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Growth arrest-specific protein 6 deficiency impairs liver tissue repair after acute toxic hepatitis in mice☆

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***Background/Aims*: Resident macrophages and myofibroblasts derived from hepatic stellate cells play a key role in liver wound healing. We previously reported that these sinusoidal cells secrete the growth arrest-specific protein 6 (Gas6) and express Axl, one of its receptors. Here we address the role of Gas6 in the healing process during acute liver injury.**

***Methods*: Toxic hepatitis was induced by a single carbon tetrachloride injection in Gas6 deficient ($Gas6^{-/-}$) mice and liver recovery was compared with wild-type animals.**

***Results*: Gas6 deficiency did not cause any change in CCl_4-induced liver damage. At 72 h, an efficient tissue repair was observed in wild-type animals whereas in $Gas6^{-/-}$ mice, we noticed a defective wound healing accounted by reduced Kupffer cell activation revealed by a decrease in the induction of CD14, TNF-α, IL6 and MCP-1. Gas6-deficiency, by limiting cytokine/chemokine release, prevents hepatocyte proliferation, recruitment of circulating monocytes and accumulation of myofibroblasts in healing areas. We also report a direct chemotactic effect of Gas6 on circulating monocytes which might explain defective macrophage infiltration in liver necrotic areas of $Gas6^{-/-}$ mice. Interestingly in $Gas6^{-/-}$ mice, we observed a high and constitutive expression of Axl and an induction of the suppressor of cytokine signaling SOCS1 after CCl_4 treatment.**

***Conclusions*: The lower level of cytokines/chemokines in $Gas6^{-/-}$ mice after CCl_4 injury, is the consequence of an inhibitory signal arising from Axl receptor overexpression, leading to delayed liver repair in deficient mice.**

***Keywords*: Liver repair; Inflammation; Kupffer cells; Growth arrest-specific protein 6; Axl**

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Abbreviations: aSMA, alpha smooth muscle actin; CCl_4, carbon tetrachloride; CCR2, chemokine C–C motif receptor 2; Gas6, growth arrest specific gene protein 6; $Gas6^{-/-}$, Gas6-deficient; HSC, hepatic stellate cell; HSC/MFB, HSC-derived myofibroblast; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; PCNA, proliferating cell nuclear antigen; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; WT, wild-type.

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1. Introduction

In response to acute injury, the liver elicits a wound healing process characterized by proliferation of unaltered hepatocytes, clearance of cell debris and matrix remodeling leading to recovery of a normal hepatic structure. In various forms of hepatitis, Kupffer cells, the liver resident macrophages, are involved at one and the same time in hepatocyte death and initiation of wound healing [1]. In addition to their phagocytic properties, Kupffer cells respond immediately to the injury by releasing proinflammatory mediators such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL6) that trigger hepatocyte proliferation [2] and monocyte chemoattractant protein-1 (MCP-1) which drives monocyte/macrophage recruitment to the lesion [3]. Evidence for a key role of liver macrophages in hepatic injury and repair has been provided by alteration in hepatic cytokine expression and delayed liver regeneration in animals depleted in Kupffer cells [4,5]. Resident and recruited monocytes/macrophages are also involved in activation of hepatic stellate cells (HSC) into myofibroblasts (HSC/MFB) [6,7], which secrete proteases and matrix proteins necessary to tissue remodeling. The central role of HSC/MFB in liver repair was clearly shown in mice where reduced HSC-myofibroblastic transformation was associated with a defective liver regeneration [8,9].

The protein product of the growth arrest-specific gene 6 (Gas6) and its tyrosine kinase Tyro-3, Axl and Mer receptors (TAM receptors) have been implicated in growth and survival processes during development and tissue repair. Gas6/Axl signaling induces accumulation of mesangial cells in kidney fibrosis [10] and vascular smooth muscle cells in response to vascular injury [11] as well as proliferation of cardiac fibroblasts [12]. Gas6 is known as a chemo-attractant protein for vascular smooth muscle cells [13], interfering with cell adhesion and stabilizing platelet aggregation [14]. A recent study also demonstrates that Gas6 plays a pivotal role in leukocyte infiltration into inflamed tissue [15]. In normal liver, we previously showed that Gas6 is expressed by resident macrophages and, after hepatic injury, in HSC along with their transformation into HSC/MFB [16]. In addition, Axl, the receptor with the highest affinity for Gas6, is expressed in liver macrophages and HSC/MFB and Gas6 protects HSC/MFB from apoptosis *in vitro* [16]. These results suggest that Gas6/Axl signaling might be involved in the wound healing response to liver injury.

In order to directly address the role of Gas6 produced by macrophages and HSC/MFB in liver repair, we studied the healing process in Gas6 deficient ($Gas6^{-/-}$) mice after a single carbon tetrachloride (CCl_4)-injection, a well-known model of acute hepatitis [8]. We report an impairment of liver repair in $Gas6^{-/-}$ mice, which was associated with delayed hepatocyte proliferation related to a default in Kupffer cell activation and reduced macrophage infiltration and HSC myofibroblastic transformation in necrotic areas. It is noteworthy that in these deficient mice, there is a high and constitutive expression of Axl leading to induction of the suppressor of cytokine signaling SOCS1 synthesis after CCl_4 injection [17]. These results might account for lower release of cytokine/chemokine in response to the injury and explain the delayed liver repair in $Gas6^{-/-}$ mice.

2. Materials and methods

2.1. Animal model of liver injury

Studies were performed on 8–12 week old male $Gas6^{-/-}$ mice bred for 18 generations in the C57BL/6 genetic background [18] and wild-type (WT) C57BL/6 mice (Janvier Animal Center, France). Mice treated with a single intraperitoneal injection of CCl_4 (4 ml/kg body weight) diluted in olive oil (1/1) and control mice injected with vehicle only, were killed at different time points (from 3 h to 7 days) after the injection. Four of 66 mice from $Gas6^{-/-}$ group died on day 2 after CCl_4 injection, whereas all WT mice (78 animals) survived to the entire experiments. Livers were collected and treated as previously described [19]. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using an Advia 1650 automate (Bayer Diasys). All animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

2.2. Histological and immunohistochemical analysis

Liver injury was assessed on 4 μm-thick paraffin-embedded liver sections stained with haematoxylin and eosin (H & E). Necrosis was graded on a 4-point scale (0, 1, 2 and 3) in 20 random fields at 100× magnification: absence of necrotic area corresponded to index 0 and index 3 corresponded to the maximal value for necrosis observed in a field (up to 50% of the field area). Immunohistochemistry was carried out on paraffin-embedded or 5 μm-thick frozen sections as previously described [19]. Primary antibodies were mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz), mouse monoclonal anti-α-smooth muscle actin (α-SMA) (Sigma), rat monoclonal directed against mouse F4/80 and CD68 (Serotec). For immunodetection with mouse monoclonal primary antibodies (PCNA, α-SMA), mouse IgG proteins were blocked using the MOM kit (Vector) and endogenous peroxidase activity by 3% H_2O_2 in 1.5% horse serum in PBS; sections were revealed by the Vectastain system (Vector) using DAB (Pierce) as peroxidase substrate [19]. For F4/80 staining, sections were revealed by the streptavidin–alkaline phosphatase conjugate (GE Healthcare) and the FastRed substrate system (Dako). Fluorescent labeling of CD68 was achieved using secondary FITC coupled anti-rat IgG STAR 80F (Serotec).

2.3. Protein analysis

Fifty μg of protein from snap frozen liver were used for Western blotting as previously described [16] using antibodies against PCNA (Sigma), phospho-Axl-Y779 (R & D systems), Axl (Santa Cruz) and phospho-NFkBp65-Ser536 (Cell Signaling). Anti-β-actin clone AC15 antibody (Sigma) was used to correct for unequal loadings. IL6 concentration was determined using a commercial ELISA kit (R & D systems) in plasma samples and in 250 μg of protein from snap frozen liver.

2.4. RNA analysis

Total RNA (2 μg) isolated from liver using the RNeasy mini kit (Qiagen) were reverse-transcribed from random hexamers using a first-stand synthesis kit (Fermentas, Life Sciences) and specific cDNA