Gas6 deficiency prevents liver inflammation, steatohepatitis, and fibrosis in mice

Agnès Fourcot,1,2 Dominique Couchie,1,2 Marie-Noelle Chobert,1,2 Elie-Serge Zafrani,1,2,3 Philippe Mavier,1,2 Yannick Lapereche,1,2 and Arthur Brouillet1,2
1INSERM, UMR-S-955, Equipe 17; 2Université Paris-Est Créteil; and 3AP-HP, Groupe Henri Mondor-Albert Chenevier, Département de Pathologie, Créteil, France

Submitted 1 July 2010; accepted in final form 18 February 2011

FOURCOT A, COUCHIE D, CHOBERT M, ZAFRANI E, MAVIER P, LAPERCHE Y, BROUILLET A. Gas6 deficiency prevents liver inflammation, steatohepatitis, and fibrosis in mice. Am J Physiol Gastrointest Liver Physiol 300: G1043–G1053, 2011. First published February 24, 2011; doi:10.1152/ajpgi.00311.2010.—The Gas6/Axl pathway has been increasingly implicated in regeneration and tissue repair and, recently, in the control of innate immunity. In liver, we have demonstrated that Gas6 and its receptor Axl are expressed in macrophages, progenitor cells, and myofibroblasts and that Gas6 deficiency reduced inflammation and myofibroblast activation, causing delayed liver repair in response to acute injury. All these data suggest a role of Gas6/Axl signaling in pathogenesis of chronic liver diseases. In the present study, we address the role of Gas6 in steatohepatitis and progression to liver fibrosis using Gas6-deficient mice fed a choline-deficient-ethionine-supplemented diet (CDE) or receiving a chronic carbon tetrachloride (CCL4) treatment. Gas6 deficiency attenuated hepatic steatosis by limiting CDE-induced downregulation of genes involved in β-oxidation observed in wild-type animals. Moreover, Gas6-deficient mice displayed reduction of hepatic inflammation, revealed by limited F4/80-positive macrophage infiltration, decreased expression of IL-1β, TNF-α, lymphotoxin-β, and monocyte chemotactic protein-1, and attenuated hepatic progenitor cell response to CDE diet. Gas6 deficiency reduced CDE-induced fibrogenesis and hepatic myofibroblast activation and decreased expression of TGF-β and collagen 1 mRNAs. After chronic CCL4 injury, Gas6-deficient mice also exhibited reduced liver fibrosis as a consequence of defective macrophage recruitment compared with wild-type animals. We conclude that improvement of steatohepatitis and fibrosis in Gas6−/− mice is linked to an inhibition of the inflammatory response that controls lipid metabolism and myofibroblast activation. This study highlights the deleterious effect of Gas6 in the progression of steatosis to steatohepatitis and fibrosis.

chronic liver diseases; macrophages; liver progenitor cells; CDE diet; Axl

THE PROTEIN PRODUCT OF THE GROWTH ARREST-SPECIFIC GENE 6 (Gas6) is a secreted ligand for Tyro3, Axl, and Mer, the TAM tyrosine kinase receptors (11). The Gas6/Axl pathway promotes hematopoietic stem cell growth (8) and fibroblast or endothelial cell survival (12, 39) and has been implicated in regeneration and tissue repair (27). In the liver, we reported that Gas6 and its high-affinity receptor Axl are expressed by macrophages, hepatic stellate cells (HSC) in their myofibroblastic phenotype (19), and liver progenitor cells (LPC) (5). We also reported that Gas6 exerts a survival effect on myofibroblasts and liver precursor cells in vitro and controls liver repair (20). Other recent studies revealed that TAM receptors have a pivotal role in the control of innate immunity (24) and that Gas6/TAM signaling is involved in inflammation by enhancing interactions between endothelial cells and leukocytes (41) as well as by facilitating the engulfment of apoptotic bodies by macrophages (38). Moreover, the induction of Axl limits cytokine synthesis in activated monocytes or dendritic cells (35). In Gas6−/− mice, a high and constitutive expression of Axl leads to delayed wound healing process after a single carbon tetrachloride (CCL4)-injection, by limiting Kupffer cell activation, macrophage infiltration, and HSC myofibroblastic transformation in necrotic areas (20). Altogether these data suggest that the Gas6/Axl pathway, through the control of inflammation and liver repair, may have a prominent role in the pathogenesis of chronic liver diseases.

Steatohepatitis is one of the leading cause of liver-related morbidity and mortality in developed Western countries. Whatever its etiology, steatohepatitis is characterized by fat storage in hepatocytes, lobular inflammation, elevated local and systemic cytokines, activation of HSC, and expansion of LPC in periporal areas reported in human as well as in animal models (31, 34). Steatohepatitis is a risk factor associated with toxic and metabolic fatty liver disease and can progress to end-stage cirrhosis (9). According to the two-hit model of steatohepatitis, steatosis is the first hit that increases hepatocyte vulnerability to any secondary insult eliciting an inflammatory response, but most probably both events are tightly interconnected since fat accumulation per se induces oxidative injury and inflammatory cytokine synthesis (6). The persistent low-grade inflammation due to chronic hepatocyte damage plays also a critical role in LPC expansion, which may play a part in fibrosis (4, 23, 31, 34).

To address the role of Gas6 in steatohepatitis, Gas6-deficient mice were fed a choline-deficient-ethionine-supplemented (CDE) diet, which is an experimental toxic model with a compensatory LPC proliferation (1), as observed in human fatty liver disease and other chronic liver diseases (4, 23, 31, 34). We show that Gas6 invalidation protects the liver from the development of CDE-induced steatohepatitis and reduces LPC expansion and the progression to fibrosis. The profibrogenic role of the Gas6/Axl pathway was also confirmed in the classical model of hepatic fibrosis induced by chronic CCL4 injection and after 18 wk of thioacetamide (TAA) intoxication.

MATERIALS AND METHODS

Animal models of liver injury. Steatohepatitis was induced by feeding mice a CDE diet consisting of choline-deficient chow (ICN Biomedicals) and drinking water supplemented with 0.15% (wt/vol) ethionine (ICN Biomedicals) for 3, 7, 14 or 21 days; control mice received normal chow and drinking water. Studies were performed on
Table 1. Mouse primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>GI No.</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACOX1</td>
<td>TGGAGCAACGGTTAACAG</td>
<td>ATCTCGGCTGGGCTTGA</td>
<td>6429155</td>
<td>195; 452</td>
</tr>
<tr>
<td>CCR2</td>
<td>CTGACCTGCTACATGCACTCT</td>
<td>CAAGGCTCCATCTCAGTTAG</td>
<td>160333416</td>
<td>164; 267</td>
</tr>
<tr>
<td>CD36</td>
<td>CAGATGCGCCATGCTTGGTC</td>
<td>ATCTCGGCTGGGCTTGA</td>
<td>142363407</td>
<td>726; 895</td>
</tr>
<tr>
<td>COL1A1</td>
<td>GGTCTTGTCAGTTGCGACT</td>
<td>CCAACGCTGACATTGCGG</td>
<td>118131144</td>
<td>129; 231</td>
</tr>
<tr>
<td>COL3A1</td>
<td>AGCTAGATGATATTGGATGCAG</td>
<td>GGTTGGGAGGCTGCTGAGT</td>
<td>226423932</td>
<td>290; 443</td>
</tr>
<tr>
<td>CPT1A</td>
<td>CTTGCGGCGAGCCCAGAA</td>
<td>CACCGAATGATGGCATTGT</td>
<td>162287141</td>
<td>148; 247</td>
</tr>
<tr>
<td>DESM</td>
<td>GCGACGACGACGCTGTAACCTTCACT</td>
<td>GCAATGTTGTCCTGGATGACAGT</td>
<td>33565249</td>
<td>970; 1100</td>
</tr>
<tr>
<td>F4/80</td>
<td>TTTTCGTCGTTGCTTCTTC</td>
<td>CCGCTGTCGTTGCTTCTTC</td>
<td>33859545</td>
<td>2196; 2418</td>
</tr>
<tr>
<td>FAS</td>
<td>GTCCTCTTGGAAATTCACTCA</td>
<td>ATCTCGGCTGGGCTTGA</td>
<td>93102408</td>
<td>767; 896</td>
</tr>
<tr>
<td>IL1β</td>
<td>CTCGACCTGCTAAGGCAAGA</td>
<td>GGCTGCTTTCTACATGACAGT</td>
<td>31560681</td>
<td>521; 669</td>
</tr>
<tr>
<td>LTB</td>
<td>AAGACTGATGACAGACAAACC</td>
<td>CCGGAAGACATGATGACATTG</td>
<td>161760766</td>
<td>216; 318</td>
</tr>
<tr>
<td>MTP</td>
<td>GATGATGTCGGAGGCCCTAG</td>
<td>AGCAGGCTGACATTGCGG</td>
<td>6678959</td>
<td>1311; 1448</td>
</tr>
<tr>
<td>PPARα</td>
<td>TGGAGCAACGGTTAACAG</td>
<td>ACAGCCGATCTGCTATTGCG</td>
<td>16466387</td>
<td>792; 916</td>
</tr>
<tr>
<td>SCD1</td>
<td>TGGGAATGTTGATGATGCGG</td>
<td>TGGGAATGTTGATGATGCGG</td>
<td>118130513</td>
<td>963; 1093</td>
</tr>
</tbody>
</table>

Sequence, GenBank GI number and positions of each mouse primer used in RT-PCR analysis.

6- to 8-week-old male Gas6−/− mice bred for 18 generations in the C57BL/6 genetic background (44) and wild-type (WT) C57BL/6 mice (Janvier Animal Center, Le Genest Saint Isle, France). Each group included 7–12 animals and the food and drink intake was not statistically different between WT and Gas6−/− mice.

Liver fibrosis was induced in 11-week-old male Gas6−/− and WT mice injected intraperitoneally with CCl4 (0.5 ml/kg body wt diluted in olive oil 1:10) twice a week for 10 days or 4 or 6 wk. Control mice received the vehicle only and each group included five to eight animals.

Mice were killed after overnight fasting and blood samples were collected from the inferior vena cava. Liver was excised and divided into five parts. One part was fixed in 10% buffered formalin and embedded in paraffin, the second one was snap-frozen for immunofluorescence studies, and three parts were lysed for RNA, protein isolation, and triglyceride extraction. All animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

Histological and immunohistochemical analysis. Liver histology was assessed on 4-μm-thick paraffin-embedded liver sections stained with hematoxylin and eosin (H&E). Necroinflammation score (including ballooning degeneration, necrosis, and inflammation) was blindly assessed on sections from three liver lobes and graded on a four-point scale as 0 (absent), 1 (mild or focal), 2 (noticeable), and 3 (severe).

Statistical analysis. All data are expressed as means ± SE. Statistical analysis were performed by Student’s t-test (CDE diet; n = 7–12) and the nonparametric Mann-Whitney U-test (chronic CCl4 intoxication; n = 5–8) with PRISM 4.0 (GraphPad). A P value of less than 0.05 indicated a significant difference between WT and Gas6−/− mice (*P < 0.05, **P < 0.01, and ***P < 0.001).

Table 2. Effects of CDE diet

<table>
<thead>
<tr>
<th></th>
<th>WT (D0)</th>
<th>Gas6−/− (D0)</th>
<th>D7</th>
<th>D14</th>
<th>D21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body</td>
<td>22.38 ± 0.25</td>
<td>21.46 ± 0.29</td>
<td>17.80 ± 0.53*</td>
<td>19.13 ± 0.56*</td>
<td>19.68 ± 0.24</td>
</tr>
<tr>
<td>D0 weight</td>
<td>21.29 ± 0.71</td>
<td>21.67 ± 0.52</td>
<td>20.6 ± 0.19</td>
<td>21.29 ± 0.71</td>
<td>21.67 ± 0.52</td>
</tr>
<tr>
<td>Liver</td>
<td>1.04 ± 0.02*</td>
<td>1.01 ± 0.05</td>
<td>1.22 ± 0.06‡</td>
<td>1.06 ± 0.07</td>
<td>1.07 ± 0.03‡</td>
</tr>
<tr>
<td>D0 weight</td>
<td>1.07 ± 0.03</td>
<td>0.99 ± 0.02</td>
<td>1.20 ± 0.08‡</td>
<td>1.07 ± 0.03</td>
<td>1.07 ± 0.03‡</td>
</tr>
<tr>
<td>ALT activity, UI/l</td>
<td>15.74 ± 3.69</td>
<td>22 ± 4.5</td>
<td>661.6 ± 107.3†‡</td>
<td>271.3 ± 77.59*</td>
<td>71.7 ± 17.6*</td>
</tr>
<tr>
<td>D0</td>
<td>26.52 ± 9.27</td>
<td>40.67 ± 14.06</td>
<td>311.8 ± 242.7*</td>
<td>216 ± 31.8</td>
<td>216 ± 31.8</td>
</tr>
<tr>
<td>Necroinflammation, arbitrary units</td>
<td>2.13 ± 0.148</td>
<td>0.86 ± 0.13</td>
<td>1.52 ± 0.25</td>
<td>1.43 ± 0.31</td>
<td>0.77 ± 0.13</td>
</tr>
<tr>
<td>D7</td>
<td>1.52 ± 0.25</td>
<td>0.52 ± 0.20</td>
<td>1.52 ± 0.25</td>
<td>1.43 ± 0.31</td>
<td>0.77 ± 0.13</td>
</tr>
</tbody>
</table>

ALT activity, UI/l

The data are means ± SE for body weight, serum alanine aminotransferase (ALT) activity, and necroinflammation scores were measured in wild-type (WT) or Gas6−/− mice on choline-deficient ethionine-supplemented diet (CDE) feeding on days 7, 14, and 21 (D7, D14, D21) or receiving control diet (D0). *P < 0.05 or †P < 0.01, significant difference from control diet; ‡P < 0.05 or §P < 0.001, significant difference between WT and Gas6−/− mice.

Data are means ± SE for Body and liver weights, serum alanine aminotransferase (ALT) activity, and necroinflammation scores measured in wild-type (WT) or Gas6−/− mice on choline-deficient ethionine-supplemented diet (CDE) feeding on days 7, 14, and 21 (D7, D14, D21) or receiving control diet (D0). *P < 0.05 or †P < 0.01, significant difference from control diet; ‡P < 0.05 or §P < 0.001, significant difference between WT and Gas6−/− mice.
RESULTS

Gas6 deficiency prevents the development of CDE-induced steatosis. In basal conditions, WT and Gas6−/− mice did not exhibit any difference in body weight, liver morphology, and triglyceride content. After CDE feeding, in both WT and deficient mice, we noticed a transient loss in body weight that was maximal at day 7 and represented up to 17% of the initial weight (Table 2). In WT CDE-treated mice, liver weight was significantly higher compared with untreated mice at day 7, an increase that was observed later at day 14 in Gas6−/− mice. Administration of CDE diet also induced hepatocellular necrosis revealed by an increase of serum ALT at day 7, which was lower and delayed in deficient animals. A rapid ballooning degeneration of hepatocytes and an infiltration of inflammatory cells, assessed by a necroinflammation score (Table 2), were observed at day 7 in WT animals and decreased thereafter. In Gas6−/− mice, this necroinflammation score reached its maximum 1 wk later. Then both genotypes recover gradually their initial body and liver weight and normal ALT level 3 wk after CDE treatment.

Feeding WT mice the CDE diet resulted in accumulation of lipid droplets within hepatocytes, as illustrated on typical sections (Fig. 1A). Steatotic hepatocytes were preferentially located in pericentral or midlobular areas, as previously reported in WT mice fed a methionine- and choline-deficient (MCD) diet (43). This macrovesicular steatosis reached its maximal level after 1 wk, as shown by semiquantitative analysis of steatosis (Fig. 1B). Consistent with the morphological changes, total triglyceride liver content at day 7 in WT mice was increased by 68% in CDE compared with control diet (Fig. 1C) but remained unchanged in Gas6−/− mice (Fig. 1, B and C). On the other hand, in deficient mice, steatosis (Fig. 1B) and liver weight (Table 2) reached the values obtained in WT animals at a later stage (day 14). In summary, Gas6 deficiency delayed hepatic accumulation of lipids and hepatomegaly during CDE diet, an effect that cannot be linked to reduced body weight, which was similar in both types of mice.

To understand the mechanism by which Gas6 interferes with CDE-induced steatosis at day 7, we further analyzed the expression of hepatic lipid metabolism genes (Fig. 2). CDE feeding was associated with increased expression of CD36 hepatic fatty acid translocase with no significant difference between both genotypes. Such an induction has also been reported in MCD-induced steatohepatitis (42). Expression of microsomal triglyceride transfer protein (MTP), essential for triglyceride export and fatty acid synthase (FAS), the rate-limiting enzyme for hepatic lipogenesis (42), were not significantly changed by the CDE diet in both genotypes (Fig. 2). As previously observed with MCD diet, CDE feeding caused a rapid and marked repression of stearoyl-CoA desaturase-1 (SCD1), the rate-limiting step in the biosynthesis of monounsaturated fatty acids, at day 3 in both genotypes. At day 7, SCD-1 mRNA expression remained significantly higher in Gas6−/− mice than WT animals. Levels of acyl-CoA oxidase-1
(ACOX1) and carnitine palmitoyltransferase-1 (CPT1) mRNA coding for two rate-limiting enzymes of mitochondrial β-oxidation (28) were significantly decreased in WT mice at day 3 and increased thereafter to recover the level found in untreated WT mice (Fig. 2). Interestingly, this CDE-induced downregulation in the expression of both enzymes did not occur in CDE-fed WT but not Gas6−/− mice. SCD-1 mRNA expression was suppressed at day 3 in both genotypes and remained significantly higher in Gas6−/− mice at day 7. *P < 0.05 and **P < 0.01.

Gas6 deficiency reduces CDE-induced liver inflammation. In parallel to steatosis, CDE treatment induced a marked infiltration of monocytes/macrophages in WT mice, as revealed by F4/80 immunolabeling that localized around steatotic hepatocytes at day 7 (Fig. 2A) and that was considerably reduced in Gas6−/− mice. Macrophage infiltration quantitated by F4/80 mRNA expression was increased by 14-fold in CDE-treated WT animals at day 7 compared with untreated controls, whereas CDE treatment was without significant effect in deficient mice (Fig. 3A). In summary, CDE-treated mice developed a steatohepatitis characterized by fat accumulation in hepatocyte and inflammatory cell infiltration, which was prevented by Gas6 deficiency. Since macrophages are the primary source of inflammatory cytokines, we analyzed liver expression of several cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP1), which is essential to the recruitment of circulating monocytes, and lymphotixin-β (LTβ), which stimulates LPC expansion (2). Hepatic expression of these cytokines was rapidly but transiently induced by CDE diet (Fig. 3B), with cytokine mRNA levels returning to initial level by day 21. The maximal expression of IL-1β was observed after
3 days on CDE diet whereas mRNA levels from other cytokines peaked later at day 7. Expression of these mRNA was largely prevented upon exposure of Gas6-deficient mice to CDE diet. Newly liver recruited macrophages were quantitated by measuring the expression of CCR2, the MCP1 receptor expressed on circulating monocytes and not on resident Kupffer cells (26). The 60-fold increase in CCR2 mRNA expression revealed a recruitment of macrophages which was maximal at day 7 in WT mice (Fig. 3B) and largely prevented in deficient mice. It should also be noted that the peak of IL-1β mRNA was obtained at day 3, before the peak of CCR2, suggesting that IL-1β mRNA induction results rather from an increase of its synthesis in resident Kupffer cells than from newly recruited macrophages. In summary, Gas6 deficiency limits CDE-induced macrophage recruitment, leading to a decrease in the production of inflammatory cytokines in liver of Gas6−/− mice.

Gas6 deficiency inhibits the accumulation of LPC in CDE-fed mice. In H&E-stained liver sections from CDE-treated WT mice, we observed a proliferative response of LPC with small ovoid nuclei (also called oval cells), organized in ductules with a poorly defined lumen, predominating in periportal areas and extending to midlobular zone (data not shown) as previously described (1, 15, 17). These LPC were easily detected by expression of CK19, a marker of bipotent oval cells. In control animals, CK19 staining revealed interlobular bile ducts and few cholangioles without any difference between WT and Gas6−/− mice (Fig. 4A). In CDE-fed WT mice, CK19-positive...
LPC located close to the portal region and their number increased with duration of treatment, forming larger clusters expanding inside the lobule at day 21 (Fig. 4A). In control WT mice, CK19 fluorescent labeling covered less than 0.5% of the liver section and reached 5% after a 3-wk CDE treatment (Fig. 4B). These data are in full accordance with LPC expansion already documented by this model (43). In Gas6−/− mice, CK19-positive cells covered less than 2% of the sections at the end of the treatment (Fig. 4B), revealing reduced LPC accumulation in response to CDE diet.

Gas6 deficiency reduces liver fibrosis. Picrosirius red staining revealed a moderate perisinusoidal collagen deposition starting from the portal region and extending into the lobule, which represented 9% of liver area in CDE-fed WT mice at day 21 (Fig. 5A). This amount of collagen was significantly attenuated in CDE-treated Gas6−/− mice (5.15 ± 0.99%). Messenger RNA expression of α chain of collagen 1 (Col1A1) was increased up to 45-fold over basal level in WT mice after 1 wk of treatment and decreased thereafter (Fig. 5B). A similar profile was obtained for the expression of profibrotic Col3A1 mRNA (data not shown). Induction of collagen synthesis was largely prevented in Gas6−/− mice, corroborating the low level of matrix deposition induced in those mice on CDE diet (Fig. 5A). Altogether, these data show that Gas6 deficiency limits fibrosis progression in response to CDE-induced steatohepatitis.

Fig. 4. Gas6 deficiency limits CDE-induced liver progenitor cell (LPC) accumulation. A: in control WT and deficient mice, CK19 immunolabeling was restricted to epithelial cells lining interlobular ducts and to few cholangioles. After 3 wk of CDE diet, labeling revealed also LPC expansion in the lobule from WT animals, which was reduced in deficient mice (original magnification ×40). B: quantitative analysis of labeled areas confirmed the reduced LPC accumulation in Gas6−/− mice. *P < 0.05.

Activation of HSC into α-SMA-positive myofibroblasts is a major fibrogenic process driven by cytokines released from activated macrophages. After 1 wk of CDE diet in WT animals, numerous α-SMA-positive cells located mainly in portal areas of the liver and infiltrated to the mid part of the lobules as recently reported (43). At later time points, α-SMA labeling decreased and was limited to few cells in close vicinity of LPC expansion (data not shown). In deficient mice, whatever the CDE time course, no staining for α-SMA was observed (Fig. 5C) except in vessels of the portal tract also labeled in control livers (data not shown). Quantitation of α-SMA mRNA confirmed the increased number of myofibroblasts in CDE-treated WT mice, which peaked at day 7 and decreased thereafter (Fig. 5D); such an induction at day 7 was not detected in treated Gas6−/− mice. Desmin labeling, a hallmark of quiescent HSC, was not modified by the diet in both type of mice (data not shown). Therefore, the very few number of α-SMA-positive HSC in CDE-treated Gas6−/− mice, not accounted by a decrease in desmin-positive HSC, suggests a defective myofibroblastic activation of HSC in those deficient mice.

Hepatic expression of transforming growth factor-β (TGF-β) mRNA, coding for the main fibrogenic mediator secreted by activated HSC and macrophages, was increased up to 16-fold over control level in CDE-fed WT mice at day 7, whereas such an induction was significantly decreased in deficient mice (Fig. 5E). This result is in accordance with CDE-induced infiltration of
macrophages (Fig. 2A) and activation of myofibroblasts upon CDE diet in WT but not in Gas6−/− mice (Fig. 5, C
and D).

To further address the role of Gas6 in hepatic fibrogenesis, deficient mice were submitted to a chronic CCl4 intoxication, a more classical model of liver fibrosis. At 4 wk of treatment, picrosirius red staining showed liver fibrosis scored F2/F3 (Metavir analysis) in six of seven WT animals, whereas in the group of deficient mice, four were scored F0/F1 and one F2 (data not shown). At 6 wk, all WT animals were scored F3 whereas 5 Gas6−/− mice where scored F1/F2 and one F3 (Fig. 6A). The fibrogenic effect of Gas6 in this model was confirmed by the significantly higher surface section stained by picrosirius (Fig. 6A) in WT mice (5.2% of liver area) than in deficient mice (2.85%) at 6 wk, whereas the serum ALT activity in both groups was not significantly different (Fig. 6B). The decreased inflammatory response, which limits the fibrogenesis reported above in Gas6−/− mice on CDE diet, could also account for reduced fibrosis in CCl4 model. Indeed the increase in hepatic F4/80 immunostaining and mRNA expression (Fig. 6C) in WT mice 10 days after the induction of the fibrogenic process, was not observed in deficient mice. At this stage, we also noticed a lower level of α1 chain of collagen 1 and 3 mRNAs in Gas6-deficient mice (data not shown). Thus early defective macrophage infiltration in Gas6−/− mice slows down CCl4-induced liver fibrosis progression. In another toxic model (Supplemental Fig. S1; the online version of this article contains supplemental data) using TAA feeding in drinking water (200 mg/l) for 18 wk (37), we also observed that Gas6
deficiency significantly reduced progression of fibrosis (Supplemental Fig. S1A) without modifying TAA-induced liver injury (Supplemental Fig. S1B). In conclusion, these results provide a new insight to the importance of Gas6 in progression of chronic liver diseases to fibrosis.

Liver Axl receptor is overexpressed in Gas6−/− mice in CDE and CCl4-treated mice. In untreated animals, Gas6 deficiency induced a ninefold increase in AXL protein level (Fig. 7) but remained without effect on Axl mRNA (data not shown), indicating a negative control exerted by Gas6 on its receptor Axl at a posttranscriptional level, as previously reported (20). In CDE-fed WT mice, AXL protein level was increased up to 10-fold over basal at days 3, 7, and 14 (Fig. 7), an induction that could be accounted for, at least partially, by liver recruitment of Axl-positive inflammatory cells including macrophages (22). In CDE-fed Gas6−/− mice, the AXL protein content was further increased and represented five times the level found in CDE-treated WT mice at any time point. This Axl induction in treated Gas6−/− mice could not be due to the recruitment of inflammatory cell, which was blocked in those mice (Fig. 3A). A similar profile in liver AXL protein content was observed during CCl4 treatment, with a basal and an induced level always higher in Gas6−/− mice than in WT animals (Fig. 7). Since Axl tempers the inflammatory response (35), overexpression of this receptor in Gas6−/− mice could prevent progression to steatohepatitis with CDE diet and fibrosis after chronic CCl4 injury.

**DISCUSSION**

Experimental models based on choline deficiency are the most efficient models to induce steatohepatitis in rodents whereas progression of models steatosis to steatohepatitis is
barely observed by feeding rodents a high-fat diet (7, 21). Choline deficiency induces hepatocyte fat storage through an inhibition of phosphatidylcholine synthesis necessary to liver VLDL assembly and secretion (45). It can be achieved by feeding animals a MCD diet, also reported to impair mitochondrial function (14, 28), or by a CDE diet (1). Choline deficiency triggers a compensatory LPC compartment activation also reported in humans (4, 23, 34), and ethionine, an inhibitor of methionine adenosyl transferase, prevents endogenous choline synthesis from methionine and dramatically increased the hepatic accumulation of LPC (1, 34, 43). However, both of these toxic models do not recapitulate the metabolic abnormalities commonly observed in human nonalcoholic steatohepatitis (NASH), such as insulin resistance and obesity (7, 21). In this regard, high fat or high sucrose diet or genetically hyperphagic mice would appear more accurate models of NASH, but failed to ensure development of fibrosis (21).

In our study, CDE diet induces a loss of body weight, liver injury and cytokine synthesis within the first 7 days of treatment as previously shown (15, 17, 43), but all these parameters returned to basal levels thereafter, indicating that this 3-wk CDE diet could not be considered as a model of chronic liver injury. This is in contrast with a recent report also performed on a C57Bl6 genetic background (18) showing that a continuous CDE feeding for 16 wk was necessary to obtain an increase in serum ALT and steatosis. The reason for this discrepancy is still unclear but may be related to the amount of ethionine ingested by the animals. More interestingly in this later report IL-6 deficiency, which induced massive steatosis, also aggravated liver necrosis, steatohepatitis, and fibrosis. Conversely, in our study lower steatosis in Gas6−/− mice was associated with reduced ALT. Altogether, these results established that fat storage in hepatocyte has a deleterious effect and may drive the pathogenic process in CDE model.

After 3 days of CDE diet, liver from WT animals displayed a decreased expression of PPARα and its target genes CPT1A and ACOX1 involved in fatty acid β-oxidation. As reported in MCD, CDE diet induced CD36 expression favoring fatty acid uptake and suppressed SCD1 mRNA preventing synthesis of unsaturated fatty acids, indicating that the lipogenic pathway was altered in both models (32, 33, 42). Then increased fatty acid uptake and decreased triglyceride synthesis and fatty acid oxidation represent three mechanisms by which CDE diet could promote intrahepatic lipid accumulation. The failure of CDE diet to reduce hepatic β-oxidation gene expression in Gas6−/− mice could account for a more efficient fatty acid catabolism and delayed steatosis in those mice. Since SCD1 is downregulated by TNF-α (32), we could also hypothesize that decreased expression of this cytokine in deficient mice (Fig. 3B) may account for a higher SCD1 expression at day 7 in Gas6−/−. Interestingly, a role of Gas6 in fat accumulation has been reported in adipogenesis. After exposure to a high-fat diet, fat accumulation in adipose tissues was reduced in Gas6−/− mice, without any effect on liver steatosis (25). In this study, Gas6 directly promotes proliferation of Axl-positive preadipocytes and their differentiation into mature adipocytes (25). In the liver, we previously demonstrated that Gas6 and its receptor Axl are not expressed in hepatocytes (5, 19). For this reason, contribution of Gas6 to CDE-induced fat storage within hepatocytes cannot be direct but mediated by Axl-positive cells, such as macrophages, LPC, and HSC in their myofibroblastic phenotype (5, 19).

Compelling findings in both animal and human studies have emphasized the pivotal role of inflammatory cytokines in progression of steatosis to steatohepatitis (6, 13, 30, 33, 40, 42). CDE diet increased hepatic IL-1β and TNF-α mRNA in liver of WT mice as previously reported (17). The level of these cytokines synthesized mostly by Kupffer cells and recruited macrophages was largely prevented in Gas6−/− mice. Decreased macrophage recruitment in deficient mice can be accounted by reduced hepatic chemokine MCP1 expression and lack of Gas6, a chemoattractant for Axl-positive circulating monocytes (20). In addition, the reported effects of Gas6 on endothelial cell activation and leukocyte extravasation (41), as well as the constitutive high level of Axl receptor in Gas6−/− mice evidenced in this study, will contribute to hold down the inflammatory response (20, 35). However, in this CDE model, we could not observe any induction of suppressor of cytokine signaling SOCS1 and 3 (data not shown) as previously observed in an acute model of liver injury (20). Therefore, the functional relevance of Axl overexpression in Gas6-deficient mice remains unclear and it has been described that Axl, even in the absence of ligand, can also directly interact with other membrane receptors (10) including cytokine receptors (3) and modulate the inflammatory response.

Kupffer cells are the primary source of IL-1β and their depletions reduces liver steatosis during MCD (33) and chronic high-fat feeding (40). Since Kupffer cell-derived IL-1β promotes triglyceride storage in hepatocytes by reducing expression of PPARα and its target genes involved in hepatocyte fatty

Fig. 7. Effect of Gas6 deficiency on AXL receptor. Densitometric analysis of immunoblots revealed higher AXL protein contents in liver of Gas6−/− mice compared with WT at basal level (control) and after CDE diet or CCl4 intoxication (arbitrary units are values normalized to β-actin). *P < 0.05, **P < 0.01, and ***P < 0.001.
acid oxidation (40), it can be hypothesized that reduced IL-1β expression revealed in Gas6−/− mice prevents early CDE-induced downregulation of genes involved in β-oxidation in hepatocytes and steatosis. Thus this study suggests that IL-1β participates in hepatic triglyceride storage during CDE diet. Since TNF-α is also a STAT3 and a death factor for hepatocytes (6, 13, 14, 28, 33, 42), the decrease in macrophage recruitment and subsequent TNF-α synthesis in Gas6−/− mice may ameliorate the hepatocyte status (in accordance with the lower value of serum ALT), reducing the need for a regenerative LPC response. Additionally, inflammatory cytokines are also mitogenic for LPC (2, 16), exerting their effects either by an autocrine mechanism or through interactions with macrophages and HSC colocalized with LPC (36). Decreased expression of TNF-α and LTβ in Gas6−/− mice may be involved in the 60% reduction of CDE-induced LPC accumulation in those mice.

In steatohepatitis, inflammatory cytokines orchestrate a cross-talk between different liver cell types, triggering HSC activation into myofibroblasts and fibrosis (42). In CDE-treated WT animals, perisinusoidal fibrosis is associated to progression of steatohepatitis. Its reduction in Gas6−/− mice is related to both defective HSC myofibroblastic transformation and TGF-β synthesis. It has been suggested that LPC are involved in the fibrogenic response elicited in the CDE model of steatohepatitis (15), a view also supported in human by the correlation between expansion of theses cells and the degree of fibrosis (4, 23, 31, 34). Indeed, fibrosis developed in the portal area where LPC proliferate in close association with α-SMA-positive HSC, suggesting that LPC can be fibrogenic (36, 43). Obviously progression of steatohepatitis to fibrosis does not occur simply as a result of LPC expansion since fibrosis reported on MCD diet develops without any ductular reaction (42).

Considering that a 3-wk CDE-induced fibrogenic response might differ from chronic liver injury and could be controlled by LPC, we submitted Gas6-deficient mice to a chronic CCl4 treatment, a classical model of liver fibrosis without any LPC activation (29). In this model, Gas6 deficiency reduced the fibrogenic response likely as consequence of defective macrophage recruitment and overexpression of Axl receptor as observed in the CDE model. Improvement of fibrosis in Gas6−/− mice was also confirmed in the chronic TAA intoxication model. Whatever the toxic damage, the protective effect of Gas6 deficiency on liver pathology could not simply due to lower liver injury as observed during CDE feeding, because the ALT levels in Gas6−/− mice were not significantly different to that observed in WT animals after a single CCl4 injection (20) or in the two chronic models of liver fibrosis (i.e., 6-wk CCl4 and 18-wk TAA intoxication).

In summary, improvement of steatohepatitis and fibrosis in Gas6−/− mice is linked to an inhibition of the inflammatory response that controls lipid metabolism and wound healing process. This study provides a new insight to the importance of the Gas6/Axl pathway in progression of steatohepatitis and fibrosis.

ACKNOWLEDGMENTS

The authors thank Dr. F. Lafidil and S. Lotersztajn for critical reading of the manuscript, Dr. T. Nakano from Shionogi Research Laboratories, Osaka (Japan) for providing the Gas6−/− mice, and M. Bouaziz for mouse breeding and nursing.

GRANTS

This work was supported by INSERM, University Paris-East Creteil and Agence Nationale pour la Recherche (ANR-06-Physio-022-2).

REFERENCES


43. Van Hul NK, Abbarca-Quinones J, Sempoux C, Horsmans Y. The active synthesis of phosphatidylcholine is mediated by 10.220.33.1 on April 3, 2017 http://ajpgi.physiology.org/ Downloaded from