Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis

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Patella, Shane, David J. Phillips, Jorge Tchongue, David M. de Kretser, and William Sievert. Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis. Am J Physiol Gastrointest Liver Physiol 290: G137–G144, 2006—Activin A, a member of the transforming growth factor-β superfamily, is constitutively expressed in hepatocytes and regulates liver mass through tonic inhibition of hepatocyte DNA synthesis. Follistatin is the main biological inhibitor of activin bioactivity. These molecules may be involved in hepatic fibrogenesis, although defined roles remain unclear. We studied activin and follistatin gene and protein expression in cultured rat hepatic stellate cells (HSCs) and in rats given CCl4 for 8 wk and examined the effect of follistatin administration on the development of hepatic fibrosis. In activated HSCs, activin mRNA was upregulated with high expression levels, whereas follistatin mRNA expression was unchanged from baseline. Activin A expression in normal lobular hepatocytes redistributed to perisepal hepatocytes and smooth muscle actin-positive HSCs in the fibrotic liver. A 32% reduction in fibrosis, maximal at week 4, occurred in CCl4-exposed rats treated with follistatin. Hepatocyte apoptosis decreased by 87% and was maximal at week 4 during follistatin treatment. In conclusion, activin is produced by activated HSCs in vitro and in vivo. Absence of simultaneous upregulation of follistatin gene expression in HSCs suggests that HSC-derived activin is biologically active and unopposed by follistatin. Our in vivo and in vitro results demonstrate that activin-mediated events contribute to hepatic fibrogenesis and that follistatin attenuates early events in fibrogenesis by constraining HSC proliferation and inhibiting hepatocyte apoptosis.

activin; cirrhosis; hepatocytes

PROGRESSIVE HEPATIC FIBROSIS is characterized by excess deposition of extracellular matrix components after the activation of resident fibroblastic cells, such as portal tract fibroblasts, and transformation of hepatic stellate cells (HSCs) into collagen-producing myofibroblasts (1, 7, 31). Ample data support the role of transforming growth factor (TGF)-β as a major profibrogenic cytokine in cutaneous wound healing and in pathological fibrotic processes in the kidney, lung, and liver (3, 8, 11, 26). TGF-β is overexpressed during experimental liver fibrosis and is a potent regulator of HSC activation (11). Recent reports have demonstrated that the development of liver fibrosis can be attenuated by blockade of TGF-β signaling (10, 29). However, other data suggest that TGF-β is not the sole mediator of fibrosis. For example, significant overexpression of TGF-β in transgenic animals leads to only moderate degrees of fibrosis (1). Furthermore, attenuation of TGF-β signaling in animal models of hepatic fibrosis ameliorates but does not completely abrogate fibrosis (10, 29), suggesting that other factors contribute to this process.

We (25) have previously demonstrated that serum levels of activin A are significantly elevated in subjects with chronic viral hepatitis, whereas serum follistatin levels remain normal. Furthermore, hepatitis B virus replication is associated with a pronounced dysregulation in the normal activin-follistatin axis. Activin bioactivity is regulated by follistatin, an extracellular binding protein that is a potent neutralizer of activin action (27). In the normal liver, activin A is constitutively expressed by hepatocytes and acts as an autocrine inhibitor of hepatocyte DNA synthesis (39). Activin induces hepatocyte apoptosis in vitro and in vivo (12, 33) and regulates liver mass (13, 21). After partial hepatectomy, follistatin infusion increases hepatocyte regeneration (19, 20) by blocking activin inhibition of DNA synthesis (27) and profoundly alters hepatic mass. Several lines of evidence support a role for activin A in hepatic fibrosis. Activin-β3 mRNA increases significantly in hepatic fibrosis (34), and activin A localization in the fibrotic liver redistributes from the hepatocyte to HSC (5). Furthermore, cultured HSCs secrete collagen and fibronectin in response to exogenous activin A (4, 34). Until now, the potential for follistatin to diminish hepatic fibrosis has not been examined.

In the present study, we characterized the gene and protein expression of activin A and follistatin in cultured HSCs. We demonstrate that HSCs secrete activin during transformation to myofibroblasts and, during in vivo studies in the fibrotic liver, that activin and follistatin gene expression is altered and that activated HSCs express activin. Finally, follistatin administration attenuates hepatocyte apoptosis and the development of early hepatic fibrosis in CCl4-treated animals.

METHODS

Animal model and experimental design. The Monash Medical Centre Animal Ethics Committee approved this project. Cirrhosis was induced in male Wistar rats by an intraperitoneal injection of CCl4 and olive oil at a final concentration of 0.4 mg/kg three times weekly for 8 wk. Control animals received olive oil alone. Groups of five animals were killed at 2, 4, and 8 wk, and whole livers were removed for analysis.

Follistatin administration. In a separate group of animals, 2 μg of recombinant human follistatin 288 (rh-FS288) were injected intravenously every second day during concurrent CCl4 administration. Animals were killed at 2, 4, and 8 wk.

Determination of hepatic hydroxyproline content. Liver hydroxyproline content was quantified as previously described (16). Briefly, frozen liver samples were hydrolyzed in 6 N HCl at 110°C for...
were detected using confocal fluorescent microscopy. Dewaxed sections
were counted at 40 oil-immersion lens and collected using Bio-Rad Laser-Sharp
Diaphot 300 microscope (Bio-Rad; Hampstead, UK) equipped with an
142 nm and triple dichronic and 560-nm filter sets. Digital images
were collected using a Bio-Rad confocal inverted Nikon
photomicroscope. HSC coexpressing activin A and α-SMA
were detected using confocal fluorescent microscopy. Dewaxed
sections were incubated with the biotinylated activin A monoclonal
antibody (25 μg/ml, Oxford Bio-Innovations; Oxford, UK) for 1 h at
room temperature. After being washed, sections were incubated with a
streptavidin-Texas red conjugate (12.5 μg/ml, Chemicon) for a further
hour. To detect α-SMA reactivity, sections were then incubated with α-SMA (3 μg/ml, DAKO) for 1 h. Reactivity was then
determined using a sheep anti-mouse FITC-conjugated antibody (12 μg/ml, Chemicon) for a further 1 h at room temperature. Confocal
images were collected using a Bio-Rad confocal inverted Nikon
Diaphot 300 microscope (Bio-Rad; Hampstead, UK) equipped with an
air-cooled 25-mW argon/krypton laser with wavelengths at 488, 586, and
647 nm and triple dichromatic and 560-nm filter sets. Digital images
were produced from an average of eight scans of 1-s duration with a
×40 oil-immersion lens and collected using Bio-Rad Laser-Sharp
software.

Computer-assisted morphometric analysis. Image analysis was used to quantify α-SMA-positive HSCs. Twenty consecutive non-overlapping fields at ×100 magnification were analyzed by image-
analysis software (Scion Image for Windows, version 4.0.2, National
Institutes of Health) to assess the immunofluorescent area per biopsy.
For each section, the positive area for replicate fields was summed.

HSC isolation and culture. HSCs were isolated from normal Wistar
rats by sequential pronase and collagenase perfusion as previously
described (30). Cells were separated on a two-step discontinuous
gradient of nycodenz (Sigma; Sydney, Australia), and purity was
assessed by vitamin A autofluorescence and flow cytometry (9). The
presence of other contaminating nonparenchymal cells (e.g., Kupffer
cells and endothelial cells) was also assessed by flow cytometry. After
Trypan blue exclusion to determine viability, 1 × 10^6 cells/ml were cultured in M199 supplemented with 10% fetal bovine serum and 10% normal horse serum (Trace Biochemicals; Noble Park, Australia) on petri dishes to allow culture activation. Media were changed after 24 h and every 48 h thereafter. Culture-activated cells were routinely used between passages 6 and 9.

RESULTS

Establishment of cirrhosis. CCl4-treated animals showed expanded portal tracts by week 2 compared with the normal
liver (Fig. 1A), and, by week 8, most (95%) animals showed bridging fibrosis with some animals exhibiting definite cirrhosis (Fig. 1B).

Cellular expression of activin but not follistatin is modified in the fibrotic liver. Activin A immunoreactivity in the normal liver localized to hepatocyte cytoplasm and was evenly distributed throughout the lobule (Fig. 1C). By week 4, activin A was primarily localized to periscar hepatocytes (Fig. 1D) with reduced immunoreactivity in lobular hepatocytes. Activin A immunoreactivity colocalized with α-SMA within fibrotic bands, implicating activated HSCs as an activin source within the injured liver (Fig. 2).

Comparable follistatin immunolocalization studies have not been previously reported. We found that diffuse staining for follistatin was evenly distributed in hepatocyte cytoplasm (Fig. 1E) and, in contrast to activin A, did not redistribute to fibrotic septae (Fig. 1F).

Activin, but not follistatin, gene expression is upregulated in HSCs. To investigate the apparent dysregulation of activin A and follistatin highlighted by the immunohistochemical analyses, HSCs were isolated from the normal liver and activated in vitro. Typically, isolated cell populations were >90% pure HSCs, expressed HSC markers such as desmin, and were negative for von Willebrand factor (endothelial cells) and ED1 (Kupffer cells). Although freshly isolated HSCs did not express α-SMA, this activation marker was expressed abundantly after 3–5 days in culture (data not shown).

Activin A production increased as HSCs transformed to a myofibroblast phenotype (Fig. 3A) associated with increases in α-SMA expression (Fig. 3, B and C) and collagen production (Fig. 3D). We next explored changes at the mRNA level. Real-time PCR analysis of activating HSCs revealed that activin, follistatin, TGF-β1, and type I collagen mRNA all significantly increased 1 day after culture (P < 0.01), but activin mRNA increased proportionally more than other transcripts (Fig. 4). Expression of all mRNAs continued to increase as HSCs activated with the exception of follistatin, which peaked at day 1 of culture and returned to baseline by day 5.

Follistatin inhibits HSC proliferation in vitro. Because proliferating HSCs produced increasing amounts of activin A during activation without a concurrent increase in follistatin gene expression, we analyzed the effect of exogenous follistatin on proliferation of freshly isolated and activated HSCs. We observed a moderate dose-dependent decrease in the proliferation of both freshly isolated and culture-activated HSCs (Fig. 5).
A) that was greater in freshly isolated HSCs compared with cells with an activated myofibroblast-like phenotype (Fig. 5B).

**Attenuation of hepatic fibrogenesis by exogenous follistatin.** Because the above observations supported a role for dysregulation of the activin-follistatin axis during hepatic fibrogenesis, we then administered follistatin during CCl4-induced hepatic fibrosis. Follistatin-treated animals had significantly lower hepatic hydroxyproline content 2 and 4 wk after CCl4 treatment, reaching a maximal decrease of 32% at week 2. This trend continued to week 8 (Fig. 6). Histological fibrosis was clearly attenuated in follistatin-treated animals at early time points, although by week 8 most (70%) animals demonstrated establishment of fibrosis (Fig. 7). Quantification of activated HSCs

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**Fig. 2.** Co-localization of activin with α-smooth muscle actin (α-SMA). Liver sections from animals receiving 2 wk of CCl4 showed hepatic stellate cell (HSC) staining for α-SMA (A; green), a HSC activation marker, and activin A (B; red). C: confocal microscopy showed double staining (yellow), confirming that activated HSCs express activin. White arrows indicate representative colocalization. Control staining of identical sections with inappropriate primary antibody was invariably negative (not shown). Original magnification: ×50.

**Fig. 3.** Isolated HSCs produce increasing quantities of activin A as they transform to a myofibroblast phenotype. A: freshly isolated HSCs were cultured in standard conditions, and conditioned medium was analyzed for activin A by ELISA. B: expression of α-SMA by cultured rat HSCs was measured by densitometry, and the ratio of α-SMA to β-tubulin was determined. Values are expressed in arbitrary units. C: representative Western blot of α-SMA expression by freshly isolated cultured rat HSCs. β-Tubulin was measured as an internal control. D: collagen production was measured in freshly isolated cultured rat HSCs using a colorimetric assay as outlined in METHODS. All data are means ± SE of 3 separate HSC isolates.
was consistent and revealed significantly lower numbers of activated HSC at weeks 2 and 8 in follistatin-treated animals compared with untreated controls (Fig. 8).

Reduction of hepatocyte apoptosis. Apoptotic events may trigger inflammation and fibrosis (2), and, because activin is a potent inducer of hepatocyte apoptosis, we compared the extent of hepatocyte apoptosis in follistatin-treated animals with untreated controls. We found that hepatocyte apoptosis was significantly decreased by follistatin treatment at all time points and maximal by 87% at week 4 (Fig. 9). A typical example of hepatocyte apoptosis is shown in Fig. 1G.

DISCUSSION

Activation of quiescent HSCs to a profibrogenic phenotype is a critical event in the pathogenesis of hepatic fibrosis, and TGF-β is the prototypical driver of this process. We investigated the role of activin A, a member of the TGF-β superfamily, in HSC activation and fibrogenic activity. Activin is a cytokine that regulates hepatic homeostasis and mass (21), an effect antagonized by follistatin, the major activin binding protein. This interaction between activin A and follistatin is typical of most biological systems in which expression of the two molecules is tightly controlled with circulating activin irreversibly bound to follistatin and neutralized (32), such that a biological effect occurs only when activin is unopposed by follistatin (27). Previous reports (5, 34) have shown that activin A expression is upregulated in hepatocellular fibrosis. In this study, we extended these findings by investigating activin A expression during HSC activation and by determining whether inhibition of activin by exogenous follistatin administration could ameliorate activin-induced hepatic fibrosis. We found that activin, but not follistatin, gene expression is upregulated in isolated rat HSCs and that HSCs secrete activin in significant quantities during myofibroblast transformation. Furthermore, analysis of the cellular expression of activin in an animal model of CCl4-induced liver injury revealed that activin A localized to areas of periscar hepatocytes and activated HSCs.

The demonstration that exogenous follistatin attenuated early fibrogenic events further supports the biological significance of activin A during the development of hepatic fibrosis.

A striking and consistent feature in this and other reports (5, 34) has been the redistribution of activin A immunoreactivity...
from the hepatic lobule to fibrous septae. We have now shown for the first time that this redistribution is unaccompanied by changes in follistatin localization. In contrast to Sugiyama and colleagues (34), who were unable to demonstrate activin A immunoreactivity in the normal liver, we detected significant hepatocyte staining. The differences in activin A immunolocalisation may reflect different antibodies for the activin A-β subunit used in each study. The E4 monoclonal antibody used for immunohistochemical localization in this study has been extensively validated (17, 23, 24). We also observed increased hepatocyte apoptosis in the fibrotic liver, although without a specific cellular distribution pattern, and no correlation was detected between activin A immunoreactivity and the septal location of apoptotic hepatocytes, as reported previously (25).

We found that during culture activation, HSCs secreted activin A protein due to increased activin mRNA expression and that follistatin mRNA expression did not increase above basal levels, consistent with the finding that HSC-derived activin was biologically active and unopposed by follistatin. In the fibrotic liver, α-SMA-positive HSCs strongly expressed activin immunoreactivity. HSC-derived activin may have a number of local consequences, consistent with the concept that activin and follistatin act primarily in a paracrine or autocrine manner (37). Previous studies have reported that activin may stimulate the production of matrix proteins such as collagen (36) and fibronectin (4), which are produced early in HSC activation (1). Furthermore, activin A increases the expression of type I collagen mRNA in HSC cultures either alone or in synergy with TGF-β (34). We found that activin mRNA expression in HSCs increased earlier than that of type I collagen, supporting the view that activin acts in combination with TGF-β to stimulate matrix production. It is also worth noting that, whereas TGF-β is secreted as a pro-protein requiring enzymatic cleavage, activin A is secreted as an active molecule (28), consistent with the view that activin A may be an important early mediator of hepatic fibrosis.

Administration of follistatin, the most potent biological inhibitor of activin, resulted in a significant reduction in hepatic hydroxyproline content and histological fibrosis. Three interrelated mechanisms may explain these findings. First, administration of exogenous follistatin may neutralize activin-induced events. In a recent study, Wada et al. (36) studied the effect of activin A on primary cultures of rat HSCs and demonstrated that activin was able to increase HSC α-SMA expression in vitro. We have extended these findings to an in vivo model by demonstrating significantly fewer activated HSCs within the hepatic lobule in follistatin-treated animals.

Fig. 8. Quantitative computer-assisted morphometric analysis of immunofluorescent staining of activated HSCs (α-SMA-positive cells) in follistatin-treated animals compared with controls. Analysis revealed significantly fewer α-SMA-positive cells in rh-FS288-treated animals compared with controls at 2 and 8 wk (P = 0.04).

Fig. 9. Administration of rh-FS288 to CCl4-treated animals significantly reduced hepatocyte apoptosis at weeks 2, 4 (P = 0.02), and 8 (P = 0.03) compared with controls given CCl4 alone.
Thus follistatin binding to HSC-derived activin may diminish autocrine activation of HSCs, and this is consistent with our observation that follistatin administration caused a dose-dependent decrease in HSC proliferation. The concept that follistatin neutralizes activin-induced events is not restricted to the liver. A recent report (6) found that local and systemic upregulation of activin occurred in different models of colonic inflammation and that exogenous follistatin administration significantly improved disease outcome with increased survival of treated animals.

A second potential mechanism for follistatin reduction in hepatic fibrosis may relate to the prevention of hepatocyte apoptosis. Activin has previously been shown to induce hepatocyte apoptosis both in vitro and in vivo when present in excess quantities (12, 33). We found that follistatin administration significantly decreased hepatocyte apoptosis in CCl4-treated animals. The mechanistic importance of this observation arises from the observation that hepatocyte apoptosis is linked to hepatic fibrosis progression due to cholestasis and alcoholic and nonalcoholic steatohepatitis (2). A recent study (35) has confirmed this important nexus by showing that disruption of the Bcl-XL gene, an antiapoptotic Bcl-2-family member, caused ongoing hepatocyte apoptosis and the development of progressive hepatic fibrosis. With regard to activin, it has been previously demonstrated that Bcl-XL upregulation inhibited activin-induced apoptosis in a mouse hybridoma model (22). Thus inhibition of activin-induced hepatocyte apoptosis by follistatin may also contribute to the observed decrease in hepatic fibrosis in this study.

Finally, Date and colleagues (4) have proposed that activin A released from injured hepatocytes interacts with activin receptors present on HSCs to induce fibrogenic synthesis. Administration of follistatin may bind activin, preventing interaction with HSC activin receptors and preventing downstream signaling events leading to extracellular matrix synthesis. This mechanism is supported by previous findings that showed decreased HSC collagen synthesis in vitro (36) and by our findings demonstrating decreased deposition of the extracellular matrix in vivo after follistatin administration. Thus several lines of evidence exist to explain how follistatin can inhibit activin A-mediated hepatic fibrogenesis.

We recognize that there is variability among some of the experimental outcomes regarding fibrosis in follistatin-treated animals, such as the morphometric data in Fig. 7, which shows less attenuation of fibrosis at week 8 compared with hydroxyproline content (Fig. 6) and the decrease in the proportion of activated HSCs (Fig. 8). These differences in outcome may reflect the use of a constant dose of follistatin rather than one corrected for body weight in growing animals, or a differential time course for morphological alteration that lags behind the decline in HSC activation and collagen production. However, a more likely explanation lies in the multifactorial nature of hepatic fibrogenesis, which involves multiple mediators such that inhibition of one factor is unlikely to prevent hepatic fibrosis completely. For example, inhibition of TGF-β, the prototypical profibrogenic cytokine, does not completely abrogate fibrosis (10, 14, 29).

In conclusion, activin A expression is markedly altered during the progression of hepatic fibrosis with changes in both the cellular site of expression and in follistatin regulation. Administration of follistatin significantly attenuated early hepatic fibrogenesis. We propose that follistatin attenuated hepatic fibrosis by reducing activin-induced activation of HSCs and therefore extracellular matrix accumulation in addition to inhibiting activin-mediated hepatocyte apoptosis. Further studies are required to define the relative contribution of these mechanisms to hepatic inflammation and wound repair.

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