Moderately High Folic Acid Supplementation Exacerbates Experimentally Induced Liver Fibrosis in Rats

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Under certain clinical circumstances, folic acid can have undesirable effects. We investigated the following: (i) the effects of moderately high folic acid supplementation on the course of liver impairment in CCl_4-treated rats and (ii) the influence of folic acid supplements on the hepatic recovery following the interruption of the CCl_4-induced toxic injury. Four experimental groups of rats were used: CCl_4-treated rats (0.5 ml of CCl_4 twice a week ip) fed standard chow for up to 12 weeks (Group A); treated rats fed chow supplemented with 25 mg/kg folic acid from weeks 6 to 12 (Group B); treated rats fed a standard diet but with CCl_4 discontinued after 6 weeks to allow for tissue recovery over 4 weeks (Group C); rats as Group C but fed a diet supplemented with 25 mg/kg folic acid from weeks 6 to 10 (Group D). Liver and blood samples were obtained for biochemical, histological, and gene expression analyses. Animals that received the supplement had a higher content of collagen, activated stellate cells, and apoptotic parenchymal cells in biopsy tissue at weeks 8 and 10 of treatment and more extensive alterations in serum albumin and bilirubin concentrations (Group B vs. Group A). In some of the time periods analyzed, alterations were observed in the expression of genes related to apoptosis (B-cell leukemia/lymphoma 2, inhibitor of apoptosis 2) and to fibrosis (procollagen I, matrix metalloproteinase 7). In the recovery period (Groups C and D), folic acid administration was associated with increased hepatic inflammation and apoptosis and with a decrease in the tissue inhibitor of metalloproteinase-3 expression following 1 week of recovery. We conclude that folic acid administration aggravates the development of fibrosis in CCl_4-treated rats. Follow-up studies are needed to determine whether folic acid treatment would be contraindicated in patients with chronic liver diseases. Exp Biol Med 233:38–47, 2008

Key words: apoptosis; fibrosis; folic acid; gene expression; nutrition

Introduction

Folate is a water-soluble vitamin essential for cell replication and DNA synthesis, repair, and methylation (1–4). Folate has been implicated in the pathophysiology of several human diseases including anemia, thromboembolia, cardiovascular disease, neurologic diseases, and cancer (5). Mandatory folic acid fortification of foods was implemented in the United States and Canada in 1998 (6), and this policy has been associated with a decrease in the prevalence of neural tube defects in newborns (7, 8). Folate is thought to be safe and free of toxicity (9). However, emerging studies indicate that folic acid supplementation of the diet may have undesirable effects in some population groups not originally targeted for such dietary fortification (10, 11). For example, clinical and experimental studies suggest that folate possesses dual modulatory effects on carcinogenesis, depending on the timing and intervention dose. Folate deficiency has been reported to promote (12) or to protect (13) against colorectal cancer, whereas supplementation appears to promote the progression of established neoplasms (14, 15). At the molecular level, folate modulates DNA methylation, which is an important epigenetic determinant in the expression of many genes (15, 16). Recent data show that oral folic acid administration in

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humans increases gastric epithelial cell apoptosis by decreasing the expression of the antiapoptotic *Bcl-2* gene while increasing the expression of the proapoptotic *p53* (17).

Folic acid treatment is common in patients with chronic liver diseases (18). Nutritional support, whether by dietary modification or by oral nutritional supplements including folic acid, is usually employed in the treatment of these patients (19), and folic acid supplements are sometimes used for the correction of anemia. Liver diseases are associated with an increase in hepatic parenchymal cell apoptosis that in turn is correlated with the degree of fibrosis (20), probably because both phenomena reflect different aspects of the same inflammatory and matrix remodeling response to hepatic injury (21). Therefore, it is likely that factors enhancing parenchymal cell apoptosis have an exacerbating effect on hepatic impairment. It is well documented that folate deficiency is associated with a greater degree of liver impairment in patients with liver disease and in experimental models (22, 23). However, the possibility of a deleterious effect of folic acid excess on the course of this disease has not been sufficiently investigated. This possibility may have clinical relevance given the risk of patients who receive an overdose over a protracted period of treatment.

One of the most commonly used models of experimental fibrosis and cirrhosis is the chronic administration of CCl_4 to rats. This treatment results in hepatocyte damage, necrosis, inflammation, and fibrosis that spreads to link the portal tract with the central vein and, over a period of 8 to 12 weeks, results in the development of cirrhosis (24). Treated animals develop changes in key biochemical factors related to matrix deposition and degradation. These include procollagen and matrix metalloproteinases and their inhibitors (25) and systemic hemodynamic alterations similar to those of human patients (26).

In the present study, we investigated the following: (i) the effects of moderately high folic acid supplementation on the course of the disease, fibrosis, and apoptosis pathways in rats with carbon tetrachloride–induced fibrosis and (ii) its influence on the recovery of hepatic function following the cessation of the toxic injury induction in this experimental model.

Materials and Methods

Animal Model and Experimental Design. The handling of animals and the procedures described were approved by the Ethical Committee of the Rovira i Virgili University. The study was performed in 90 male Wistar rats weighing 200 ± 10 g (Panlab, Barcelona, Spain). Experimental fibrosis was induced by intraperitoneal (ip) injections of 0.5 ml of CCl_4 diluted 1:1 (v/v) in olive oil twice a week (27). Animals were subdivided into four groups (Fig. 1). Group A consisted of 24 CCl_4-treated rats that were fed standard rat chow (Harlan Interfauna, Barcelona, Spain; folic acid content: 1.5 mg/kg) *ad libitum* and were killed in 4 subgroups of 6 animals each at 6, 8, 10, and 12 weeks of treatment. Group B consisted of 18 CCl_4-treated rats that were fed *ad libitum* rat chow supplemented from week 6 with 25 mg/kg chow of folic acid (Harlan Interfauna) and were killed in 3 subgroups of 6 animals each at 8, 10, and 12 weeks of treatment. Group C consisted of 24 rats fed a standard diet *ad libitum* and treated with CCl_4 over 6 weeks; toxic injury induction with CCl_4 was then stopped, and subgroups of 6 animals each were killed after 1, 2, 3, and 4 weeks of recovery. Group D consisted of 24 rats that were treated the same way as rats in Group C, but the rats in Group D were fed a 25 mg/kg folic acid–supplemented diet *ad libitum*. An additional group of six untreated rats was used as a control for biochemical measurements. These animals were fed standard rat chow *ad libitum* for 6 weeks before sacrifice and received ip injections of the excipient (0.5 ml of olive oil) twice a week. The livers were removed from all experimental and control animals under anesthesia. Small portions of the livers were fixed in 4% formaldehyde and embedded in paraffin for histologic examination. The remaining portions of the livers were frozen in liquid nitrogen and stored at –80°C for subsequent batched RNA expression analyses.

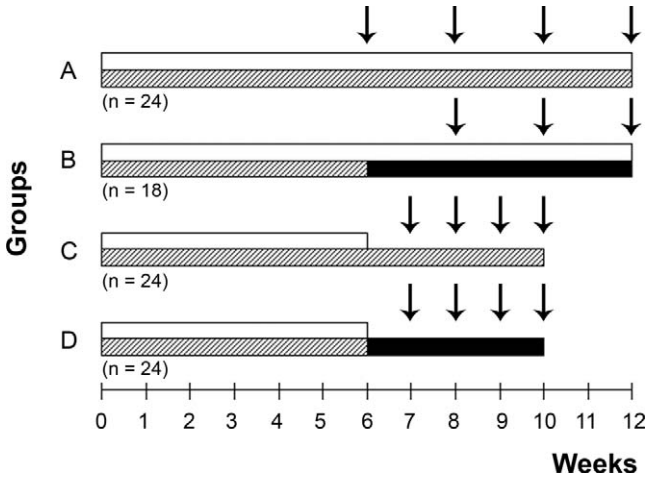

Figure 1. Scheme of the experimental design. White bars indicate carbon tetrachloride treatment. Striped bars indicate the standard diet. Black bars indicate the folic acid–supplemented diet. The arrows indicate the dates (time points) of animal sacrifice.

Biochemical Measurements. Blood obtained by cardiac puncture was collected into EDTA-containing tubes for homocysteine measurement and into tubes with no anticoagulant for the other biochemical analyses. After centrifugation at 4°C, serum and plasma samples were distributed in aliquots and stored at –80°C until they were required for analysis. Aspartate aminotransferase (AST) activity, albumin, bilirubin, folate, vitamin B_{12}, and homocysteine concentrations were analyzed by using reagents purchased from Beckman-Coulter (Fullerton, CA) in a Synchron Lxi automated analyzer (Beckman-Coulter).

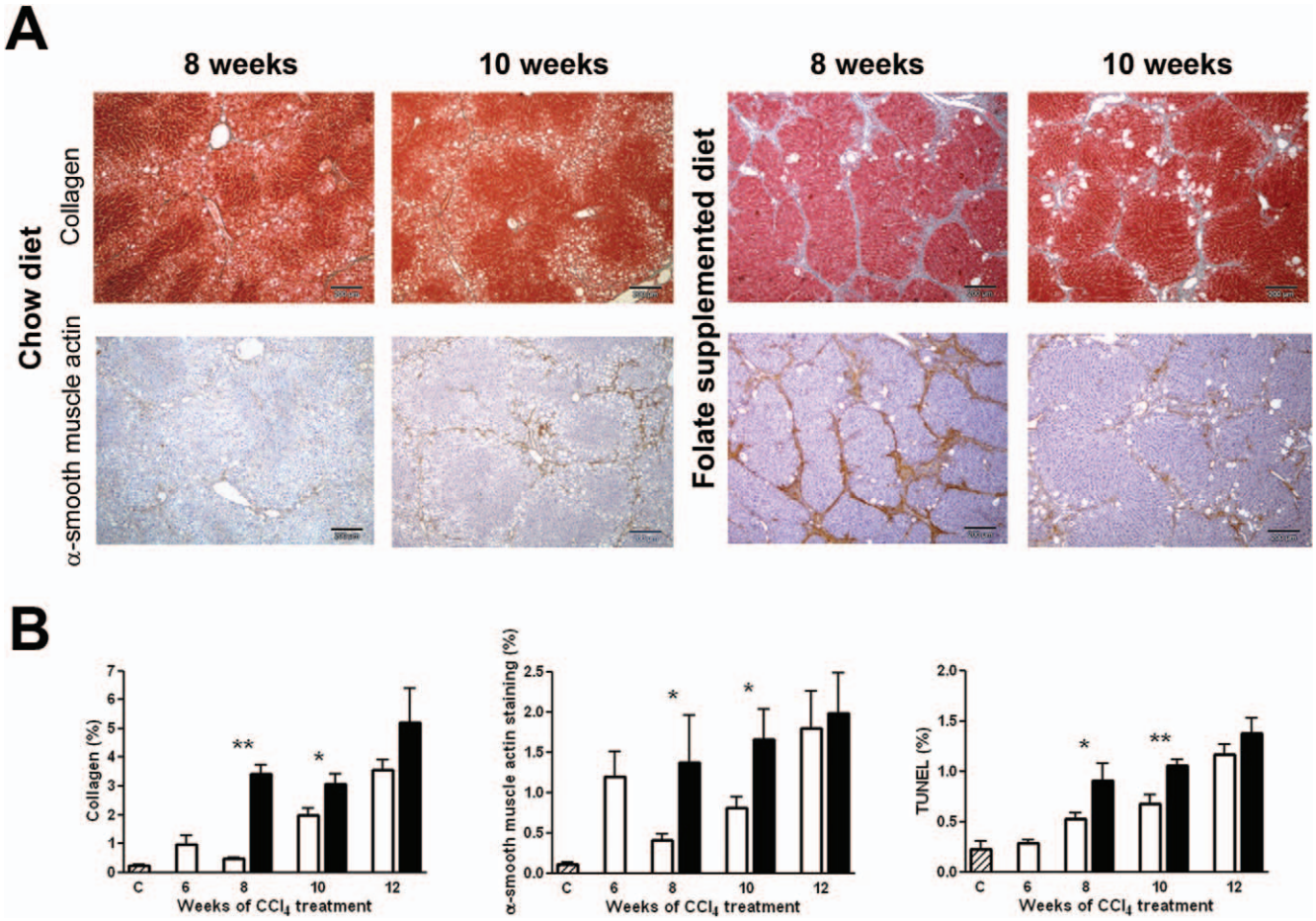

Figure 2. Effect of folic acid administration on the histopathologic variables. (A) Representative micrographs of the Masson trichrome stain (upper panels) and α-smooth muscle actin immunohistochemistry (lower panels) in rats following 8 or 10 weeks of a standard diet or a folic acid–supplemented diet. Original magnification: ×40. (B) Chronologic changes of selected histologic variables in CCl_4-treated rats between 6 and 12 weeks of treatment. White bars represent rats that received a standard diet (Group A). Black bars represent rats that received a folic acid–supplemented diet (Group B). Striped bars represent the control group. $P < 0.001$ by ANOVA (Group A vs. Group B) for all the analyzed variables. $^*P < 0.05$; $^{**}P < 0.01$ by the Mann-Whitney U test relative to animals that received a standard diet and at the corresponding test-animal time point. Results are expressed as means and SEM ($n = 6$ per group). A color figure is available in the online version of this article.

Quantitative Real-Time PCR. Total RNA was isolated from frozen hepatic tissue obtained from rats killed at weeks 6, 8, and 12 of treatment and processed as described in the literature. In brief, 30 mg of liver were lysed with 650 µl of 1× Nucleic Acid Purification Lysis Solution (Applied Biosystems, Darmstadt, Germany). RNA extraction was performed in an ABI Prism 6100 nucleic acid prep-station (Applied Biosystems). RNA quality and concentration was determined as the $OD_{260/280}$ ratio in a GeneQuant Pro spectrophotometer (Cambridge, UK). One microgram of total RNA was reverse-transcribed to cDNA by using random hexamer primers obtained from Invitrogen (Carlsbad, CA). Real-time PCR analysis (28) was performed by using TaqMan Low Density Arrays (Applied Biosystems). Real-time PCR primers and probes (TaqMan Gene Expression Assays) were spotted onto a 384-well card (Applied Biosystems). Each sample was measured in triplicate. Thermal cycling and fluorescence detection was performed on the Applied Biosystems ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS Software 2.2. Forty cycles were run with the following parameters: 2 mins at 50°C and 10 mins at 94.5°C and for each cycle 30 secs at 97°C for denaturation and 1 min at 59.7°C for transcription. Analyses of gene expression were performed by using the $2^{-\Delta\Delta CT}$ method (28). In total 29 genes were studied in relation to folate metabolism (S-adenosyhomocysteine hydrolase, cystathionine beta synthase, phosphatidylethanolamine N-methyltransferase); apoptosis (B-cell leukemia/lymphoma 2; baculoviral IAP repeat–containing 2, 4, and 5; caspases 3 and 9, DNA fragmentation factor, α and β subunits; programmed cell death 8; v-rel reticuloendotheliosis viral oncogene homolog A; tumor necrosis factor receptor superfamily, member 6; CD36 antigen; and tumor necrosis factor (ligand) superfamily, member 6); fibrosis (procollagen type I, α 2; procollagen type IV, α 4; matrix metalloproteinases 3, 7, 8,

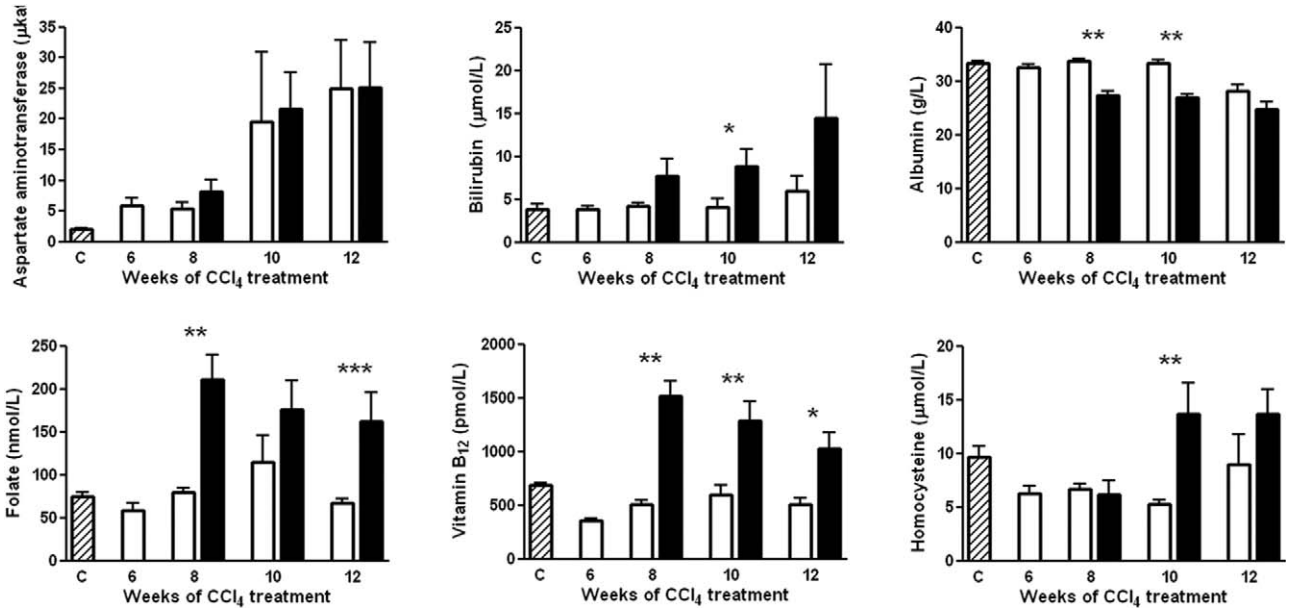

Figure 3. Effect of folic acid administration on biochemical variables. Chronologic changes of selected biochemical variables in CCl_4-treated rats between 6 and 12 weeks of treatment. White bars represent rats that received a standard diet (Group A). Black bars represent rats that received a folic acid–supplemented diet (Group B). Striped bars represent the control group. $P < 0.001$ by ANOVA (Group A vs. Group B) for all the analyzed variables, except AST activity. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by the Mann-Whitney U test relative to animals that received a standard diet and at the corresponding test-animal time point. Results are expressed as means and SEM ($n = 6$ per group).

and 9; tissue inhibitor of metalloproteinase 1, 2, and 3); peroxisome proliferator–activated receptor α, δ, and γ; and tumor protein p53. β-glucoronidase (*Gusb*) gene expression was used for normalization because its threshold cycle is similar to that of the other genes investigated and because *Gusb* expression has been reported to not change in rats and to have a range of fibrosis similar to that obtained in the present investigation (29). All primers were purchased from Applied Biosystems (TaqMan Gene Expression Assays; https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=600689).

Histologic and Immunohistochemical Analyses. Paraffin-embedded liver sections (2 µm) were used for all the histologic analyses. Livers were fixed in 10% phosphate-buffered formalin for 24 hrs at room temperature, washed twice with water, stored in 70% ethanol at 4°C, and embedded in paraffin. Sections were stained with hematoxylin and eosin and Masson trichrome. Collagen

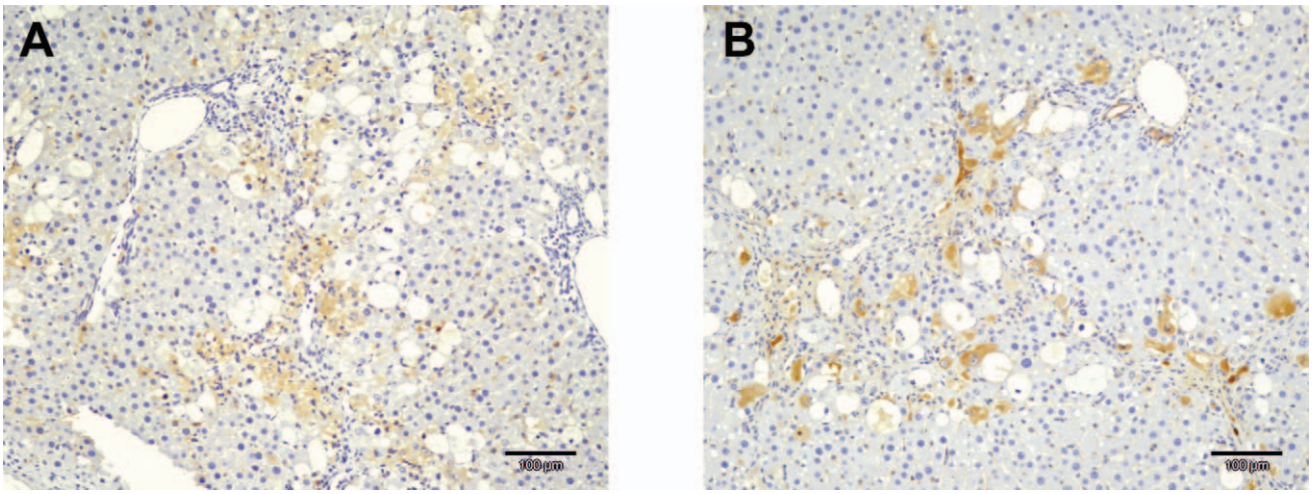

Figure 4. Representative micrographs of the hepatic lipid peroxidation measured by the expression of 4-hydroxy-2-nonenal-protein adducts. (A) Rats received a standard diet over 10 weeks. (B) Rats received a folic acid–supplemented diet over 10 weeks. Lipid peroxidation staining was consistently stronger in animals that received folic acid supplementation. Original magnification: ×100. ($n = 6$ per group). A color figure is available in the online version of this article.

content of biopsies was estimated by image analysis of the Masson trichrome stain. The amount of activated stellate cells in the liver was estimated by α-smooth muscle actin immunohistochemistry (30) using an anti–smooth muscle α–actin antibody (Novocastra, Menarini, Florence, Italy) and a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. To quantify the amount of collagen and α-smooth muscle actin–positive cells, the positively stained areas were measured by image analysis in a total of 40 fields at ×200 magnification and expressed as a percentage of the total area examined. Hepatic 4-hydroxy-2-nonenal-protein adducts were assessed as an index of lipid peroxidation by using a monoclonal antibody from the Japan Institute for the Control of Aging (Shizuoka, Japan). DNA fragmentation in parenchymal liver cells was measured as an indirect estimation of apoptosis by using the TUNEL method (ApopTag Peroxidase *in situ* apoptosis detection kit, Chemicon Int., Temecula, CA). Results of all the histologic and histochemical methods were analyzed by using the AnalySIS image software system (Soft Imaging System, Munster, Germany).

Statistical Analyses. Two-way ANOVA was employed to assess the effects of folic acid administration over the weeks of treatment (or recovery). The Mann-Whitney *U* test was used to compare the differences between treatments in each individual time period. The Spearman test was used to evaluate the degree of association between any two variables. Results are shown as means ± SEM. Statistical analyses were performed with the SPSS 14.0 statistical package (SPSS Inc., Chicago, IL).

Results

Folic Acid Administration Increases Liver Injury in CCl_4-Treated Rats. Folic acid was not associated with mortality and was well tolerated. We did not observe any significant differences in relation to body weight or liver weight between rats given the standard diet and those receiving a folic acid supplement (data not shown). Folic acid administration to CCl_4-treated rats was associated with a higher severity of the hepatic histopathologic and biochemical alterations (Figs. 2 and 3). Serum bilirubin concentration was significantly higher, and serum albumin concentration was significantly lower in rats with a folic acid– supplemented diet ($P < 0.001$ by ANOVA). At weeks 8 and 10, the hepatic histology of animals receiving a standard diet predominantly consisted of steatosis and thin collagen bands. Conversely, rats receiving a folic acid–supplemented diet had a lower number of lipid droplets, but the collagen septa were thicker and formed regeneration nodules. Hepatic collagen content measured by histomorphometry was significantly higher in rats with a folic acid–supplemented diet than in animals receiving a standard diet ($P < 0.001$ by ANOVA, Group A vs. Group B). The investigation into whether folic acid affected the accumulation of α-smooth muscle actin–positive cells indicated that the rats receiving the folic acid–supplemented diet had a significantly higher content of positive cells ($P < 0.001$ by ANOVA); this result suggested a higher content of activated stellate cells. In addition, the percentage of TUNEL-positive cells (a marker of apoptosis) was also significantly higher in animals with folic acid supplementation ($P < 0.001$ by ANOVA). Essentially the TUNEL-positive cells were parenchymal cells, and staining was restricted to the nuclei. Serum folate, vitamin B_{12}, and plasma homocysteine concentrations were higher in animals receiving the folic acid–supplemented diet ($P < 0.001$ by ANOVA). These changes were associated with an increase in expression of the 4-hydroxy-2-nonenal-protein adducts in the hepatic tissue (Fig. 4).

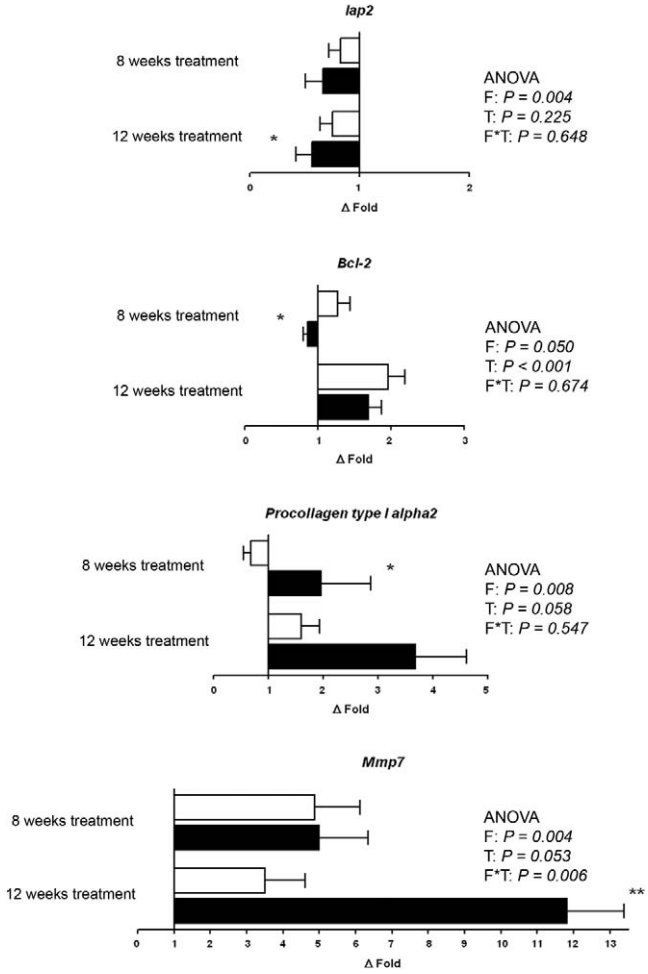

Figure 5. Genes differentially expressed in relation to folic acid administration. White bars represent rats that received a standard diet (Group A). Black bars represent rats that received a folic acid–supplemented diet (Group B). For ANOVA, F represents an effect of folic acid administration and time effect T. *$P < 0.05$; **$P < 0.01$ by the Mann-Whitney *U* test with respect to animals that received a standard diet and at the corresponding test-animal time point. Results (means and SEM) are expressed as the ratios relative to week 6 ($n = 6$ per group).

Folic Acid Administration Influences Hepatic Apoptosis and Fibrosis Gene Expression. We quantified the hepatic expression of several genes involved in folate metabolism, apoptosis, and fibrosis. In Groups A and B, dietary folic acid administration was associated with significant changes in four of these genes (Fig. 5): the inhibitor of apoptosis 2 (IAP_2 or $Birc_2$) and the B-cell leukemia/lymphoma 2 (*Bcl-2*) were decreased, and procollagen type I α 2 (*Col1a2*) and the matrix metalloproteinase 7 (*Mmp7*) were increased with respect to animals receiving a standard diet. $Birc_2$ expression was inversely related to the percentage of α-smooth muscle actin–positive cells ($r = -0.70$; $P < 0.001$), the percentage of collagen ($r = -0.54$; $P < 0.01$), and *Col1a2* expression ($r = -0.53$; $P < 0.01$). *Col1a2* expression was directly associated with the percentage of collagen ($r = 0.72$; $P < 0.001$), the percentage of α-smooth muscle actin–positive cells ($r = 0.81$; $P < 0.001$), and *Mmp7* expression ($r = 0.61$; $P < 0.001$). We did not observe any significant changes associated with folic acid administration in any of the other genes evaluated.

Folic Acid Administration Impairs the Recovery of Liver Function After CCl_4 Cessation. To test whether dietary folic acid supplementation may influence the recovery of hepatic function, the CCl_4 administration was suspended at 6 weeks of treatment (Groups C and D). Results are shown in Figure 6. We did not observe any significant difference in the collagen content or the number of activated stellate cells between animals receiving the standard diet and animals given the supplemented diet. However, folic acid administration was associated with a significantly higher percentage of hepatic TUNEL-positive cells ($P < 0.001$ by ANOVA), serum AST activities, and bilirubin concentrations and a lower serum albumin concentration ($P < 0.01$ by ANOVA). As expected, rats receiving folic acid had higher serum concentrations of folate and vitamin B_{12} ($P < 0.001$ by ANOVA). There were no significant differences in plasma homocysteine concentrations. During the recovery period, dietary folate administration was associated with a mild but significant decrease in the expression of the tissue inhibitor of metalloproteinase 3 (*Timp3*) gene at week 1 of recovery (Fig. 6B) but was not associated with changes in expression of the other genes evaluated. There was a higher content of inflammatory cell clusters in the liver biopsies of animals receiving the folic acid–supplemented diet (Fig. 6C).

Discussion

Folate plays a critical role in the *de novo* synthesis of purines and thymidine and influences the cell cycle, DNA stability, and apoptosis (31). The effects of folic acid dietary manipulation have been extensively studied in experimental models of cancer and cardiovascular disease (10), but there is a relative paucity of data regarding the effects of folic acid supplementation on liver function and associated diseases. Chronic liver impairment is associated with alterations in hepatic parenchymal cell apoptosis (20), regeneration (32), and the composition of the extracellular matrix (33). As such, the hypothesis of an influence of folic acid on the course of the disease has validity and warrants further investigation. Our results indicate that a diet with moderately high folic acid supplementation has an exacerbating effect on the development of liver impairment in carbon tetrachloride–treated rats. Animals receiving the dietary supplement had a higher content of collagen, a higher number of activated stellate cells, a higher number of apoptotic parenchymal cells in liver biopsies, and greater alterations in the serologic markers of liver impairment. Differences were in general more evident at weeks 8 and 10 of treatment; this finding suggests that the dose of folic acid employed accelerates the development of liver disease. Results from previous studies showed that the period between 6 and 10 weeks is critical in the inflammatory and fibrogenetic processes in CCl_4-induced liver impairment, including sensitivity to lipopolysaccharide toxin and Kupffer cell activation (34, 35). The present study shows that the mechanisms underlying these effects may include changes in the expression of genes involved in the apoptosis and the fibrosis pathways in the liver.

Apoptosis (or programmed cell death) is characterized by distinct morphologic and biochemical changes in the cell (36–38). Various receptors embedded in cell membranes respond to death-signaling ligands and induce the activation of a series of cytoplasmatic proteases termed caspases. One of these ligand-receptor systems is the Fas ligand–Fas receptor system. Caspase activation plays a crucial role in the proteolytic processes at specific aspartate cleavage sites during apoptosis. The mitochondria release proapoptotic and antiapoptotic proteins that effectively modulate the caspase-induced proteolytic reactions. A major regulation of apoptosis is the Bcl-2/Bax family of mitochondrial proteins. Bcl-2 inhibits and Bax promotes the formation of the proapoptotic apoptosomes that in turn induce further caspase activation and nuclear chromatin condensation. Apoptosis is also regulated by a family of caspase inhibitors: the inhibitors of apoptosis (IAPs). The end result is DNA fragmentation and phagocytosis of the apoptotic cell by macrophages. Our data indicate a significant decrease in *Iap2* and *Bcl-2* gene expression in carbon tetrachloride–treated rats receiving folic acid as dietary supplementation. We observed significant inverse relationships between *Iap2* expression and hepatic collagen content, *Col1a2* expression, and the amount of activated stellate cells. These results are similar to those reported in patients with premalignant gastric lesions (17) in which folic acid therapy decreased *Bcl-2* expression in gastric mucosa. They are also consistent with other studies, indicating that Bcl-2 may play a pivotal role in the regulation of hepatic cell apoptosis (39, 40).

In our study, folic acid administration was associated with a 4-fold increase in the hepatic *Mmp7* expression

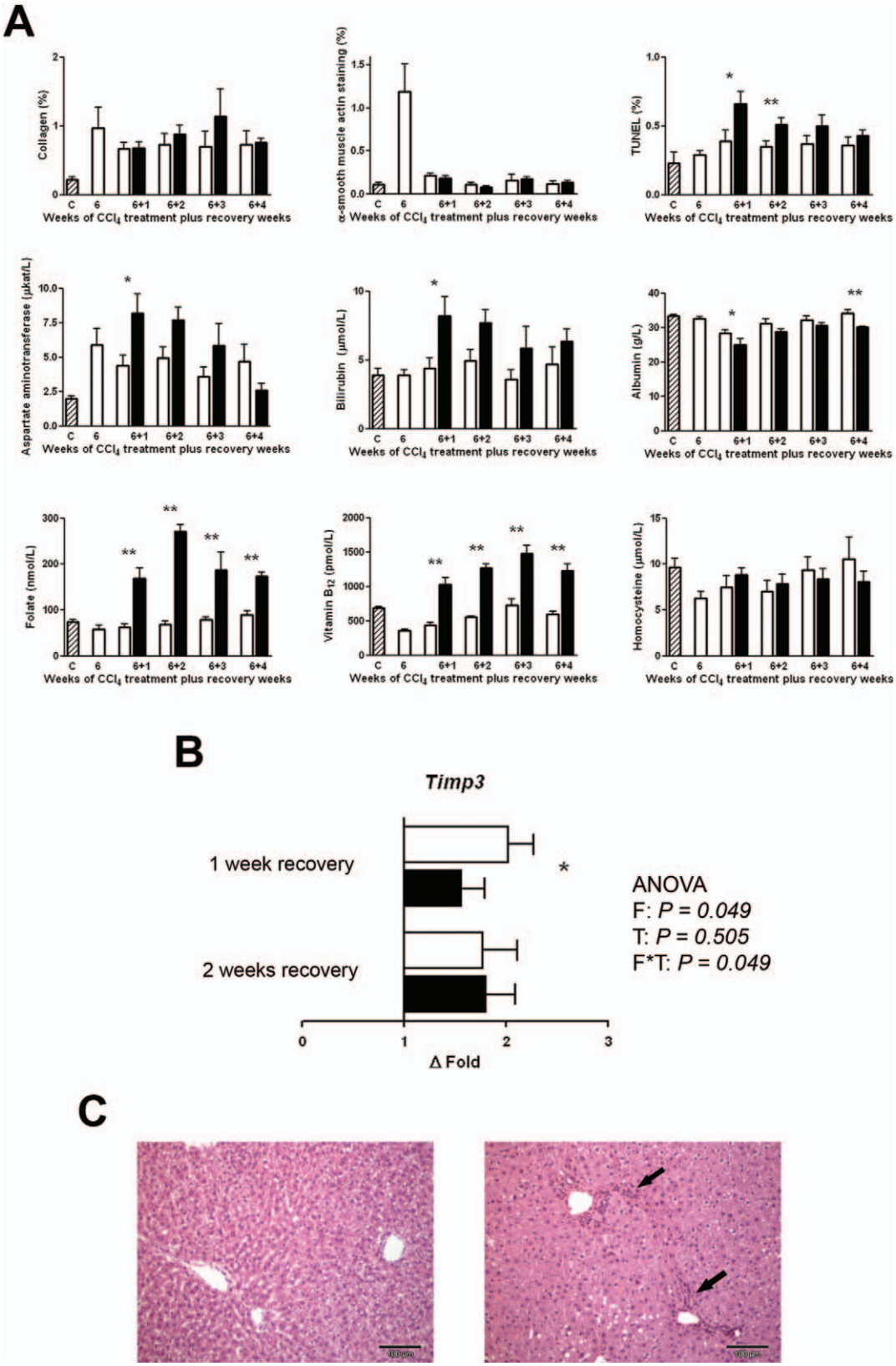

Figure 6. Effect of folic acid on the recovery of liver function. (A) Results represent the chronologic changes of selected biochemical variables in treated rats in which CCl_4 was suspended at 6 weeks, and the rats proceeded to receive different diets from weeks 6 to 10. White bars represent rats that received a standard diet (Group C). Black bars represent rats that received a folic acid–supplemented diet (Group D). Striped bars represent the control group. $P < 0.001$ by ANOVA (Group C vs. Group D) for all analyzed variables except for collagen and α-smooth muscle actin. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$ by the Mann-Whitney *U* test with respect to animals that received a standard diet at the corresponding

following 12 weeks of the dietary supplementation. The main physiologic substrates for Mmp7 are entactin, gelatin, elastin, fibronectin, vitronectin, laminin, and fibrinogen, but not collagen (41). Increased Mmp7 synthesis is probably not related to fibrinolysis but to the degradation of the hepatic extracellular matrix during the process leading to fibrogenesis and collagen deposition. Indeed, previous studies have shown that *Mmp7* is strongly expressed in fibrotic liver tissue and that it correlates with the degree of fibrosis and with standard serologic tests of liver dysfunction (42). All these suggest that the hepatic disease process itself is related to the transcriptional activity of this protein.

An additional factor influencing the deleterious effects of folic acid supplementation is the observed increase in plasma homocysteine concentrations. Hyperhomocysteinemia increases *Col1a2* expression in cultured smooth muscle cells and stellate cells, suggesting a fibrogenetic role for homocysteine in chronic liver diseases (43). The observation of an increased homocysteine concentration following folic acid intake is intriguing because folic acid is expected to cause the opposite effect on homocysteine levels. Reasons for this apparently abnormal response cannot be ascertained from the present study, but an altered function in the methionine-transsulfuration cycle in liver disease, secondary to impaired synthesis of several enzymes, could lead to the paradoxical accumulation of homocysteine in a response to folate. Indeed, an earlier study by Malinow et al. (44) described an increase in plasma homocysteine concentrations following folic acid supplementation in about 20% of the participants in a population-based study.

Current evidence indicates that liver fibrosis is a dynamic process with phases of either matrix deposition or net degradation, that fibrosis does not inevitably lead to an irreversible stage, and that if the original cause of the liver injury is removed, regression of liver fibrosis can often occur (41). This is one of the reasons why we decided to investigate the influence of folic acid supplementation on the recovery of the hepatic structure and function after suspending the administration of the injury-inducing carbon tetrachloride. Again, we observed a deleterious effect of folic acid administration (i.e., the folic acid–treated animals had a significantly higher degree of apoptosis in hepatic parenchymal cells and more severe alterations in serum bilirubin and albumin concentrations). We did not observe, however, any significant differences either in the collagen content (there was a trend, but the differences did not reach statistical significance) or in the amount of activated stellate cells in both groups of animals. Our results also suggest that the molecular mechanisms of the effects of folate on the recovery differ from those obtained in the initial section of our study. In this second experiment, we did not find any significant alterations in *Iap2* and *Bcl-2* gene expression, but we did observe a significant decrease in *Timp3* expression in animals given folic acid supplementation. This decrease was associated with a higher degree of hepatic inflammation as evidenced in hematoxylin and eosin staining. Compared with other members of the TIMP family, TIMP3 is unique in that it inhibits activation of TNF-α convertase (45, 46). Reduced *Timp3* expression results in an increased concentration of circulating TNF-α and is associated with inflammation (47). Recent studies have shown that $Timp3^{-/-}$ mice develop hepatic necrosis and periportal lymphocytic infiltrates (48), as was observed in our animals with lower *Timp3* expression (Fig. 6C).

A caveat of the present study is the dose of folic acid used for dietary supplementation. In the literature, the amount of folic acid used for dietary supplementation in rats is variable, ranging from 4 to about 200 mg/kg (49–52). The dose used in the present investigation can be considered "moderately high" (53). However, we decided to use this dose because it was demonstrated to increase DNA methylation, which plays a key role in the regulation of gene expression, and to prevent the development of several diseases. This dose of folic acid is considerably higher than that commonly employed in humans (54). Extrapolation from experimental animals to humans is always tenuous and fraught with doubt and, as such, we prefer not to be categorical regarding the extent our findings in the rat could be generalized to human patients with liver disease. However, our study could act as a cautionary signal for the clinical practitioner because our findings suggest that folic acid overdose during treatment of patients with anemia resulting from liver diseases may not be innocuous and may even be contraindicated. In our opinion, long-term follow-up studies are needed to determine whether moderately high folic acid supplementation can cause adverse effects in patients with chronic liver diseases.

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test-animal time point. (B) Effect of folic acid on gene expression. White bars represent rats that received a standard diet (Group C). Black bars represent rats that received a folic acid–supplemented diet (Group D). For ANOVA, F represents an effect of folic acid administration and a time effect T. * $P < 0.05$ with respect animals that received a standard diet at the corresponding test-animal time point. Results are expressed as ratios of the week-6 values. (C) Representative micrographs of hematoxylin and eosin stains of liver tissue of rats that received a standard diet (left panel) or a folic acid–supplemented diet (right panel) at 1 week of recovery. The arrows indicate areas of inflammatory cell clusters. Original magnification: ×100. *Timp3* expression was 2.67 in the animal shown in the left panel and 0.39 in the one shown in the right panel. Results are expressed as means and SEM ($n = 6$ per group). A color figure is available in the online version of this article.

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