Galectin-3 modulates phagocytosis-induced stellate cell activation and liver fibrosis in vivo

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GALACTINS ARE MEMBERS OF THE family of β-galactoside-binding lectins (1, 3, 20). Galectin-3 (known also as MAC-2) is a unique 30-kDa molecule with a chimeric structure (21). It is known to be involved in the regulation of inflammatory processes through its role in macrophage activation (22), migration (8, 13), and cell survival (33). Galectin-3 also has a critical role in the phagocytosis of opsonized red blood cells (29) and apoptotic neutrophils by macrophages (6). Galectin-3 was thought to play an important role in fibrogenic processes in different tissues (10, 11, 24) mainly through its immunomodulatory effects. Galectin-3 is both an extra- and intracellular lectin; however, the respective mechanisms by which these forms exert fibrogenic activity are yet undefined.

In our previous studies, we have shown that hepatic stellate cells (HSC) are able to phagocytose apoptotic bodies (AB) of dead hepatocytes (17, 34). Engulfment of apoptotic cells leads to phenotypic changes, transdifferentiation of HSC to myofibroblasts with production of collagen I and transforming growth factor (TGF)-β, and reactive oxidative species via the NADPH oxidase 2 (NOX2) activation (19). Phagocytosis also induced myofibroblast survival, further contributing to the fibrogenic injury (18). Because galectin-3 binding is an important step in phagocytosis, our aim was to elucidate the role of galectin-3 in the regulation of phagocytosis-mediated HSC activation. Here we have shown that extracellular galectin-3 plays a significant role in AB tethering and engulfment via cross-linking integrin αvβ3, and in the subsequent activation of HSC. Engulfment of AB also induced galectin-3 upregulation and secretion from HSC suggestive of a novel autocrine pathway of HSC activation. In vivo, we found a decrease in fibrosis stage in galectin-3−/− mice following bile duct ligation (BDL), indicating that galectin-3 is an important regulator in liver fibrogenesis.

MATERIALS AND METHODS

Animals. Galectin-3−/− mice in a C57/B6 background were generated previously by gene targeting technology, as described (16). As controls, age- and sex-matched wild-type (wt) littermates were used. HSC isolation from mice was performed according to Geerts et al. (7), by sequential in situ perfusion with collagenase and pronase. BDL was performed with the mice under pentobarbital anesthesia (60 mg/kg ip). The common bile duct was ligated in two locations. The animals were killed 3 wk following surgery, and the liver specimens were fixed. Control animals underwent sham operations. The animals were housed in facilities approved by the National Institute of Health. All procedures were reviewed and approved by the Animal Welfare Committee of the University of California Davis.

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condition, and the rate of phagocytosis was obtained from dividing the number of phagocytosing cells by the total number counted.

Cell culture and transfection of primary HSC. To study phagocytosis, HSC were cultured in serum-free medium for 16 h as above and then treated with lactose (50 mM, 1 h) followed by AB exposure in the presence or absence of recombinant galectin-3 (1 μM). Primary HSC were exposed to the combination of integrin αvβ3 antibodies (10 μg/ml; Millipore, Billerica, MA) before the AB, and phagocytosis was evaluated. To study galectin-3 binding to integrins, primary rat HSC were cultured in chamber slides and transiently transfected with pBJ1Integrin β3 (Intβ3wt) or a binding-deficient mutant (Intβ3D119A) using GeneJuice transfection reagent (EMD Chemicals, Gibbstown, NJ) following the instructions. The transfection efficiency was 40%.

To study the role of nuclear factor (NF)-κB signaling in the phagocytosis-mediated galectin-3 expression, primary rat HSC were also isolated and incubated with AB with or without the NF-κB inhibitor caffeic acid phenethyl ester (CAPE, 1 ng/ml; Sigma-Aldrich) for 24–48 h. In parallel, rat HSC were transfected with a dominant-negative IkB expression vector (DN-IkB) using GeneJuice transfection reagent. Twenty four hours after the transfection, the cells were exposed to AB for 48 h and then harvested to study mRNA expression.

Cell adhesion assay. We used the protocol modified from Eto et al. (5). A 96-well enzyme-linked immunosorbent assay (ELISA) plate was coated with recombinant human galectin-3 and then blocked with 1% polyvinylpyrrolidone (Sigma-Aldrich) in PBS. The HSC-expressing Intβ3wt and Intβ3D119A were detached and resuspended in serum-free DMEM; 10^3 cells/well were added to the wells and incubated at 37°C for 1 h. The unbound cells were removed by rinsing with DMEM, and the attached cells were quantified by measuring endogenous phosphatase activity. In brief, 100 μl of the substrate/lysis buffer (1% Triton X-100, 6 mg/ml p-nitrophenylphosphate, and 50 mM sodium acetate, pH 5.0) were added to the wells. After 45 min of incubation at 37°C, the reaction was stopped by 50 μl of 1 M NaOH. The optical density at 405 nm wave length representing the cell adhesion intensity was read. The percentage of the phosphatase activity from the adherent cells over that from the total cell input was used to represent cell adhesion.

Quantitative RT-PCR. Total RNA from cells or tissue was extracted by using an RNA purification kit (Qiagen, Valencia, CA). Reverse transcription was performed by using the Superscript III kit based on the random hexamer method (Superscript III first-strand synthesis supermix for qRT-PCR; Invitrogen). For real-time PCR reactions, the primer sequences used are listed in Table 1.

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ELISA. The plates were coated with affinity-purified goat anti-mouse galectin-3 capture antibodies, in PBS. After washing and blocking with 1% BSA/PBS, the appropriately diluted standards from recombinant galectin-3 and samples (conditioned medium form HSC and Kupffer cells exposed to AB) were added and incubated overnight at 4°C. After washing in Tween 20/PBS, affinity-purified rabbit-anti-mouse galectin-3 was added at room temperature for 1 h and then, after wash, a horseradish peroxidase-conjugate antirabbit antibody (Invitrogen) was applied. 

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) studies confirmed that phagocytosis induced NF-κB activation, which was significantly blocked by CAPE (A, #P < 0.01). Real-time PCR showed a significant induction of galectin-3 mRNA by phagocytosis, and this was abolished by CAPE (A, #P < 0.005) or DN-IκB (B, mean ± SE, n = 3, **P < 0.005). Electrophoretic mobility shift assay (EMSA) studies confirmed that phagocytosis induced NF-κB activation, which was significantly blunted by CAPE (C and D, densitometry data, control denotes no AB exposure). Arbp, acidic ribosomal phosphoprotein P0. Data are means ± SE, n = 3. **P < 0.01.
mobility shift assay (EMSA) using a Biotin 3'-prime DNA labeling kit and lightshift chemiluminescent EMSA kit (Thermo Scientific). Briefly, the nuclear extract was mixed with the biotin-labeled NF-kB oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') in a buffer containing 1 mg/ml of poly(dI-dC). The lysate mixture was subjected to electrophoresis on 6% polyacrylamide gel and then electrotransferred to a nylon membrane. The membrane was then cross-linked, followed by application of a chemiluminescent substrate, and the resulting chemiluminescence was detected by autoradiography.

**Hydroxyproline assay.** The liver tissue (400–500 mg) from BDL and sham-operated mice was denatured in 6 N HCl for 16 h after washing away the acid, and the tissue was incubated with 50 mM chloramine-T (Sigma-Aldrich) at room temperature for 20 min. Perchloric acid (3.15 M; Sigma-Aldrich) and 20% p-dimethylaminobenzaldehyde (Sigma-Aldrich) were added sequentially. The samples were incubated at 60°C for 20 min, and 557 nm absorbance was recorded. A standard curve was generated with a series of hydroxyproline solution with known concentrations, by which the hydroxyproline amounts of liver tissue were calculated. The data were expressed as microgram of hydroxyproline per gram of wet liver.

**Statistical analysis.** All data represent at least three experiments and are expressed as the mean ± SE. Differences between groups were compared using one-way ANOVA associated with the Dunnett’s test. Statistical significance was assumed when P < 0.05.

## RESULTS

Galectin-3 is required for the phagocytic activity of HSC. Because galectin-3 was shown to regulate phagocytosis in other systems, first we tested if the efficiency of phagocytosis was reduced in stellate cells isolated from galectin-3−/− mice. The wt and galectin-3−/− HSC were cultured in serum-free medium overnight and then exposed to AB for 16 h (Fig. 1A). The engulfment of AB has decreased to 12.0 ± 1.9% in galectin-3−/− HSC compared with wt HSC (26.3 ± 3.7%, P < 0.05). When galectin-3−/− HSC were incubated with the recombinant galectin-3, this was reversed (33.0 ± 1.1%, P < 0.0001), suggesting that extracellular galectin-3 participates in the engulfment process. To confirm that the increase in phagocytosis was indeed galectin-3-mediated, HSC were incubated in the presence or absence of the galectin-3-binding competitor lactose. The phagocytosis rate was significantly reduced after lactose exposure both in wt (13.9 ± 3.0%, P < 0.05) and galectin-3−/− HSC (14.5 ± 3.6%, P < 0.01) after

Table 2. ALT and bilirubin values do not change significantly in the galectin-3−/− mice

<table>
<thead>
<tr>
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<th>WT Sham</th>
<th>WT BDL</th>
<th>Gal-3−/− Sham</th>
<th>Gal-3−/− BDL</th>
</tr>
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<tbody>
<tr>
<td>ALT, U/l</td>
<td>24.4 ± 4.3</td>
<td>346.5 ± 73.4*</td>
<td>64.0 ± 2.0</td>
<td>408.0 ± 44.0*</td>
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<td>TBIL, mg/dl</td>
<td>0.66 ± 0.03</td>
<td>21.19 ± 6.00*</td>
<td>3.67 ± 1.67</td>
<td>18.00 ± 3.95*</td>
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Data are presented as means ± SE; n = 4 mice in each group. WT, wild type; BDL, bile duct ligation; ALT, alanine aminotransferase; TBIL, total bilirubin. *P < 0.05 compared with WT sham.
recombinant galectin-3 treatment, indicating that extracellular galectin-3 may play a role in phagocytic tethering and engulfment.

Integrins are well-known to mediate phagocytosis of apoptotic cells by facilitating tethering and inducing signaling pathways leading to actin reorganization and phagosome formation (4, 32). To show that HSC phagocytosis is regulated by integrins, primary HSC were incubated with the integrin αvβ3 antibodies (Fig. 1B). This heterodimer was described as inducing HSC activation and proliferation (35) and also the engulfment of apoptotic cells by phagocytes (4). After blocking the integrins, the phagocytic activity decreased in HSC. Because galectin-3 is known to cross-link integrins, thus modulating cell adhesion (25, 26), next we tested if galectin-3 binding to integrin αvβ3 occurs in HSC. Primary HSC were transfected with Intβ3wt or Intβ3D119A, and cell adhesion assay was performed to assess binding to galectin-3. We found decreased binding of the cells transfected with Intβ3wt and exposed to the recombinant galectin-3, there is membrane colocalization of the signals (Fig. 1Da), whereas, in the Intβ3D119A-transfected cells, no such colocalization was seen, and the overall intensity of signals diminished, suggesting that intact integrin αvβ3 are required for the binding of galectin-3 (Fig. 1Db). These findings indicate that extracellular galectin-3

![Image](http://ajpgi.physiology.org/)

**Fig. 6.** Liver fibrosis is decreased in galectin-3−/− mice. Picrosirius staining demonstrates decreased fibrosis following BDL in galectin-3−/− mice (A). The areas of fibrosis were significantly lower in the galectin-3−/− mice following BDL (mean ± SE, 5 different areas counted in 5 mice, *P < 0.05, B). The collagen level in the liver was assessed by hydroxyproline assay (C). In wt mice, increased levels of hydroxyproline were seen, whereas in the galectin-3−/− mice this was significantly decreased (*P < 0.05). The expression of procollagen α1(I) and α-SMA was examined by real-time PCR (D). In wt mice following BDL, the expression of both transcripts has significantly increased, whereas in the galectin-3−/− mice the increase was significantly blunted (*P < 0.05). The expression of TGF-β1 and tissue inhibitor of matrix metalloproteinase-1 was also significantly reduced in the galectin-3−/− mice. Data are means ± SE, N = 3 (**P < 0.01, E). The inflammatory markers tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1β, and IL-6 have decreased in galectin-3−/− BDL mice; however, only the reduction of MCP-1 was significant. Data are means ± SE, n = 3 (*P < 0.05, F).
plays a role in binding and cross-linking integrin $\alpha_\beta_3$, facilitating the uptake of AB.

Stellate cell activation in galectin-3$^{-/-}$ cells is decreased. To assess if extracellular galectin-3 mediates HSC activation, wt or galectin-3$^{-/-}$ cells were incubated with AB with or without the recombinant galectin-3 (Fig. 2). Engulfment of AB by wt HSC induced their activation with production of TGF-$\beta_1$ (2.8 ± 0.5-fold, $P < 0.001$) and procollagen $\alpha_1$ (I) (2.6 ± 0.6-fold, $P < 0.05$), consistent with our previous data (34), whereas recombinant galectin-3 alone did not induce activation. In HSC isolated from galectin-3$^{-/-}$ livers, however, the activation of HSC was blunted, with the TGF-$\beta_1$ mRNA expression decreasing to 1.3 ± 0.1-fold ($P < 0.05$) and procollagen $\alpha_1$ (I) to 1.0 ± 0.2-fold ($P < 0.05$), in parallel with the decrease of the phagocytic activity in these cells (Fig. 1A). Galectin-3$^{-/-}$ HSC were then treated with recombinant galectin-3 before exposing them to AB, and we found that the expression of TGF-$\beta_1$ and procollagen $\alpha_1$ (I) both increased (1.7 ± 0.2-fold, $P < 0.05$, and 1.4 ± 0.5), albeit not to the level observed in wt phagocytosing HSC. This suggests that both intra- and extracellular galectin-3 play a role in the regulation of phagocytosis-induced activation of HSC.

Galectin-3 is upregulated in activated HSC via an NF-$\kappa$B-mediated pathway. Because galectin-3 expression was found to be increased in cirrhotic livers (14), next we explored if galectin-3 was induced upon myofibroblastic transdifferentiation and whether this was phagocytosis-mediated. HSC were cultured in the presence or absence of the NF-$\kappa$B inhibitor CAPE (Fig. 3A), since NF-$\kappa$B is known to regulate the galectin-3 promoter (15, 22). Galectin-3 was upregulated following phagocytosis by $5.3 \pm 0.9$-fold ($P < 0.005$), and this was significantly inhibited in the presence of CAPE (by $0.7 \pm 0.2$-fold, $P < 0.005$, compared with AB-treated cells). To confirm the role of NF-$\kappa$B activation in the regulation of galectin-3 expression, HSC were transfected with the DN-$\kappa$B before phagocytosis (Fig. 3B). Galectin-3 expression was significantly decreased (0.3 ± 0.03-fold, $P < 0.005$), similar to the cells exposed to CAPE. In control vector-expressing cells, phagocytosis induced a 1.8 ± 0.2-fold increase in galectin-3 expression ($P < 0.01$). To confirm NF-$\kappa$B activation, EMSA was performed on phagocytosing HSC in the presence or absence of CAPE (Fig. 3C). NF-$\kappa$B was activated following AB engulfment. Taken together, galectin-3 is upregulated in HSC following phagocytosis involving the activation of NF-$\kappa$B. This suggests that galectin-3 may participate in a feedforward induction of phagocytosis-mediated fibrogenic processes. To confirm that these HSC can indeed secrete galectin-3 upon induction, next we performed ELISA assays.

Phagocytosis induces secretion of galectin-3 from HSC and Kupffer cells. Galectin-3 expression and secretion are closely linked to macrophage function during inflammation and renal fibrogenesis (10); however, it is not known whether myofibroblasts can secrete it and induce activation in an autocrine or paracrine manner. Primary HSC or Kupffer cells in serum-free medium were exposed to AB, and ELISA was performed. After phagocytosis, galectin-3 concentration in the culture medium increased significantly ($P < 0.0001$) in HSC (Fig. 4A) and in Kupffer cells (Fig. 4B).

Galectin-3 is upregulated during BDL in activated HSC. To confirm that galectin-3 indeed is upregulated in active HSC in vivo, BDL was performed on rats, and HSC were isolated from both sham-operated and BDL animals. Galectin-3 expression was significantly induced (3.9 ± 0.4-fold, $P < 0.05$) in HSC isolated from BDL animals (Fig. 5A). Immunohistochemistry and confocal microscopy indicated that, in the BDL mouse livers, the $\alpha$-SMA-positive myofibroblasts coexpressed galectin-3 ($n = 4$, Fig. 5B).

Fig. 7. The role of galectin-3 in HSC activation. Galectin-3 induces integrin-mediated uptake of AB, NF-$\kappa$B activation, and an increase in galectin-3 production and secretion. Thus galectin-3 may regulate fibrosis by a feedforward mechanism.

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Dissection

In this study, we described a novel mechanism addressing the profibrogenic effects of galectin-3. This lectin is known to be expressed traditionally by neutrophils and macrophages and plays a major role in the phagocytic clearance of pathogens such as Streptococcus pneumoniae (6) and in alternative macrophage activation (23). Galectin-3 is also shown to be expressed in activated myofibroblasts in liver, and intracellular galectin-3 is thought to play a role in Smad2/3-independent TGF-$\beta$ signaling and collagen production (11). However, the source and the mechanism by which extracellular galectin-3 modulates fibrosis is not well described. These are important areas of research, since extracellular secreted galectin-3 has...
distinct effects on immunomodulation (28, 31) and cell adhesion (25), all of which are key events during fibrogenesis.

HSC are described to be phagocytic, and phagocytosis induces their activation (34); thus, it is tempting to speculate that extracellular galectin-3 is required to tether and engulf AB from damaged hepatocytes. Indeed, we have shown that phagocytosis of AB does not occur in galectin-3−/− HSC but only in the presence of recombinant galectin-3. To investigate the mechanism by which galectin-3 contributes to the engulfment of apoptotic cells, we studied its binding to integrins. The integrin heterodimer αvβ3 expressed by HSC and linked to its activation and proliferation (35) is also known to be a major regulator for phagocytosis (9). Our data indicate that blocking integrin αvβ3 did suppress HSC phagocytosis. The binding of galectin-3 to the integrin αvβ3 leading to ligand clustering on the surface is often referred to as the “galectin lattice” (27). This may increase the efficiency of surface adhesion and tethering of the apoptotic cell to the phagocyte. Integrin engagement is also necessary for actin remodeling, which is required for phagosome formation and maturation (4). Rac 1 GTPase activation is an essential step in actin remodeling following integrin binding, and, in fact, we have shown in an earlier study that in phagocytosing HSC the GTPase activity of Rac1 is induced (17). In wt HSC, we found an induction of profibrogenic transcripts following phagocytosis; however, no significant induction of TGF-β or procollagen α1(I) was seen in galectin-3−/− HSC corresponding to the decrease in profibrogenic activity. Recombinant galectin-3 only partially reversed this, suggesting that intracellular galectin-3 also plays a role in fibrogenic signaling as this was proposed by Henderson et al. (11). In that study, liver fibrosis was based on the CCI4 model where the toxic injury, apoptosis/necrosis of hepatocytes are the initiating events. BDL is a distinct model of fibrosis, and according to previous studies, besides HSC, portal myofibroblasts also play a major role (2). Indeed we observed intense galectin-3 and α-smooth muscle actin co-staining in periportal myofibroblasts.

In this study, we also provided a detailed analysis of the cellular source of galectin-3 and investigated the autocrine activation of HSC by galectin-3. We have demonstrated that galectin-3 is upregulated in HSC isolated from BDL rats. The induction of galectin-3 expression in active HSC was NF-κB-mediated, and galectin-3 is secreted from active HSC following phagocytosis (Fig. 7). Galectins are known to be stored in the cytoplasm in a quiescent state, but, upon tissue injury, cytosolic galectins could be actively secreted by activated cells through a nonclassical pathway (1). As we have shown here, activated HSC also produce galectin-3 and thus could potentially induce autocrine and paracrine activation of HSC and also further modulate immune responses such as inducing macrophage chemotaxis and their alternative activation (12, 23). Indeed, we found decreased inflammatory cytokine production in the galectin-3−/− BDL mice. According to recent data, galectins are also classified as damage-associated molecular pattern candidates (30), lending support to the theory that they regulate innate and adaptive immune responses.

In conclusion, in the present study, we have shown a novel role of extra- and intracellular galectin-3 modulating stellate cell phagocytosis and activation during liver fibrosis by a feedforward mechanism. Galectin-3 is required for the elimination of apoptotic cells during chronic liver injury, and both extracellular and intracellular galectin-3 play important roles. In vivo, galectin-3−/− mice developed an attenuated fibrogenic response. Based on these findings, targeting galectin-3 production during liver injury and fibrosis may become a successful treatment strategy.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


