Alteration of Intrahepatic Cytokine Expression and AP-1 Activation in Transgenic Mice Expressing Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) infection often leads to the development of hepatocellular carcinoma (HCC), but its molecular mechanism has not been clearly elucidated. Previously, transgenic mice constitutively expressing HCV core protein have been shown to develop HCC, suggesting a pivotal role of the core protein in hepatocarcinogenesis. Here, we analyzed the expression of cytokines associated with a variety of cellular processes, including cell proliferation, in the mouse model for HCV-associated HCC to define the molecular events prior to oncogenesis. The expression of tumor necrosis factor-α and interleukin-1β was increased at both protein and mRNA levels. In addition, the activities of c-Jun N-terminal kinase and activator protein-1 (AP-1), downstream effectors, were enhanced, while IκB kinase or nuclear factor-κB activities were not enhanced. Thus, the altered in vivo expression of cytokines with AP-1 activation in consequence to the core protein expression may contribute to hepatocarcinogenesis in persistent HCV infection.

Key Words: HCV; core protein; transgenic mouse; TNF-α; IL-1β; AP-1; JNK; NF-κB; HCC.

Hepatitis C virus (HCV) is a causative agent of acute and chronic non-A non-B hepatitis (Choo et al., 1989). HCV infection frequently persists and leads to liver cirrhosis and hepatocellular carcinoma (HCC) (Saito et al., 1990). However, the mechanism of hepatocarcinogenesis in HCV infection is not clearly understood.

The core protein of HCV, by interacting with its viral RNA, forms a viral nucleocapsid (Shimoike et al., 1999). In addition to this genome-packaging function, the core protein has various functions. The core protein transforms fibroblasts with or without the cooperation of the ras oncogene, suppresses or enhances apoptosis of cultured cells, modulates the transcription of some genes, and binds to some cellular proteins (Lai and Ware, 2000; McLauchlan, 2000; Suzuki et al., 1999). In addition, we have reported that transgenic mice expressing the core protein in the liver develop HCC (Moriya et al., 1999). These results indicate that the core protein is closely associated with hepatocarcinogenesis in HCV infection.

Cytokines are important mediators of tissue injury and inflammation. The cytokine signal is transmitted to cells via membrane-bound receptors. This cytokine–receptor interaction activates intracellular signaling pathways. Cytokine-inducible transcription factors, including activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), interact with their responsive elements in the promoter regions of various genes. Cytokines like tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 are closely associated with liver regeneration. Treatment with antibodies to TNF-α before partial hepatectomy resulted in decreased DNA synthesis and an abrogation of increases in c-Jun N-terminal kinase (JNK), c-jun mRNA, and nuclear AP-1 activity (Diehl et al., 1994). In addition, mice lacking the type I TNF receptor were deficient in liver regeneration (Yamada et al., 1997). Concerning IL-6, hepatocyte DNA synthesis during liver regeneration was suppressed in mice carrying a homozygous deletion of the IL-6 gene (Cressman et al., 1996). Taken together, the cytokines described above are considered as playing important roles in cell proliferation and, possibly, hepatocarcinogenesis.

TNF-α, IL-1, and IL-6 are mainly synthesized by blood cells like lymphocytes and monocytes, but the liver is also an important producer of these cytokines. Human and murine hepatocytes as well as Kupffer cells produce TNF-α, IL-1, and IL-6 (Andus et al., 1991; Gonzalez-Amaro et al., 1994; Hunt et al., 1992; Wordemann et al., 1998).

Since TNF-α signaling plays important roles in a wide range of cellular functions, including inflammation, cell proliferation, differentiation, and apoptosis, it is rational to consider that the core protein contributes to these
pathogeneses by influencing TNF-α signaling pathways. In fact, several previous reports showed an association or interaction of the core protein with TNF-α signaling pathways (Chen et al., 1997; Marusawa et al., 1999; Matsumoto et al., 1997; Shrivastava et al., 1998; Zhu et al., 1998). However, carcinoma or transformed cell lines were used to express the core protein, and some conflicting evidence was observed in these studies.

Here we demonstrate the enhancement of TNF-α and IL-1β expression in the liver of core-transgenic mice in the absence of inflammation in the liver. In these mice, the activation of JNK and transcription factor AP-1, which are downstream effectors of these cytokines, was also demonstrated. These results suggest that the core protein potentially enhances the hepatic expression of TNF-α and IL-1β and activates AP-1 in vivo, contributing to hepatocarcinogenesis.

RESULTS

HCV core protein modulates intrahepatic cytokine expression in the liver of core-transgenic mice

Cytokines such as TNF-α, IL-1β, and IL-6 are closely associated not only with controlling proliferation of hepatocytes, but also with various pathogeneses of the liver, including regeneration, necrosis, inflammation, fibrosis, and, possibly, carcinogenesis (Andus et al., 1991). The liver is an important source of production of these cytokines as well as the main scavenger for them (Andus et al., 1991). Therefore, we first investigated the gene expression profiles of the above cytokines in the liver of core-transgenic mice. In this study, we used pairs of young, 2- to 3-month-old transgenic mice and their littermates for the following reasons. First, we aimed to assess the direct in vivo effect of the core protein in the liver. Second, we wanted to use core-transgenic mice with histopathological changes of the liver that were as slight as possible without advanced steatosis or HCC because these pathological changes themselves might modulate cytokine expression. The levels of the core protein in the liver of core-transgenic mice were within the range of those in livers of HCV-associated HCC patients (Koike et al., 2002).

We determined intrahepatic mRNA expression of cytokines by RT–PCR. Figure 1A shows the mRNA expression of TNF-α and IL-1β in the liver of core-transgenic mice of 2 to 3 months old and that of nontransgenic control mice of the same ages. The expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was also examined. In spite of a lack of inflammation in the liver of core-transgenic mice, gene expression of these cytokines was enhanced in core-transgenic mice compared to that of nontransgenic littermates. The expression levels of the GAPDH gene were similar in all of the samples, indicating that the amount of mRNA used and the efficiency of the amplification were almost equal. A similar tendency was also observed when we decreased the cycles of the PCR by five, indicating that the PCR products were not saturated. On the other hand, the gene expression of IL-6 as well as of β-actin in the liver of core-transgenic mice was principally similar to that of nontransgenic mice (Fig. 1B).

Such modulation of the cytokine gene expression was observed in the mice from two independent lines. We further performed TaqMan real-time PCR to quantify TNF-α and IL-1β mRNA in the liver of mice. For statistical analysis, we examined three more pairs of mice in addition to those used for the above experiment. One hundred nanograms of total RNA was amplified, and the amount of the PCR products was recorded at each cycle. mRNA copy numbers calculated from the calibration curves were normalized against GAPDH, the expression of which was not altered by the core protein in mice (data not shown). As shown in Table 1, the mRNA levels of TNF-α and IL-1β in the liver of core-transgenic mice were increased 2- to 2.5-fold and 1.5- to 2-fold, respectively. These results suggest that the expression of the core protein enhances intrahepatic gene expression of the cytokines TNF-α and IL-1β in core-transgenic mice.

To assess whether these enhanced gene expressions reflected the intrahepatic protein levels, we next determined the protein expression of TNF-α and IL-1β in the liver of these mice. The amount of TNF-α and IL-1β was determined by ELISA using mouse liver lysates and divided by the amount of liver total proteins. As shown in Table 2, both the TNF-α and IL-1β protein levels were...
significantly increased in the livers of core-transgenic mice compared to nontransgenic littermates. The ratios (core-transgenic/nontransgenic) of the TNF-α and IL-1β protein levels were 1.9 and 2.3, respectively, which were slightly different from those of the mRNA levels (2.4 and 1.8, respectively). We cannot clearly show the reason for this, but one possible explanation is that possible post-transcriptional modifications work for the protein expression of the cytokines as reported previously (Chantry et al., 1989; Moller et al., 1998). We also determined the protein expression levels of these cytokines in the livers of the mice expressing HCV envelope proteins under the same regulatory region as that of the core-gene-transgenic mice (Koike et al., 1997) and found similar expression levels in envelope-transgenic and control mice (data not shown).

### TABLE 1

<table>
<thead>
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<th>Transgenic mice (n=6)</th>
<th>Nontransgenic mice (n=6)</th>
<th>P</th>
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<tr>
<td>TNF-α</td>
<td>$1.30 \times 10^{-2}$</td>
<td>$0.54 \times 10^{-2}$</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-1β</td>
<td>$2.25 \times 10^{-2}$</td>
<td>$1.24 \times 10^{-2}$</td>
<td>&lt;0.05</td>
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* Mann–Whitney’s U test.
* Values are expressed in mRNA copy numbers quantified by real-time PCR, followed by normalization against levels of GAPDH (means ± SE).

### TABLE 2

<table>
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<th>Transgenic mice (n=7)</th>
<th>Nontransgenic mice (n=7)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>$600.5 \pm 311.0$</td>
<td>$323.4 \pm 114.6$</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-1β</td>
<td>$1387.3 \pm 565.8$</td>
<td>$610.6 \pm 160.6$</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Mann–Whitney’s U test.
* Picograms/milligrams total proteins (means ± SE).

**HCV core protein activates the JNK–AP-1 pathway in the liver of core-transgenic mice**

TNF-α and IL-1β bind to their specific receptors on the cell surface and activate intracellular signaling cascades. One of the well-known pathways is JNK–AP-1; JNK is activated by TNF-α or IL-1β stimulation and phosphorylates c-Jun, leading to AP-1 activation (Angel and Karin, 1991). Therefore, we examined whether this pathway was modulated in the liver of core-transgenic mice. First, we determined JNK activity by *in vitro* kinase assay. As shown in Fig. 2A, the JNK activity was increased in the livers of core-transgenic mice. Densitometry showed that the JNK activity was increased approximately 2.5-fold compared to that of nontransgenic mice. This was almost consistent with the result that the expression of both TNF-α and IL-1β was about 2-fold increased in the liver of core-transgenic mice (Table 2). We also determined the activity of extracellular signal-regulated kinase (ERK) and found similar activation between core-trans-
genic and nontransgenic mice (Fig. 2B). This indicates that JNK was selectively activated in core-transgenic mice.

We next determined the AP-1 activation in the liver of core-transgenic and nontransgenic mice. As shown in Fig. 3A, the activation of AP-1 in core-transgenic mice was markedly enhanced compared to that in nontransgenic littermates. Densitometry showed that the level of AP-1 activity was about two times higher in these transgenic mice. The same amount of nuclear proteins used in the assay was stained by Coomassie brilliant blue after separation by SDS–PAGE (Fig. 3A, lower panel), indicating that a similar amount of nuclear proteins was used between core-transgenic and nontransgenic mice. We confirmed the specificity of this band by adding 50-fold, unlabeled, identical or different oligonucleotides (Fig. 3B). These results indicate that the JNK–AP-1 pathway was activated in the liver of core-transgenic mice.

AP-1 consists of c-Jun and c-Fos, and the expression of c-jun and c-fos is regulated mainly by JNK–AP-1 and ERK-SRE, respectively. Therefore, we determined the gene expression of c-jun and c-fos by RT–PCR. As shown in Fig. 3C, c-jun but not c-fos expression was increased in the liver of core-transgenic mice. In addition, the expression of the collagenase gene, which is known to be regulated by AP-1 (Angel et al., 1987), was increased in the liver of core-transgenic mice (Fig. 3C). Semiquantitative comparisons of the mRNA abundance revealed that the expression of c-jun and collagenase was increased about two- to threefold in the liver of core-transgenic mice, whereas the expression of c-fos as well as β-actin was similar. These results indicate that AP-1 activation actually modulated the responsible gene expression in core-transgenic mice.

HCV core protein does not activate the IKK–NF-κB pathway in the liver of core-transgenic mice

TNF-α and IL-1β are known to activate IκB kinase (IKK). IKK phosphorylates IκB, leading to degradation by proteasome. Loss of IκB results in nuclear translocation and activation of NF-κB (Baeuerle, 1991). Therefore, we determined the kinase activity of IKK derived from the
liver of core-transgenic mice. IKK was immunoprecipitated by anti-IKK antibody from lysates of mouse liver and incubated with GST–IκB-α in the presence of \(\gamma^{-32}\text{P})\text{ATP}. The lower panel shows densitometric analysis of the blots. Values were normalized by taking the density of the nontransgenic mice as 1 (relative kinase activity). Data shown are means ± SE (\(n = 3\) for each sample group). N. S., no statistically significant difference. (B) The expression of IκB-α determined by Western blotting. Top, IκB-α; bottom, β-actin. The intensity of the bands corresponding to IκB-α analyzed by densitometry and the ratio of the intensity of IκB-α to that of β-actin is shown below. (C) The NF-κB activation determined by EMSA.

FIG. 4. The activity of IKK and NF-κB in the liver of core-transgenic (T) and nontransgenic (NT) mice. (A) The activity of IKK determined by \textit{in vitro} kinase assay. IKK was immunoprecipitated by anti-IKK-α/β antibody, and an \textit{in vitro} kinase assay was performed using the GST–IκB-α (aa 1–54) fusion protein as a substrate. As shown in Fig. 4A, the IKK activity was almost equal in core-transgenic and nontransgenic mice. We also determined the expression of IκB-α in the liver of the mice. As mentioned above, IκB-α was phosphorylated by IKK and rapidly degraded by the ubiquitin–proteasome pathway, so IKK activity should correlate negatively with IκB-α expression. As shown in Fig. 4B, IκB-α expression in the liver of core-transgenic mice was similar to that of nontransgenic mice, further confirming that IKK activity was not activated by the core protein in core-transgenic mice.

DISCUSSION

We previously demonstrated that HCV core-transgenic mice develop steatosis and later HCC, indicating an important role of the core protein in hepatocarcinogenesis (Moriya \textit{et al.}, 1997b, 1998). In core-transgenic mice, the degree of the core protein expression, determined by Western blotting and fluorescence enzyme immunoassay, was comparable with that in the liver of patients with HCV-associated HCC (Koike \textit{et al.}, 2002). However, in some works from other laboratories, transgenic mice harboring HCV genes showed only mRNA expression but not detectable levels of core protein. Reasons for this may be technical issues in producing transgenic mice, HCV and mouse strains, or the sensitivity of detection systems, particularly antibodies, as discussed previously (Koike \textit{et al.}, 2002). In our core-transgenic mice, (i) HCCs were observed later in their lives, (ii) there was a gender difference in HCC incidence, and (iii) histologically, no inflammation was observed. These characteristics, except for (iii), are analogous to HCV-related HCC in humans. Changes of some host factors may be involved in HCC development as a consequence of the core protein expression. We thus proceeded to investigate the differ-
ential expression of several genes related to cell proliferation in the livers of core-transgenic mice.

In this study, we showed that the expression of TNF-α and IL-1β was significantly enhanced in core-transgenic mice compared to that in nontransgenic littermates. The enhancement of IL-6, in contrast, was not observed. Furthermore, the JNK–AP-1 pathway was activated in the liver cells, but the IKK–NF-κB pathway, another downstream effector of these cytokines, was not enhanced. These modifications of expression of cytokines and transcription factors involved in the signal transduction pathway were observed at as early as 2 months of age in core-transgenic mice, as a consequence of the core protein expression in the liver.

There are conflicting data concerning the association of the core protein with NF-κB activation in previous studies using various cultured cells. Some reports demonstrate that the core protein enhances NF-κB activation (Chung et al., 2001; Marusawa et al., 1999; You et al., 1999), whereas other reports demonstrate that the core protein suppresses TNF-α-induced NF-κB activation (Shrivastava et al., 1998) or does not modulate NF-κB activation in human-derived cells (Zhu et al., 1998). This discrepancy may be due to the differences in the assay systems or cell cultures used. The cells used in the above studies were carcinoma cell lines or transformed cell lines, and their expression levels of the core protein seemed to be considerably higher than that in the liver of HCV-infected patients. Recently, two studies demonstrated that NF-κB was activated in the liver tissue of HCV-infected patients in contrast to that in normal healthy adults (Boya et al., 2001; Tai et al., 2000). However, in these cases one cannot exclude the association of cytokines released by leukocytes, which could contribute to NF-κB activation in the liver, since HCV-infected liver tissue is generally accompanied by inflammatory changes. Our core-transgenic mice expressed the core protein in the liver at a level comparable to that of HCV-infected patients (Koike et al., 2002; Moriya et al., 1998) and exhibited no inflammatory changes throughout their lives. Thus, our core-transgenic mouse model is suitable for investigating the activation of transcription factors, including NF-κB, in the liver by the core protein, and the core protein does not significantly enhance NF-κB activation in the liver, which was otherwise normal.

There are several reports demonstrating the association of the core protein with TNF-α signaling pathways (Chen et al., 1997; Marusawa et al., 1999; Matsumoto et al., 1997; Shrivastava et al., 1998; Zhu et al., 1998). The core protein binds to the TNF receptor superfamily lymphotixin-β receptor and the cytoplasmic domain of TNF receptor 1 (TNFR1) (Matsumoto et al., 1997; You et al., 1999; Zhu et al., 1998). Such binding implies that the core protein may be involved in the apoptosis signaling pathway and mechanisms of host immune defenses. The present study, for the first time, demonstrates the in vivo enhancement of TNF-α expression in the liver by the core protein, which was expressed in otherwise normal liver. Hepatocytes as well as Kupffer cells produce TNF-α (Gonzalez-Amaro et al., 1994; Wordemann et al., 1998); according to a previous study, significant TNF-α production was observed in hepatocytes with chronic HCV infection, whereas TNF-α was detected at low levels in hepatocytes from normal individuals or patients with alcoholic liver diseases (Gonzalez-Amaro et al., 1994). In view of this report and our result here, the core protein may up-regulate TNF-α expression in hepatocytes of core-transgenic mice, as the core protein was detected mainly in hepatocytes of the liver tissues (Moriya et al., 1997b). Alternatively, the core protein expression in hepatocytes may indirectly up-regulate the expression of the cytokines in nonparenchymal cells, such as Kupffer or Kupffer-like cells. Further studies are necessary to clarify this point.

We showed here that the expression of TNF-α and IL-1β, but not IL-6, was enhanced in the liver of core-transgenic mice. Although IL-6 expression is positively regulated by several factors, such as TNF-α, IL-1, AP-1, and NF-κB (Seigel, 1992), the cooperative interaction of mediators, including negative cross-talk, could be required to achieve the level of IL-6 expression. Indeed, according to a previous report investigating the levels of cytokines in sera in chronic liver diseases, the levels of circulating IL-1β were closely correlated with that of TNF-α, whereas the IL-6 level correlated only weakly with that of TNF-α and did not correlate with that of IL-1β (Tilg et al., 1992). It is noteworthy that a vitronectin gene, which was reported to be regulated positively by IL-6 and negatively by TNF-α and IL-1 (Seiffert et al., 1996), is down-regulated in the liver of core-transgenic mice of all ages (data not shown). Interestingly, a recent report showed that gene expression of vitronectin was also down-regulated in the liver of patients with chronic hepatitis C (Honda et al., 2001). This is compatible with the enhanced expression of TNF-α and IL-1β in the transgenic mice noted in our study. However, contrary to the situation with HCV-infected human livers, our transgenic mice expressed only the core protein. No HCV replication took place. Therefore, it may not be reasonable to directly extrapolate the events that occur in the core-transgenic mice with those in human livers. However, several studies showed that the serum and intrahepatic levels of TNF-α and IL-1β were increased in HCV-infected patients compared to those in healthy controls (Larrea et al., 1996; Tilg et al., 1992). In view of such reports and the fact that there is no in vitro culture system for HCV, our core-transgenic mice are regarded as one of the appropriate models for investigating HCV pathogenesis. In addition, it should be noted that our results were not obtained from cultured cells, which already have properties of cancer cells, but from normal
liver cells, which constitute the liver organ in living animals. Future work will be aimed at investigating the mechanism of differential expression of these cytokines in core-transgenic mice.

Our data suggest that the core protein enhances activation of AP-1, a downstream effector of TNF-α and IL-1β. Increased expression of these cytokines in core-transgenic mice possibly contributes to AP-1 activation, since JNK, which phosphorylates and activates c-Jun, a component of AP-1, was also activated. In addition, enhanced expression of c-jun but not c-fos by AP-1 may also contribute to AP-1 activation. Of course, several other factors may also be associated with AP-1 activation. As reported previously, the core protein enhances basal JNK activity by increasing the phosphorylated form of MAPK kinase (Shrivastava et al., 1998). In addition, two recent studies demonstrated contradictory effects of the core protein on JNK activation; the core protein suppressed TNF-α-induced JNK activation by disrupting the TNFR1–TRADD–TRAF2 complex (Zhu et al., 2001), or enhanced basal and TNF-α-induced JNK activation by forming a ternary complex with TNFR1 and TRADD (Park et al., 2001). In view of these findings, it is possible that the core protein also associates with this complex in core-transgenic mice and influences the downstream pathways.

NF-κB as well as AP-1 are downstream effectors of these cytokines, but NF-κB activation was not obviously enhanced in the core-transgenic mice. Although the reason for this inconsistency remains to be determined, one possibility may be the alteration in the oxidant/antioxidant status in core-transgenic mice (Shrivastava et al., 1998). Previous studies have reported that overexpression of antioxidant enzymes like catalases and superoxide dismutase impairs NF-κB activity (Manna et al., 1998; Nilakantan et al., 1998). It has also been demonstrated that antioxidants like pyrrolidine dithiocarbamate and N-acetyl-L-cysteine activate AP-1 and suppress NF-κB (Meyer et al., 1993). Notably, biochemical analyses revealed that our core-transgenic mice had enhanced antioxidative enzyme activity compared to non-transgenic mice of the same age. For example, the livers of core-transgenic mice had almost normal livers. The blood cells were removed as much as possible from the livers of mice when the mice were sacrificed.

**RT–PCR**

Mice were sacrificed and total RNA was extracted from the liver using RNAzolB (TEL-TEST, Friendswood, TX). Three micromgrams of total RNA were reverse transcribed by Superscript II (GIBCO BRL, Gaithersburg, MD) using oligo(dT) primer. PCR of TNF-α, IL-1β, and GAPDH was performed using the Quantitative PCR Detection Kit (BioSource International, Camarillo, CA). This process can detect multiple gene expression by amplifying all the genes under the same conditions because the PCR primers in this kit have similar melting temperatures and no obvious 3’-end overlap to enhance multiple amplification. Primers for RT–PCR of IL-6 and β-actin were synthesized according to a previous report (Faulkner et al., 1995). Primers for the RT–PCR of c-jun, c-fos, and collagenase were as follows: c-jun: 5’-GCATGAGGAACCGCATGCCCGCCTCCAAGT-3’ and 5’-TCAAAACGTTTGCAACTGCTGTCTTAT-3’; c-fos: 5’-TCCTTTGAGCATGCCGGGTGTCTTAT-3’ and 5’-AGCACAAGGAAAGCTGTAAATGTGCAGCC-3’; collagenase: 5’-ATTCCCACAGAGAGTTGGAGACACT-3’ and 5’-TATG-GAATTTTGGCGATGACTCT-3’. For the RT–PCR, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification so that semiquantitative comparisons of the mRNA abundance between different samples were possible.

**Southern blotting**

Five microliters of PCR products was separated on a 1.5% agarose gel containing 1× TBE buffer, denatured and 0.2% formaldehyde. Five microliters of PCR products was separated on a 1.5% agarose gel containing 1× TBE buffer, denatured and 0.2% formaldehyde. The DNA was blotted to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN). The membrane was hybridized with the cDNA probe and autoradiographed on X-ray film. The membrane was hybridized with a probe for the core protein, and the blot was probed with a probe for the core protein. The hybridization conditions were as follows: prehybridization at 65°C for 3 hours, hybridization at 65°C for 16 hours, and washing at 65°C for 15 minutes. The membrane was then probed with a probe for the core protein. The hybridization conditions were as follows: prehybridization at 65°C for 3 hours, hybridization at 65°C for 16 hours, and washing at 65°C for 15 minutes. The blot was then probed with a probe for the core protein.
twice in denaturing buffer (0.5 N NaOH, 15 M NaCl) for 15 min, neutralized twice in neutralization buffer [0.5 M Tris–HCl (pH 7.5), 3 M NaCl], and transferred onto a Hybond-N+ membrane filter (Amersham, Buckinghamshire, UK) in 20X SSC [3 M NaCl, 0.3 M trisodium citrate dihydrate (pH 7.0)]. The filter was dried, and DNAs were fixed on the membrane by irradiation with UV light and then hybridized with an appropriate heat-denatured probe in hybridization buffer [50% deionized formamide, 5X SSC, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.02% SDS, 0.1% N-lauryl sarcosin, 0.1 mg/ml of salmon sperm DNA] overnight after prehybridization in the same buffer without a probe for 3 h. Probes were prepared by amplifying and digoxigenin (DIG) labeling the positive control DNA provided in the kit used for PCR. After hybridization, the filter was washed twice with 2X wash buffer (2X SSC, 0.1% SDS) for 5 min and then twice with 0.1X wash buffer (0.1X SSC, 0.1% SDS) for 15 min. DIG-labeled DNA probes were detected using the DIG Luminescent Detection Kit (Boehringer Mannheim).

Real-time PCR

TaqMan probes were labeled with a reporter fluorescent dye [6-carboxy-fluorescein (FAM)] at the 5'-end and a quencher fluorescent dye [6-carboxy-tetramethyl-rhodamine (TAMRA)] at the 3'-end. Primers and the TaqMan probes for TNF-α and IL-1β were as follows: TNF-α forward primer, 5'-CAGACCTCAGACTCGATCATCT-3'; reverse primer, 5'-CCACTTGGGTGTGTTGCTACGA-3'; probe, 5'-FAM-TCGAGTGCAAGCCTGTAGCCCACGT-TAMRA-3'. IL-1β forward primer, 5'-TGCCCCTCAAAAGGAAAGAAGTCAATC-3'; reverse primer, 5'-GACAAAGGTCTTCCATCTTC-3'; probe, 5'-FAM-TGCAGCTGGAGAGTGATGTGGATCCCAAA(TAMRA)-3'. Fifty microliters of reaction mixture was used, and it contained 100 ng of extracted total RNA, 0.3 mmol/L forward and 0.9 mmol/L reverse primers, 0.2 mmol/L TaqMan probe, and the TaqMan One-Step RT–PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA). The conditions of one-step RT–PCR were as follows: 30 min at 48°C, 10 min at 95°C, and then 60 cycles of amplification for 15 s at 95°C and 1 min at 60°C. The assay used an instrument capable of measuring fluorescence in real time (ABI Prism 7700 Sequence Detector; Applied Biosystems). The calibration curve, covering the range from 1.6 ng to 1 μg total RNA/50 μl reaction, was created using Mouse Liver Total RNA (Ambion, Austin, TX). The same procedure was performed using TaqMan Rodent GAPDH Control Reagents (Applied Biosystems) as an internal control.

ELISA

ELISAs for TNF-α and IL-1β were performed using Mouse TNF-α and IL-1β ELISA Kits (BioSource). Samples were prepared according to a previous paper (Castagliuolo et al., 1998). The ELISA was performed by tripling for each sample. The amount of total proteins in each sample was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL), and the concentrations of the cytokines in the livers were calculated.

In vitro kinase assay

The activities of JNK and ERK were determined using the SAPK/JNK and the p44/42 MAPK Assay Kit (Cell Signaling Technology, Beverly, MA), respectively. Liver tissues were homogenized in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 0.5 mM β-glycerophosphate, and Protease Inhibitor Cocktail (Complete; Roche Molecular Biochemicals, Indianapolis, IN). Homogenates were sonicated four times for 5 s and then centrifuged at 14,000 rpm for 10 min. Supernatant containing 500 μg of proteins was incubated overnight with anti-IKKα/β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) bound to Protein Sepharose 4B beads (Amersham). Immunoprecipitates were washed three times and the kinase reaction was performed in the same buffer with 10 μCi [γ-32P]ATP, 2 mM MgCl2, 1 mM DTT, and 1 μg of GST–IKKβ fusion protein as a substrate. GST–IKKβ was prepared in Escherichia coli and purified using GST Sepharose Beads (Amersham). The reaction mixture was then separated by SDS–PAGE followed by autoradiography.

Electrophoretic mobility shift assay

Nuclear extracts from the livers of core-transgenic and nontransgenic mice were prepared according to the procedure in a previous report (Deryckere and Gannon, 1994). The double-stranded oligonucleotides (AP-1, 5'-CGGTTCATGAGTGTCGCGAGAAGAAGAAGGTT-CCCAAA; NF-κB, 5'-AGTTGAAGGGAGTTTCCAGGC-3') were end-labeled with [γ-32P]ATP. Labeled oligonucleotides were incubated on ice for 30 min, along with 5 μg of nuclear extracts, in 30 μl of binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 2 μg of poly(dI–dC)]. The binding reactions detecting the AP-1 activity contained 5 mM MgCl2 (Meyer et al., 1993). The reaction mixture was analyzed by electrophoresis on a 7% nondenaturing polyacrylamide gel containing 4% glycerol in 0.25X TBE. For competition experiments, a 50-fold excess of unlabeled, identical oligonucleotides or unrelated oligonucleotides (the NF-κB sequence for AP-1 assay, and the AP-1 sequence for NF-κB assay, respectively) was added 20 min before the addition of labeled oligonucleotides. AP-1 activity was determined by measuring the intensity of the shifted band by densitometry (NIH Image; National Institutes of
Health, Bethesda, MD). A serial 2-fold dilution of purified c-Jun protein (rhAP1; Promega, Madison, WI) was used as a control, and the intensity of each band was measured for standard curves.

Western blotting

Tissue lysates were mixed with SDS sample buffer and sonicated for 5 min. Boiled samples were separated by 10% SDS–PAGE and electrotransferred to a PVDF membrane. After blocking, the membrane was probed with anti-IκB-α antibody (Cell Signaling Technology) or anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO), followed by anti-rabbit IgG conjugated with horseradish peroxidase, and visualized by the Phototope-HRP Western Detection System (Cell Signaling Technology).

Statistical analysis

Results are expressed as means ± SE. The significance of the differences of means was determined using Mann–Whitney’s U test.

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