

Activin A and follistatin in acute liver failure

Robin D. Hughes^a and Lee W. Evans^b

Background Liver regeneration may be impaired in acute liver failure due to either inhibition of the proliferative response or ongoing liver cell death. Activin A, a member of the TGF β superfamily, inhibits hepatocyte DNA synthesis and induces apoptosis.

Methods Levels of activin A and its binding protein follistatin in the serum of 23 patients with acute liver failure were determined by enzyme-linked immunosorbent assay.

Results Serum activin A was significantly increased in acute liver failure patients (median 2.15 ng/ml, range 0.28–6.87 ng/ml) compared to normal controls (median 0.25 ng/ml, range 0.19–0.53 ng/ml; $n = 10$; $P < 0.001$). However, this was not linked to the final disease outcome. Higher levels of activin A were found in the serum of patients with acute liver failure due to paracetamol overdose (median 2.87 ng/ml, range 0.72–6.87 ng/ml; $n = 17$) than in patients with acute liver failure due to non-A to E hepatitis (median 1.10 ng/ml, range 0.28–2.70 ng/ml; $n = 6$; $P < 0.05$). Serum follistatin was also increased in

acute liver failure patients (median 2.84 ng/ml, range 0.57–13.24 ng/ml) compared to normal controls (median 0.68 ng/ml, range 0.32–3.70 ng/ml; $P < 0.01$).

Conclusion Serum activin A is increased in acute liver failure and could be a factor in the inhibition of liver regeneration. *Eur J Gastroenterol Hepatol* 15:127–131 © 2003 Lippincott Williams & Wilkins

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Introduction

Liver regeneration is essential for recovery from acute liver failure (ALF). In some aetiological sub-groups, e.g. non-A, non-B hepatitis, a poor prognosis has been associated with a lack of liver regeneration [1]. A high level of hepatocyte growth factor (HGF) in the blood indicates that stimulatory activity is not reduced in such patients. This has led to the suggestion that inhibitors of liver regeneration are present in the circulation of these patients. Increased blood levels of transforming growth factor (TGF)- β_1 , an endogenous inhibitor of hepatocyte DNA synthesis, were found in ALF patients [2]. Activin A is a pluripotent 24 kDa homodimeric member of the TGF β superfamily consisting of two inhibin- β_A subunits. It is produced by hepatocytes, inhibits hepatocyte DNA synthesis [3] and induces apoptosis [4,5]. Although the biological functions of activin and TGF- β_1 are similar, it has been shown that they act differently and bind to different cell membrane receptors. Follistatin, a monomeric glycoprotein structurally unrelated to activins, is known to antagonize the biological action of activin by binding to it in a stoichiometric manner [6,7]. These three cytokines interact with each other [8].

The relationship between TGF- β_1 , activin and follistatin in patients with liver failure has not been studied previously. The aim of this study was to determine the

concentrations of activin A and its binding protein follistatin in relation to TGF- β_1 in the serum of patients with ALF, and to assess their possible inhibitory effects on liver regeneration.

Patients and methods

We studied 23 patients (12 men and 11 women) with ALF (Table 1). In 17 of these patients, liver failure was due to paracetamol overdose and in six it was due to non-A to E hepatitis. Eleven patients survived, six underwent liver transplantation and six died. Ten normal subjects were studied as controls.

Blood samples were collected soon after admission. We stored serum for activin A and follistatin or ethylenediamine tetraacetic acid (EDTA) plasma for TGF- β_1 at -70°C until the assay. In 11 patients (nine with paracetamol overdose and two with non-A to E hepatitis), samples were taken daily for 4 days.

The procedures used were approved by King's College Hospital Research Ethics Committee. Informed consent was obtained from the patients or their relatives.

Activin A assay

The assay for the measurement of activin A was adapted from that of Knight *et al.* [9]. A Nunc Maxisorp

Table 1 Clinical details and liver function tests of patients with ALF (n = 23)

	Age (years)	Coma grade	International normalized ratio	Aspartate aminotransferase (IU/l)	Bilirubin ($\mu\text{mol/l}$)
Median	30	II	7.6	4528	132
Range	14–60	0–IV	2.5–15	80–10 200	37–719

(Invitrogen Ltd, Paisley, UK) plate was coated with E4, a monoclonal antibody raised to a synthetic peptide, which corresponded to a region of the activin β_A -subunit. Test samples were diluted in 5% (weight/volume) bovine serum albumin (BSA)/phosphate-buffered saline (PBS); an equal volume of sodium dodecyl sulphate (10% weight/volume) was then added. Triton assay diluent (25 μl 0.1 M tris-buffered saline containing 10% weight/volume BSA, 5% volume/volume Triton X-100 and 0.1% weight/volume sodium azide) was added to each well. We used bovine follicular fluid calibrated against rh-activin A as the standard. Duplicate 100 μl /well standard/samples were added, followed by 25 μl diluted biotinylated E4. The plate was incubated overnight in a sealed, moist box. The following day the plate was washed and 50 μl streptavidin alkaline phosphatase conjugate was added per well. After incubation for 1 h, the plate was washed and 50 μl alkaline phosphatase substrate solution (ELISA Amplification System, Life Technologies Ltd, Paisley, UK) was added to each well, followed by a further 50 μl /well amplifier solution after 1 h. The absorbances were read at 490 nm.

Follistatin assay

The assay for the measurement of follistatin was as described previously [10]. A Nunc Maxisorp plate was coated with 29/9, a monoclonal antibody raised to recombinant human FS288. Test samples were diluted in dissociating buffer (3.5 g sodium deoxycholate, 3.4 g Tween 20, 1 g BSA, 5 ml mouse serum and 100 ml PBS) to break up the follistatin–activin complexes. The standard used in the follistatin assay was immunopurified human follistatin from ovarian follicular fluid, calibrated against rh-FS288.

Duplicate 50 μl /well standard/samples were added to the plate, which was then incubated overnight in a sealed moist box. The plate was washed and 50 μl diluted Fab fragment of clone 17/2, also raised to human FS288, coupled with alkaline phosphatase was added to each well and incubated for 2 h. The plate was washed and the alkaline phosphatase substrate solution (ELISA Amplification System, Life Technologies Ltd) was added, followed after 2 h by the amplification solution from the kit. The absorbances were read at 490 nm.

TGF- β_1 assay

The total TGF- β_1 was measured after acid activation of the plasma samples, as the majority of TGF- β_1 is produced in a latent form [11]. The acid activation and enzyme-linked immunosorbent assay (ELISA) were performed according to the manufacturer's instructions for the kit (Promega, Southampton, UK). Briefly, for acid activation, 50 μl , 5 \times diluted samples of plasma in PBS were acidified with 1 μl 1N HCl and then neutralized by 1 μl 1N NaOH. Dynatech Immulon-4 (Dynex Technologies, Ashford, Middlesex, UK) plates were coated with anti-TGF- β_1 monoclonal antibody. Duplicates of the test and standard samples (100 μl /well), diluted ($\times 33$) by the sample buffer provided by the manufacturer were incubated on the plate. Primary anti-TGF- β_1 antibody (10 μl) was added, followed by antibody–peroxidase enzyme conjugate (10 μl). Finally, the level of TGF- β_1 was determined using a microplate reader (Dynatech MRX) from the absorbance at 450 nm.

Thymidine incorporation in HepG2 cells

HepG2 cells (ECACC, Porton Down, Salisbury, UK) were plated out into 96-well tissue culture plates (Nunc/D) to give approximately 30 000 cells per well. Human serum was added (eight replicate wells) at a final concentration of 10% in the culture medium, with medium-only controls on each plate for reference. The tissue culture medium used was Dulbecco modified Eagle medium (DMEM), containing 10% volume/volume fetal calf serum (FCS), Hepes buffer (0.7% volume/volume), glutamine (0.005 mg/ml) and the antimicrobials streptomycin (0.1 mg/ml), penicillin (100 U/ml) and amphotericin B (250 $\mu\text{g/ml}$), all obtained from Life Technologies Ltd, Paisley, UK. The plates were incubated for 24 h at 37°C in 5% CO₂/95% air. The media were replaced with fresh medium containing [6-³H]thymidine (925 GBq/mmol; 37 MBq/ml, Amersham International plc, Amersham, Bucks, UK) to give a concentration of 0.5 μCi per well. The plate was incubated for 4 h and the cells harvested (FilterMate, Packard Instruments, Pangbourne, Berkshire, UK) onto a glass fibre membrane. The filters were counted (MATRIX 9600, Packard Instruments) to determine the incorporation of [³H]thymidine.

Statistics

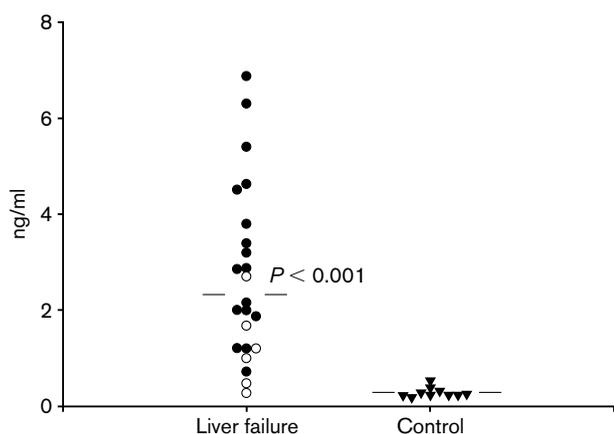
The statistical significance of the results was determined using the Mann–Whitney *U* Test and the Pearson correlation test.

Results

Activin A

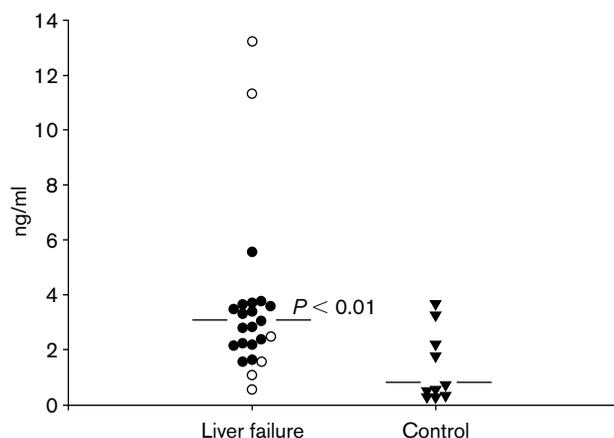
Activin A was significantly increased in the serum of patients with ALF (median 2.15 ng/ml, range 0.28–6.87 ng/ml; *n* = 23) compared to normal controls (median 0.25 ng/ml, range 0.19–0.53 ng/ml; *n* = 10; *P* <

Fig. 1



Serum levels of activin A in patients with acute liver failure. Closed circles, paracetamol overdose; open circles, non-A to E hepatitis; inverted triangles, normal control subjects; horizontal bar, median value. There was a significant difference between paracetamol overdose and non-A to E hepatitis ($P < 0.05$).

Fig. 2



Serum levels of follistatin in patients with acute liver failure. Closed circles, paracetamol overdose; open circles, non-A to E hepatitis; inverted triangles, normal control subjects; horizontal bar, median value.

0.001; Fig. 1). Higher levels of activin A were found in the serum of patients with ALF due to paracetamol overdose (median 2.87 ng/ml, range 0.72–6.87 ng/ml; $n = 17$) than in sera from non-A to E hepatitis patients (median 1.10 ng/ml, range 0.28–2.70 ng/ml; $n = 6$; $P < 0.05$). Serial measurements of activin A in these patients ($n = 11$) showed no significant changes over 3 days from admission: day 1 values were median 2.15 ng/ml, range 0.48–6.30 ng/ml; day 3 values were median 2.40 ng/ml, range 0.75–7.50 ng/ml. There was no significant relationship between serum activin A and

the outcome of the disease. Ten of the 17 patients with paracetamol overdose (59%) and one of the six non-A to E hepatitis patients (17%) survived without liver transplantation.

Follistatin

Serum follistatin was increased in ALF patients (median 2.84 ng/ml, range 0.57–13.24 ng/ml) compared to normal controls (median 0.68 ng/ml, range 0.32–3.70 ng/ml; $P < 0.01$; Fig. 2). There was no difference in the levels of serum follistatin between ALF patients with paracetamol overdose and those with non-A to E hepatitis. There were no significant changes in serum follistatin over the first 3 days after admission: day 1 values were median 3.31 ng/ml, range 1.10–13.24 ng/ml; day 3 values were median 2.78 ng/ml, range 1.79–5.02 ng/ml. There was no significant relationship between serum follistatin and the outcome of the disease.

There was a significantly higher activin A to follistatin ratio in ALF patients (median 0.77, range 0.04–2.05) compared to controls (median 0.35, range 0.07–0.89; $P < 0.01$).

TGF- β_1

Plasma TGF- β_1 was increased in ALF patients (median 37.8 ng/ml, range 13.7–108 ng/ml) compared to normal controls (median 23.0 ng/ml, range 8.5–34.9 ng/ml; $P < 0.01$). Significantly higher plasma levels of TGF- β_1 were found in ALF patients with non-A to E hepatitis (median 77.7 ng/ml, range 38.8–108 ng/ml) compared to those with paracetamol overdose (median 36.5 ng/ml, range 13.7–67.3 ng/ml; $P = 0.001$). There was no relationship between plasma TGF- β_1 levels and the outcome of ALF.

Thymidine incorporation into HepG2 cells

HepG2 cells exposed to ALF serum incorporated significantly less [^3H]thymidine (median 37.5% of normal control, range 0.2–169%) than those exposed to normal serum (median 100%, range 76–133%; $P < 0.05$). A greater inhibitory effect was observed with serum from ALF patients with paracetamol overdose (median 18.8% of normal control, range 6.50–169%) than with serum from ALF patients with non-A to E hepatitis (median 83.2% of normal control, range 0.2–113%; $P = 0.03$).

Correlations between parameters and liver function tests

There was no significant correlation between the serum levels of activin A and follistatin or activin A and plasma TGF- β_1 . In relation to liver function tests, serum levels of activin A correlated positively with the serum aspartate aminotransferase (AST) ($r = 0.603$; $P = 0.002$) and negatively with the serum bilirubin ($r = -0.546$; $P = 0.007$). There was no correlation between follistatin and liver function tests.

Discussion

This study has shown that serum levels of activin A are considerably increased in patients with ALF, particularly when it is caused by paracetamol overdose. As activin A was lower in non-A to E hepatitis patients and these patients have a worse prognosis [12], the hypothesis that liver regeneration is inhibited by activin A is not supported. However, only a small number of patients were studied. It is possible that, in non-A to E hepatitis patients, ongoing liver injury is more important than a lack of liver regeneration. Ongoing liver injury may be due to continued liver cell death as a result of necrosis or apoptosis. Apoptosis in hepatocytes could involve the effects of activin A [5].

We performed the assays on blood samples taken soon after admission and thus the lack of correlation between the cytokines measured and disease outcome is unsurprising, as many factors influence the course of ALF. Increased serum levels of activin A were recently reported in patients with chronic viral hepatitis due to hepatitis B and, to a lesser extent, hepatitis C, although not to the extent found in ALF [13]. This may reflect the much greater liver damage in ALF. No significant increase in follistatin was detected in chronic viral hepatitis. In another study from our laboratory, increased serum levels of both activin A and follistatin were found in patients with hepatocellular carcinoma and alcoholic cirrhosis, but again the increases were not as great as in ALF [14].

The sera of the ALF patients studied had inhibitory effects on DNA synthesis in HepG2 cells *in vitro*. Greater inhibition was observed with serum from patients with ALF due to paracetamol overdose; this serum had higher levels of activin A. The inhibitory potential of ALF serum on hepatocytes has been reported previously [15,16], but this could be due to factors other than activin A. As with hepatocytes, activin A has been shown to inhibit proliferation of HepG2 cells [17] as well as inducing apoptotic cell death [18]. However, as HepG2 cells are continuously dividing tumour cells, this may not be a good *in-vitro* model for liver regeneration in normal liver. Further experiments are required to determine whether the inhibitory effects found with ALF serum are directly related to activin A. This could be done using a blocking antibody to activin A to determine whether this reduces the inhibitory activity of the serum. Other evidence for the presence of substances inhibitory to liver regeneration in ALF patients has been obtained in experiments with animals both *in vivo* and *in vitro*. Serum from patients with ALF was shown to reduce hepatic DNA synthesis when administered to partially hepatectomized rats [19] and was shown to reduce DNA synthesis in primary rat hepatocytes *in vitro* [20]. In addition, TGF- β_1 , which is also an inhibitor of

hepatocyte DNA synthesis, was increased to a small extent in ALF plasma. Levels were higher in ALF patients with non-A to E hepatitis than in those with paracetamol overdose, as found previously [2]. It is possible that this, in combination with increased activin A, could add to the inhibitory potential of the sera; such an effect has recently been suggested from experiments on rat livers [21].

The serum levels of follistatin were also increased in patients with ALF, but not to the same extent as with activin A. This is reflected in the significant increase in the ratio of activin to follistatin that, when estimated as a molar ratio, exceeded 1:1 in half of the sera studied. In patients with hepatocellular carcinoma and alcoholic cirrhosis, both cytokines were increased to a similar extent [14]. Follistatin binds to activin and, as a result, blocks its inhibitory effects on DNA synthesis; therefore, the relative lack of follistatin in ALF may be detrimental. Administration of follistatin to rats after partial hepatectomy has been shown to stimulate liver regeneration [22] and it is possible that administration of recombinant follistatin would be beneficial in patients with ALF to overcome the inhibitory effects of the increased levels of activin.

The serum levels of activin A were greatest in ALF patients with paracetamol overdose, where liver injury tends to be greater and occurs more rapidly. This is reflected in the correlation between activin A levels and serum AST, suggesting that the source of activin may be damaged hepatocytes. This was not the case with follistatin, which is considered to be mainly released from vascular endothelial cells [23]. There is evidence that both activin and follistatin are involved in the systemic inflammatory response syndrome (SIRS) together with other cytokines [24]. SIRS is frequently observed in ALF [25], where increased cytokine production, particularly of TNF- α [26] and IL-6 [27], leads to multi-organ failure. In preliminary unpublished observations on the samples used in the present study, the increased activin A in ALF patients correlated with plasma IL-6 but not plasma TNF- α , providing support for the involvement of activin in SIRS. However, in a study of patients with acute *Salmonella* infection, levels of follistatin rather than activin A were increased [24], which differs markedly from patients with ALF. Further studies will help to clarify the relationship between and roles of activin A and follistatin to the inflammatory cytokine response in ALF and their involvement in liver injury and regeneration.

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