 3 Binding activity of NF-κB in acute liver damage. Wistar rats were intoxicated acutely with CCl_4 and then sacrificed at different time points. Nuclear proteins were extracted from the livers and Gel-shift assay was performed for NF-κB transcription factor. **A**: Binding DNA activity of NF-κB after CCl_4 intoxication; **B**: supershift assay using 1-h sample, 1 μL of polyclonal antibody against p65 subunit was added and incubated for 1 h before usual EMSA assay; **C**: densitometric analysis of results of EMSA assay.

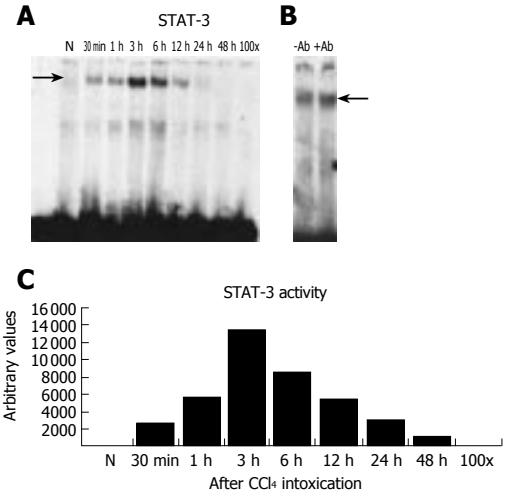

Figure 4 Binding activity of STAT-3 in acute liver damage**.** Wistar rats were intoxicated acutely with CCl_4 and then sacrificed at different time points. Nuclear proteins were extracted from the livers and Gel-shift assay was performed for STAT-3. **A**: Binding DNA activity of STAT-3 after CCl_4 intoxication; **B**: supershift assay similar using 6-h sample; **C**: densitometric analysis of results of EMSA assay.

activated during hepatic regeneration in different hepatectomy models[9,22,23]. To determine the role and kinetic of activation of transcription factors critically involved in hepatic regeneration after acute CCl_4 injury, we performed gel-shift assays to evaluate the binding activity of NF-kB, 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[24]. Transcriptional

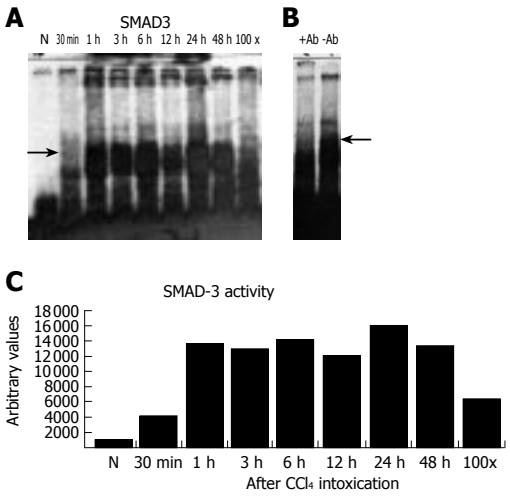

Figure 5 Binding activity of SMAD3 in acute liver damage. Gel-shift and supershift assays were performed to analyze SMAD3 DNA binding activity. **A**: Binding DNA activity of SMAD3 after CCl_4 intoxication; **B**: supershift assay using 24-h sample; **C**: densitometric analysis of results of EMSA assay.

factor NF-κB presented strong DNA binding activity from 30 min after CCl_4-induced injury, peaking at 1 h and decreasing thereafter. Nevertheless, 48 h after CCl_4-induced injury, NF-κB activity did not return to normal levels (Figure 3A). To confirm the specific binding activity of NF-κB, we performed supershift assay using specific anti-NF-κB antibodies. The use of a polyclonal antibody against the p65 subunit of NF-κB decreased the binding of this transcription factor to the labeled probe, suggesting the binding of the antibody to the DNA binding site of the transcription factor (Figure 3B). Furthermore, almost complete elimination of the radiolabeled band, when a 100-fold cold DNA probe was used, confirmed the specificity of our results. These results were confirmed by densitometric analysis (Figure 3C).

With respect to STAT-3 activity, our results showed an increase at 30 min after intoxication, presenting the maximum activity at 3 h, and then decreasing and disappearing completely by 48 h (Figure 4A). We also made a supershift assay for this transcription factor obtaining a clear DNA binding reduction. The data suggest that, in our experimental conditions, this antibody preferentially binds to the DNA binding site on the transcriptional factor, thus hindering formation of STAT-3-DNA consensus site complex. This piece of data verifies that the results corresponded to this transcription factor (Figure 4B). These results were confirmed by densitometric analysis (Figure 4C).

On the other hand, SMAD3 showed a strong binding activity in this animal model at all analyzed times, as was confirmed by densitometric analysis at 1 h after CCl_4 intoxication until 48 h (Figures 5A and C). The specificity of the binding activity was analyzed by supershift assay (Figure 5B).

The transcription factor AP-1 showed a basal activity in normal animals. This activity increased as early as 30 min after CCl_4-induced injury and increased progressively showing the maximum peak in the last analyzed time in

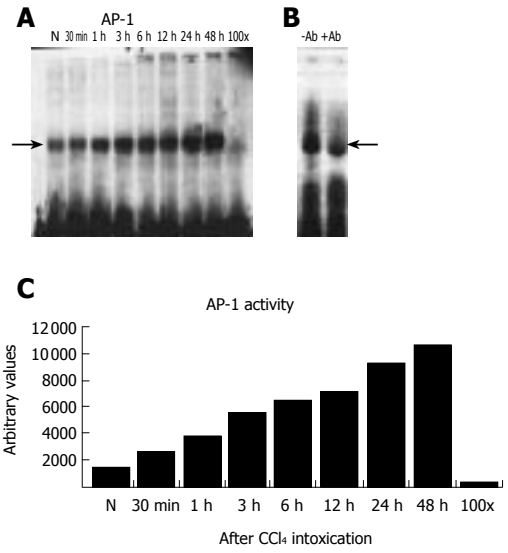

Figure 6 Binding activity of AP-1 in acute liver damage. EMSA and supershift assays, similar to Figures 3 and 4, were performed to analyze AP-1 activity in the same samples. **A**: Binding DNA activity of AP-1 after CCl_4 intoxication; **B**: supershift assay using 24-h sample; **C**: densitometric analysis of results of EMSA assay.

this study, 48 h after intoxication. Figures 6A-B clearly show the specificity of AP-1 DNA-binding activity when an excess of cold probe and specific anti-AP-1 antibody were used to override the binding of nuclear factor to the radiolabeled DNA-consensus sequence. These results were confirmed by densitometric analysis (Figure 6C).

DISCUSSION

The increase in DNA binding activity of the transcription factors NF-κB, STAT-3 and AP-1 analyzed in this study indicates that hepatic regeneration process in response to CCl_4-induced acute liver damage requires the switch on and the switch off of many genes. These genes include cytokines, growth factors, kinases and cyclins which regulate cell cycle and induce hepatocyte proliferation. Our previous study demonstrated that expression of IL-6 is strongly associated with hepatic regeneration[8]. We observed IL-6 gene expression in acute liver damage between 6 and 24 h, and disappeared thereafter. Other pro-inflammatory cytokines like IL-1β and TNF-α also show strong expression around 24 h after liver damage[24,25]. Because NF-κB is required for TNF-α and iNOS production[12], our results suggest that the strong inflammatory response present in CCl_4-intoxicated animals causes oxidative stress manifested as an increase in NF-κB activity which induces iNOS expression. Although the maximum activity of NF-κB was detected at 1 h post-CCl_4 intoxication, and iNOS expression was not observed before 6 h, suggesting that some hours are required for iNOS RNAm to be detected. This inflammation is accompanied with damage and death followed by hepatocyte proliferation where TNF-α plays an important role[26]. The activation of NF-κB, STAT-3, SMAD3 and AP-1 allows their migration to the nucleus, where they can bind to their consensus sequence and induce the expression of several genes involved in inflammation and cell proliferation[27]. TNF-α promoter contains multiple binding sites for NF-κB being a vital component for its expression and a ubiquitous oxidative stress-sensitive transcription factor[28]. NF-κB is found in almost all cell types, including hepatocytes and non-parenchymal cells[29]. In absence of NF-κB, TNF-α functions as an apoptotic agent in liver development. The NF-κB inhibitor (Iκ-B) degradation is enhanced by reactive oxygen species that can be generated by TNF and many other agents and cellular processes[30].

Evidence indicates that blockade of NF-κB in the regenerating liver by expression of NF-κB super-repressor in an adenovirus vector leads to apoptosis after the cells have replicated their DNA[31]. Similarly, introduction of I-κB in an adenovirus vector after partial hepatectomy in mice results in increased liver injury and decreased hepatocyte cell proliferation.

On the other hand, transcription factor STAT-3 is also activated after partial hepatectomy but its activation is delayed compared to NF-κB. STAT-3 becomes activated mainly by IL-6 type cytokines[32]. Binding of IL-6 causes dimerization of the receptor, activation of tyrosine kinases which phosphorylate gp130 and create docking sites for STAT-3 binding. IL-6/STAT-3 signaling pathway is involved in cell proliferation through the induction of cyclins D1, D2, D3, A, cdc25A and concomitant down-regulation of cyclin-dependent kinase (cdk) inhibitors p21 and p27[33,34]. p53, mdm2, p21, cyclins and cdks genes are also activated. STAT-3 activation observed in this study is in agreement with previous reports which demonstrated binding activity as early as 30 min using partial hepatectomy model to induce liver regeneration[1,23]. SMAD3 have been found to be involved in hepatic stellate cell activation and collagen production after liver damage[34], since high level of transcription factor SMAD3 could be detected even after 48 h of CCl_4 intoxication. These results suggest that liver damage caused by CCl_4 intoxication has not resolved at this time and some genes involved in the damage resolution activated by SMAD3 like TGF-α and collagen I are being expressed.

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[23]. 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 CCl_4-induced liver damage, which are in agreement with previous studies[35,36]. 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[1,37]. Activation of these transcription factors leads the progression to G1 phase of the cell cycle[33]. "Priming" of hepatocytes induces them to respond to extra- and intra-hepatic growth factors, such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and HGF[1]. 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[15,17].

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 CCl_4 treatment. In contrast, lack of signaling through TNFR-1 greatly inhibites DNA replication after partial hepatectomy and cause significant mortality 24-40 h after the operation[39] 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 CCl_4-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.

ACKNOWLEDGMENTS

We thank to Dr. Pedro Diaz for providing care to experimental animals and to Nuclear Medicine Department, Hospital Civil de Guadalajara for providing radioactivity facilities.

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