Protection against Hepatocyte Mitochondrial Dysfunction Delays Fibrosis Progression in Mice

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Accumulating evidence indicates that oxidative stress is involved in the physiopathology of liver fibrogenesis. However, amid the global context of hepatic oxidative stress, the specific role of hepatocyte mitochondrial dysfunction in the fibrogenic process is still unknown. The aim of this study was to determine whether a targeted protection of hepatocytes against mitochondrial dysfunction could modulate fibrosis progression. We induced liver fibrogenesis by chronic carbon tetrachloride treatment (3 or 6 weeks of biweekly injections) in transgenic mice expressing Bcl-2 in their hepatocytes or in normal control mice. Analyses of mitochondrial DNA, respiratory chain complexes, and lipid peroxidation showed that Bcl-2 transgenic animals were protected against mitochondrial dysfunction and oxidative stress resulting from carbon tetrachloride injury. Picrosirius red staining, α -smooth muscle actin immunohistochemistry, and real-time PCR for transforming growth factor- β and collagen α -I revealed that Bcl-2 transgenic mice presented reduced fibrosis at early stages of fibrogenesis. However, at later stages increased nonmitochondrial/nonhepatocytic oxidative stress eventually overcame the capacity of Bcl-2 overexpression to prevent the fibrotic process. In conclusion, we demonstrate for the first time that specific protection against hepatocyte mitochondrial dysfunction plays a preventive role in early stages of fibrogenesis, delaying its onset. However, with the persistence of the aggression, this protection is no longer sufficient to impede fibrosis

progression. (*Am J Pathol* 2009, 175:1929–1937; DOI: 10.2353/ajpath.2009.090332)

Liver fibrosis is the result of a complex interplay among different cell types. It is characterized by the recruitment of inflammatory cells in response to chronic injury and by the activation of hepatic stellate cells (HSCs), leading to the accumulation of extracellular matrix. Increased oxidative stress is a common factor in all chronic liver diseases leading to fibrosis, regardless of their etiology. It has been documented in chronic hepatitis C,¹ alcohol-induced fibrosis,² iron overload,³ nonalcoholic steatohepatitis,⁴ or biliary cirrhosis.^{5,6} Injured hepatocytes, HSCs, and infiltrating inflammatory cells are major sources of reactive oxygen species (ROS). Indeed, the oxidative stress will induce the recruitment of inflammatory cells and the activation of HSCs.^{6,7} Therefore, in a chronic liver injury context, a vicious circle of hepatocyte damage, ROS production, HSC activation, and inflammatory cell recruitment will occur, amplifying the fibrogenic answer to injury. Some studies have suggested that initial lipid peroxidation produced by injured hepatocytes could initiate a pathway leading to increased collagen production by stellate cells.^{2,8,9} However, it is still unknown whether mitochondrial dysfunction specifically in hepatocytes plays a role in the fibrogenic process. Therefore, in this article we studied the consequences on fibrosis progression of a targeted protection of hepatocytes against mitochondrial dysfunction.

With this purpose, we induced chronic liver injury in wild-type and transgenic mice overexpressing Bcl-2 specifically in hepatocytes (Bcl-2 Tg). Besides its antiapoptotic functions, Bcl-2 displays antioxidant actions at the mitochondrial level.^{10,11} We have shown previously that Bcl-2 Tg mice presented lower lipid peroxidation after

Supported by the Agence Nationale de la Recherche and the INCA (National Institute of Cancer), France.

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Accepted for publication July 22, 2009.

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ischemia-reperfusion injury,¹² a classic model of liver damage induced by oxidative stress.

We show here that Bcl-2 liver expression protected against hepatocyte mitochondrial dysfunction and oxidative stress induced by carbon tetrachloride (CCl₄). This specific protection against hepatocyte damage was correlated with a delay in the progression of fibrosis at the early steps of the process after CCl₄ administration. However, this benefit was lost at later stages. Our results demonstrate that hepatocyte mitochondrial dysfunction and subsequent oxidative stress play a pathophysiological role in the early steps of fibrogenesis. Hence, the preservation of mitochondrial integrity delays the progression of the disease, but it is not sufficient to block fibrogenesis.

Materials and Methods

Animal Models

Heterozygous Bcl-2 transgenic male mice were generated as described previously¹³ and back-crossed into the C57BL/6 background. These mice express the Bcl-2 transgene under the control of the liver pyruvate kinase promoter. It has been shown previously that our transgenic mice express Bcl-2 mainly in the liver but also in the kidney and in the intestine. In the liver, only hepatocytes express the transgene.¹³ Wild-type littermate C57BL/6 males were used as controls. CCl₄-induced liver injury was obtained by intraperitoneal injection of 3.5 ml/kg CCl₄ dissolved in a 1:10 ratio with mineral oil. Control animals (oil) were injected with the same volume of mineral oil. Animals were treated twice a week for 3 or 6 weeks and were sacrificed 2 days after the last injection ($n = 6$ animals per group). Blood was collected 24 hours after the last CCl₄, and the activity of serum aminotransferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) was measured on a Hitachi 747 analyzer. Livers were harvested either into 10% buffered formalin for histological evaluation or snap-frozen into liquid nitrogen for mRNA or cellular lysate preparations. All experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Histological Analysis

Five-micrometer-thick paraffin-embedded liver sections were stained with H&E or with picosirius red. For α -smooth muscle actin immunohistochemistry, 5- μ m paraffin sections were incubated with α -smooth muscle actin antibody (M 0851, Dako, Glostrup, Denmark). For morphometric analysis, 10 images per animal from three different lobes were taken at $\times 100$ magnification. The areas of staining were quantified using the software ImageJ 1.37v (NIH, Bethesda, MD).

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick-End Labeling Assay

A terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) assay was used to label apoptotic

nuclei in paraffin-embedded liver sections. Tissues were predigested for 5 minutes (100 U of pepsin/0.2 N HCl) and incubated in CaCo/cobalt buffer (pH 7.2) containing 10% biotin-11-dUTP (Sigma-Aldrich, St. Louis, MO) and 2.5% deoxynucleotidyl transferase (GE Healthcare, Uppsala, Sweden) for 1 hour at 37°C. Slides were washed in 2 $\times$ standard saline citrate buffer and endogenous phosphatases were inhibited by 2% levamisole (Sigma-Aldrich) before the incubation in 0.1 M Tris (pH 7.6) and streptavidin-alkaline phosphatase (1:1000, Vector Laboratories, Burlingame, CA) for 30 minutes at 37°C. Revelation was done by incubation of slides for 20 minutes in the dark with 0.1 M Tris (pH 9.4) buffer containing 100 mmol/L NaCl, 50 mmol/L MgCl₂, 0.32% nitro blue tetrazolium (Invitrogen, Carlsbad, CA), and 0.44% 5-bromo-4-chloro-3-indolyl phosphate (Invitrogen). Methyl green (1.5%) staining was performed, and slides were mounted in a warm glycerin-gelatin solution.

Caspase Activity

To assess liver caspase activity, livers were minced and homogenized in 1 mmol/L EDTA, 5 mmol/L dithiothreitol, 50 mmol/L Hepes, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, 4 mg/ml leupeptin, and 4 mg/ml pepstatin, pH 7.4. After centrifugation at 14,000 $\times g$ for 10 minutes at 4°C, the supernatant was recovered. Caspase 2, 8, 9, and 3 activities were measured with the fluorescent ApoAlert Caspase Assay plate (Clontech, Mountain View, CA) following the manufacturer's instructions.

Real-Time RT-PCR

RNA was purified from livers using the TRIzol method (Invitrogen). cDNA synthesis was undertaken using a Transcriptor First Strand cDNA synthesis kit (Roche, Mannheim, Germany). Quantitative RT-PCR was performed in duplicate with the QuantiTect SYBR Green PCR kit (Qiagen, Mainz, Germany) using the LightCycler apparatus. Predesigned QuantiTect Primer Assays (Qiagen) were used to amplify all analyzed genes using the standard QuantiTect protocol. Relative expression was calculated and normalized to the control gene *HPRT*. Wild-type mice injected with excipient (oil) served as reference for the calculations of fold inductions in gene expression.

Activity of Mitochondrial Enzymes

Liver homogenates were resuspended in 0.1 mmol/L phosphate buffer and activity of mitochondrial enzymes was measured by spectrophotometry at 30°C, as described previously.¹⁴ In brief, complex I (NADH-ubiquinone oxidoreductase) activity was measured by following the decrease in absorbance due to oxidation of NADH to NAD⁺ at 340 nm for 4 minutes in the presence of decylubiquinone and antimycin A. The reaction mixture contained 40 mmol/L potassium phosphate (pH 7.5), 2 mmol/L KCN, 5 mmol/L MgCl₂, 100 μ mol/L decylubiqui-

none in dimethyl oxide, 100 $\mu\text{mol/L}$ NADH, and 2 μg of antimycin A with or without 5 $\mu\text{g/ml}$ of rotenone. Complex IV (cytochrome *c* oxygen oxidoreductase) activity was measured by following the decrease in absorbance due to the oxidation of cytochrome *c* at 550 nm for 4 minutes. The assay mixture consisted of 10 mmol/L potassium phosphate (pH 7.0) and 0.1% cytochrome *c*. Lastly, citrate synthase activity was measured at 412 nm for 4 minutes in Tris buffer containing 0.1 mmol/L 5,5'-dithio-bis-2-nitrobenzoic acid, 0.3 mmol/L acetyl-CoA, and 0.5 mmol/L oxaloacetate.

Lipid Peroxidation, Aconitase Activity, and Reduced Glutathione

To evaluate hepatic lipid peroxidation, we measured thiobarbituric acid reactants. In brief, the reaction mixture consisted of 0.2 ml of an 8.1% sodium lauryl sulfate solution, 1.5 ml of a 20% acetic acid solution, pH 3.5, and 1.5 ml of a 0.8% aqueous solution of thiobarbituric acid. To this mixture was added 0.2 ml of liver homogenate prepared in 0.1 mmol/L phosphate buffer containing 3 mmol/L EDTA. The mixture was brought up to 4.0 ml with 0.5 ml of distilled water and 0.1 ml of 10 mmol/L butylated hydroxytoluene and was heated at 95°C for 60 minutes. After cooling, the absorbance was measured at 532 nm. Thiobarbituric acid reactant contents were expressed as nanomoles of malondialdehyde equivalents per milligram of protein.

We also assessed the activity of aconitase, an enzyme highly sensitive to oxidative damage and located both in cytosol and mitochondria.¹⁵ To this end, liver samples were resuspended in a 50 mmol/L Tris-HCl, pH 7.4, 0.2 mmol/L sodium citrate, and 0.05 mmol/L MgCl_2 buffer. Homogenates were then centrifuged at $800 \times g$ at 4°C for 10 minutes, and supernatants were sonicated for 20 seconds. Mitochondrial and cytosolic fractions were prepared from livers as described previously.¹⁶ Aconitase activity was measured on 200 μg of proteins in the presence of 1 mmol/L sodium citrate, 1 mmol/L NADP^+ , and 2 U of isocitrate dehydrogenase. Absorption was measured at 340 nm at 37°C for 5 minutes. The activity is expressed as nmol of $\text{NADPH} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

Reduced glutathione (GSH) levels were determined by a method adapted from Griffith, as described previously.¹⁶

Long PCR Mitochondrial DNA Experiments

Total hepatic DNA was recovered using the standard phenol/chloroform method. We used long PCR experiments to detect mitochondrial DNA (mtDNA) oxidative lesions hampering the progression of polymerases, such as abasic sites and DNA strand breaks.¹⁷ This PCR technique, based on the amplification of a short and a long mtDNA fragment, exploits the rationale that randomly distributed blocking lesions are more likely to hamper amplification of a long stretch of mtDNA than a very short fragment. Primers A (5'-CGACAGCTAAGACCCAACTGGG-3') and B (5'-CCCATTCTTCCCATTTCATTGGC-3') amplify a 316-bp mtDNA fragment, whereas primers

C (5'-CATGAGCTCATCATAGCCTTAT-3') and D (5'-GGGTGATCTTTGTTTGCGGGT-3') amplify a 4567-bp mtDNA fragment. PCR reactions were performed with the Expand Long PCR System (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's recommendations using 40 pmol of primers. The thermocycler profile included initial denaturation at 94°C for 2 minutes, 22 cycles of 95°C for 45 seconds, 61°C for 30 seconds and 68°C for 10 minutes, and final extension at 68°C for 7 minutes. Photographs were scanned to determine the respective intensity of the short and long PCR products.

Statistical Analysis

Statistical analyses were performed using Statview 4.5. Data sets were compared with Student's *t*-test. Data are presented as means $\pm$ SEM. *P* < 0.05 were considered significant.

Figure 1. Bcl-2 hepatocyte expression protects against mitochondrial dysfunction caused by chronic CCl_4 injury. Activity of mitochondrial respiratory chain complexes. **A:** Complex I. **B:** Complex IV. **C:** Mitochondrial DNA integrity was assessed by long PCR. **P* < 0.05; ***P* < 0.001.

Figure 2. Apoptosis is a minor event during CCl₄ treatment. **A:** Caspase 3, 8, 2, and 9 activities measured in the liver of wild-type (WT) and Bcl-2 Tg mice after 3 weeks of CCl₄ injections compared with oil-injected mice and animals submitted to Fas-induced liver injury (0.15 mg/kg of Jo2 antibody was injected i.v. and livers were harvested 24 hours after injection; at this time point, animals presented ALT levels equivalent to CCl₄-injected mice). **B:** TUNEL assay on liver sections of representative wild-type and Bcl-2 Tg mice after 3 weeks of CCl₄ injections (arrows indicate positively stained cells) and of an animal submitted to Fas-induced apoptosis (positive control for staining). Original magnification, $\times 200$.

Results

Overexpression of Bcl-2 Prevents CCl₄-Induced Mitochondrial Dysfunction

To determine whether Bcl-2 overexpression could prevent hepatocyte mitochondrial dysfunction induced by CCl₄, we submitted wild-type and Bcl-2 Tg mice to 3 or 6 weeks of biweekly injections of CCl₄ and analyzed several markers of mitochondrial integrity. We first confirmed, by measuring liver citrate synthase activity, that the mitochondrial mass was unchanged either before injection or after chronic CCl₄ administration in both groups of animals (data not shown). We then measured the activity of complexes I and IV of the mitochondrial respiratory chain and found them to be significantly decreased in wild-type mice but not in Bcl-2 transgenic mice (Figure 1, A and B) after both 3 and 6 weeks of CCl₄ administration. Mitochondrial DNA is also known to be vulnerable to damage induced by ROS and lipid peroxidation products, which can generate DNA alterations (eg, abasic sites and strand breaks) able to block the progression of the DNA polymerases.¹⁸ Although the amplification of the 316-bp mtDNA fragment was equivalent in all groups of mice, we found that the long 4567-bp mtDNA fragment was less efficiently amplified in wild-type mice compared with Bcl-2 Tg mice, thus indicating

significantly less hepatic mtDNA oxidative lesions in mice overexpressing Bcl-2 (Figure 1C). Taken together, these data demonstrate that Bcl-2 overexpression has protected hepatocytes against mitochondrial respiratory chain dysfunction and loss of mtDNA integrity induced by chronic administration of CCl₄.

Bcl-2 Does Not Protect against Apoptosis after CCl₄-Induced Injury

Bcl-2 is a well known antiapoptotic protein and has been shown to block Fas-induced apoptosis in the liver.¹³ To determine whether Bcl-2 expression has protected hepatocytes against apoptosis, the activities of several caspases were measured in liver samples after 3 weeks of chronic CCl₄ injections. In this model, we found an activation of caspases 2, 3, 8, and 9, as described previously,¹⁹ but this induction was minor compared with the positive control liver isolated from a mouse injected with a Fas agonist antibody reaching equivalent ALT levels (Figure 2A). These results confirmed that CCl₄ liver injury primarily involves necrotic cell death. Only a few scattered cells, which were mainly nonparenchymal cells, stained positive for a TUNEL assay (Figure 2B). Importantly, both caspase activity analyses and the TUNEL

Figure 3. Neco-inflammatory reaction induced by chronic CCl₄ injury. **A:** H&E-stained representative liver sections (original magnification, ×100) showing an equivalent level of inflammatory reaction and liver injury in wild-type (WT) and Bcl-2 Tg mice after 3 and 6 weeks of CCl₄ injury. **B:** Quantification of necro-inflammatory areas in the livers of wild-type and Bcl-2 Tg mice at the two time points after CCl₄ treatment. **C:** Levels of serum ALT and serum AST measured 24 hours after the last CCl₄ injection. ALT and AST levels are given in IU/L.

assay showed that there was no difference in apoptosis between transgenic and wild-type mice.

Delayed CCl₄-Induced Fibrosis in Bcl-2 Transgenic Mice

We assessed liver injury by quantifying the necro-inflammatory area on liver sections (Figure 3, A and B) and measurement of serum transaminase levels (Figure 3C). We found no significant difference in the extension of the necro-inflammatory area between Bcl-2 and wild-type mice or in ALT or AST levels 24 hours after the last injection. Finally, we measured the inflammatory reaction in the liver by real-time RT-PCR for various cytokines (Table 1), and no significant difference in the expression of inflammation markers could be detected between Bcl-2 and wild-type mice. To investigate the consequences of Bcl-2 overexpression on fibrosis progression, liver sections were stained at the peak of fibrosis (2 days after the last CCl₄ injection) with picrosirius red. Fibrotic areas were quantified and found to be significantly decreased by 41% in Bcl-2 Tg mice compared with wild-type mice after 3 weeks of chronic injections (Figure 4, A and B). However, after 6 weeks, Bcl-2 Tg mice presented the same degree of fibrosis as wild-type mice. We next analyzed the activation of fibrogenic cells through the expression of α -smooth muscle actin and demonstrated that Bcl-2 Tg mice presented a decrease in this marker compared with wild-type mice after 3 weeks but not after 6 weeks of CCl₄ treatment (Figure 5A). The reduced expression in mRNA of early markers of fibrogenesis, such as transforming growth factor- β 1 and collagen α -I, seen in Bcl-2 Tg mice after 3 weeks, confirmed these

observations (Figure 5B). Overall, these results demonstrated that Bcl-2 overexpression in hepatocytes delays the onset of liver fibrosis but is not enough to block hepatic stellate cell activation and the progressive collagen deposition induced by long-term CCl₄ injections.

Bcl-2 Expression Is Not Sufficient to Block the Global Oxidative Stress Induced by CCl₄ Liver Injury

To verify that the lack of protection at 6 weeks of treatment was not due to a decrease in Bcl-2 transgene

Table 1. Evaluation of Neco-Inflammatory Reaction after CCl₄ Injections

	Wild-type	Bcl-2	P
TNF- α			
3 weeks	3.49 $\pm$ 1.39	1.94 $\pm$ 1.67	0.35
6 weeks	1.76 $\pm$ 1.63	1.06 $\pm$ 0.41	0.19
IL-6			
3 weeks	1.17 $\pm$ 0.55	0.49 $\pm$ 0.30	0.1
6 weeks	0.40 $\pm$ 0.06	0.56 $\pm$ 0.35	0.33
MCP-1			
3 weeks	7.51 $\pm$ 2.32	4.20 $\pm$ 1.59	0.12
6 weeks	2.11 $\pm$ 0.69	2.08 $\pm$ 0.53	0.93
MIP-2			
3 weeks	3.11 $\pm$ 2.47	0.59 $\pm$ 0.77	0.06
6 weeks	2.86 $\pm$ 1.44	1.52 $\pm$ 1.23	0.33
IP-10			
3 weeks	0.58 $\pm$ 0.15	0.73 $\pm$ 0.27	0.54
6 weeks	0.49 $\pm$ 0.12	0.85 $\pm$ 0.24	0.08

Fold induction of liver RNA expression of inflammation markers in wild-type or Bcl-2 Tg mice after CCl₄ administration compared with their respective oil-injected controls. P values show no significant difference between wild-type and Bcl-2 Tg mice at either time point.

TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage-derived inflammatory mediator-2; IP-10, interferon-inducible protein-10.

Figure 4. Bcl-2 mice present delayed fibrogenesis after chronic CCl₄ injury. **A:** Sirius red staining of liver sections (original magnification, $\times 100$) showing less collagen deposit in Bcl-2 Tg mice after 3 weeks of biweekly injections of CCl₄. **B:** Quantification of Sirius red staining in oil- or CCl₄-injected wild-type (WT) or Bcl-2 Tg animals, showing a significant reduction in fibrosis at 3 weeks in Bcl-2 Tg mice compared with wild-type mice. ** $P < 0.01$.

expression, we confirmed by real-time RT-PCR that the transgene expression was equivalent at both time points analyzed (data not shown). We then evaluated the global oxidative stress induced by CCl₄ administration during the progression of fibrosis to understand why fibrosis was no longer prevented at 6 weeks despite the preservation of mitochondrial integrity. We first quantified lipid peroxidation (malondialdehyde) in liver homogenates. After 3 weeks of CCl₄ treatment, there was a significant induction of lipid peroxidation in wild-type mice, whereas malondialdehyde levels in the liver of Bcl-2 Tg mice were still low. The difference between the two groups was still significant after 6 weeks, although the difference between wild-type and Bcl-2 Tg mice was reduced because of increased lipid peroxidation in this latter group (Figure 6A). We then measured total hepatic GSH content and liver aconitase activity. We found no modification of either

indicator of oxidative stress in the whole homogenates after 3 weeks of CCl₄ compared with oil-injected animals (Figure 6, B and C). However, it was noteworthy that when aconitase activity was assessed separately in isolated mitochondria and cytosol, this activity was 61% lower in the mitochondrial fraction of wild-type livers compared with Bcl-2 livers, whereas no significant difference was found for the cytosolic fraction between both genotypes (data not shown). After 6 weeks of CCl₄ treatment, a 40% reduction in the total hepatic GSH content and a 50% decrease in aconitase activity were noticed in wild-type mice (Figure 6, B and C). At this later stage of fibrosis, there was no significant difference between Bcl-2 Tg mice and wild-type mice for these markers. Taken together, these results correlate with the lack of fibrosis protection and suggest that Bcl-2, while still protecting the hepatocyte mitochondrial DNA and respira-

Figure 5. Other markers of fibrosis confirm delayed fibrogenesis in Bcl-2 Tg mice. **A:** α -Smooth muscle actin staining of liver sections (original magnification, $\times 100$) after 3 weeks of CCl₄ treatment show less staining in Bcl-2 mice. **B:** Expression of fibrosis markers by real-time RT-PCR confirming the lower induction of transforming growth factor- β 1 (TGF- β 1) and collagen α -I at 3 weeks in Bcl-2 Tg mice. * $P < 0.05$.

Figure 6. Analysis of global oxidative stress in the liver of Bcl-2 Tg and wild-type (WT) mice after chronic CCl_4 treatment, as assessed by malondialdehyde (MDA) levels (A), glutathione levels (B), and aconitase activity (C). * $P < 0.05$; ** $P < 0.01$.

tory chain, cannot prevent global liver oxidative stress at later stages.

Discussion

There is accumulating evidence that oxidative stress and ROS play a major pathogenic role in liver fibrogenesis independent of the disease etiology. The most accepted theory proposes that ROS derived from damaged hepatocytes activate Kupffer cells, infiltrating neutrophils, as well as HSCs.²⁰ Indeed, damaged hepatocytes generate lipid peroxidation products that have been shown to stimulate collagen α -I expression by stellate cells in culture.^{9,21,22} However, the role of hepatocyte mitochondrial dysfunction in the progression of fibrosis had not been established until now. We therefore investigated whether protection against mitochondrial dysfunction exclu-

sively in hepatocytes could have an impact on fibrosis progression.

With this purpose, we induced liver fibrosis by CCl_4 chronic administration, which is associated with hepatic mitochondrial dysfunction and oxidative stress.²³ We confirmed that the activity of the mitochondrial respiratory chain was impaired and that lipid peroxidation was increased in this model. We also showed that hepatocyte Bcl-2 overexpression protects against these alterations. Bcl-2 is a guardian of the mitochondrial membrane, avoiding its permeabilization by blocking lipid pores formed by Bax/Bak oligomerization.²⁴ Therefore, we hypothesized that, by protecting the mitochondria, Bcl-2 could reduce mitochondrial dysfunction and subsequent lipid peroxidation in our transgenic animals. Zimmermann et al²⁵ have shown that Bcl-2 overexpression could also directly increase the levels of GSH, particularly in neurons. However, we did not observe higher GSH liver content in Bcl-2 transgenic mice either before or after liver injury compared with that in wild-type mice. GSH content was not depleted after 3 weeks of treatment in wild-type mice, whereas there was already an increase in liver lipid peroxidation. Malondialdehyde and other toxic derivatives are slowly removed by the hepatic detoxification system and are likely to accumulate with time.^{26–28} In contrast, GSH is rapidly consumed after CCl_4 injection, thus causing its depletion, but this can be followed by a compensatory increased GSH synthesis, which restores the GSH pool after liver injury.^{29–31} We can therefore hypothesize that after 3 weeks of CCl_4 injections, total GSH had already recovered. After 6 weeks, however, the greater number of CCl_4 injections could have induced higher oxidative stress responsible at this time for a significant reduction of liver GSH stores. Indeed, the reduction in GSH noticed after 6 weeks of chronic CCl_4 administration both in wild-type and in Bcl-2 transgenic mice was accompanied by diminished aconitase activity. The absence of protection by Bcl-2 against GSH depletion and reduced aconitase activity in the later stages of fibrogenesis suggests that this global rise in oxidative stress could be due to nonparenchymal cells and/or to stress derived from nonmitochondrial compartments of hepatocytes.

A link between reduced oxidative stress and diminished liver fibrosis has been suggested in various experimental models of cirrhosis.^{19,32–35} However, in all these studies, the antioxidant effect was not restricted to hepatocytes. For example, NADPH oxidase that generates superoxide anions and ROS particularly in phagocytic cells plays a key role in the development of fibrogenesis induced either by bile duct ligation³³ or by CCl_4 .¹⁹ Our model, with its Bcl-2 expression exclusively in hepatocytes, provided us with a unique tool to specifically investigate the role of hepatocyte mitochondrial dysfunction during fibrogenesis.

In the present article, we demonstrate that the protection against hepatocyte mitochondrial damages correlates with a delayed progression at early stages of the disease. After 3 weeks of CCl_4 treatment, fibrosis was significantly less extensive in Bcl-2 transgenic mice than in wild-type mice. However, after 6 weeks of CCl_4 treat-

ment, even though the mitochondria of Bcl-2 Tg mice were still protected, as attested to by the sustained activities of complexes I and IV and by the protection against mitochondrial DNA alterations, the extent of fibrosis was no longer different between the two groups of mice.

A recent article showed that IGF-I administration reduced oxidative liver damage, improved mitochondrial function, and alleviated fibrosis induced by CCl₄ chronic administration.³⁶ In comparison, although it protected hepatocyte mitochondria and reduced lipid peroxidation, Bcl-2 overexpression could only delay fibrosis. This difference could be attributed to the lack of protection of nonparenchymal cells such as HSCs, endothelial cells, and macrophages, in which the transgene is not expressed. We can therefore conclude that protecting hepatocytes exclusively is not sufficient to block the vicious circle of ROS production and HSC activation and thus prevent fibrogenesis.

In conclusion, we show for the first time that the protection against hepatocyte mitochondrial dysfunction can delay early stages of fibrogenesis, but this protection is eventually overwhelmed when ROS are further produced by extramitochondrial compartments and nonhepatocytic cells.

Acknowledgment

We thank Dr. Patrick Jaffray for serum transaminase measurements.

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