β A- and β C-activin, follistatin, activin receptor mRNA and β C-activin peptide expression during rat liver regeneration

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Abstract

The mRNA expression of two activin growth factor subunits (β A- and β C-activin), activin receptor subunits (ActRIIA, ActRIIB) and the activin-binding protein follistatin, and peptide expression of β A-activin and β C-activin subunits, were examined in regenerating rat liver after partial hepatectomy (PHx). Liver samples were collected from adult, male Sprague–Dawley rats, 12–240 h ($n=3$ –5 rats per time point) after PHx or from sham-operated controls at the same time points. Hepatocyte mitosis and apoptosis were assessed histologically and by *in situ* cell death detection. RT and PCR were used to assess relative gene expression. β A- and β C-activin peptide immunoreactivity was assessed in liver and serum samples by western blotting, whereas cellular expression was investigated by immunohistochemistry, using specific monoclonal antibodies. β A- and β C-activin mRNA dropped to < 50% of sham control values 12 h after PHx and remained at this level until 168 h post-PHx, when β A-activin expression increased to three times sham control values and β C-activin mRNA returned to pre-PHx levels. A peak in follistatin expression was observed 24–48 h post-PHx, coincident with an increase in hepatocyte mitosis. No changes were observed in ActRIIA mRNA, whereas ActRIIB expression paralleled that of β A-activin mRNA. β C-activin immunoreactive homo- and heterodimers were observed in regenerating liver and serum. Mitotic hepatocytes frequently contained β C-activin immunoreactivity, whereas apoptotic hepatocytes were often immunoreactive for β A-activin. We conclude that β A- and β C-activin subunit proteins are autocrine growth regulators in regenerating liver and when expressed independently lead to hepatocyte apoptosis or mitosis in a subset of hepatocytes.

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Introduction

Cell proliferation and cell death are subject to control by stimulatory and inhibitory factors. Simplistically, tropic factors will stimulate mitosis and at the same time inhibit cell death, while negative growth signals will inhibit mitosis and cause apoptosis. In contrast to the many heterotropic factors that have been identified, very few growth inhibitors or apoptosis inducers have been characterised in the liver (Fausto *et al.* 1995). The activins belong to the transforming growth factor- (TGF- β) gene family. Members of this family share structural similarities, but are diverse in their biological activities (Kingsley 1994). Activins are hetero- or homodimers of inhibin β -subunits held together by disulphide bonds (Ying 1988). Activins control cell proliferation and programmed cell death in a variety of tissues including the liver (Schwall *et al.* 1993, Yasuda *et al.* 1993, Hully *et al.* 1994). Activin A is a homodimer of the inhibin β A-activin subunit. It is expressed in

hepatocytes and has been shown to be a negative regulator of hepatocyte cell growth (Schwall *et al.* 1993, Yasuda *et al.* 1993, Hully *et al.* 1994). In the normal liver the expression of activin A is relatively low (Yasuda *et al.* 1993). Activin A is involved in the maintenance of constant liver mass, since blocking activin A action by administering the activin-binding protein follistatin leads to hepatocyte proliferation (Kogure *et al.* 1995, 1996, 1998, 2000, Kozian *et al.* 1997, Zhang *et al.* 1997a). Activin A is also up-regulated in the development of liver cirrhosis concomitant with an increase in hepatocyte apoptosis (Gold *et al.* 2003).

β C-activin mRNA appears to be predominately expressed in the liver (Hotten *et al.* 1995, Lau *et al.* 1996, 2000, Schmitt *et al.* 1996, Gold *et al.* 2004) and is down-regulated 12 h after partial hepatectomy (PHx) in the rat, suggesting that it too may be a negative regulator of liver cell growth (Esquela *et al.* 1997, Zhang *et al.* 1997b). Two recent studies show that β C-activin

inhibits DNA synthesis of hepatic cells *in vivo* (Chabicovsky *et al.* 2003) and induces apoptosis in hepatoma cells *in vitro* (Vejda *et al.* 2003). Stimulation of hepatocyte proliferation by administration of di-*n*-butyl phthalate was accompanied by a rise in β B- and β C-activin mRNA and a decrease in β A-activin mRNA (Kobayashi *et al.* 2002). The β C-activin subunit can form heterodimers with the β A-, β B- and β E-activin subunits *in vitro* (Mellor *et al.* 2000, 2003, Vejda *et al.* 2002) and acts at the intra- cellular level to modulate the synthesis of activin A or activin B via the formation of activin AC or BC heterodimers (Mellor *et al.* 2000, 2003, Kobayashi *et al.* 2002). Recent research demonstrates that β C-activin homodimer can be growth promoting and confirms that intracellular AC dimerisation prevents the formation of activin A (Wada *et al.* 2004). Thus β C-activin has been shown to be either growth inhibitory (Esquela *et al.* 1997, Zhang *et al.* 1997b) or growth promoting (Kobayashi *et al.* 2002, Wada *et al.* 2004) in liver. A balance between cell proliferation and apoptosis is crucial for regulating normal liver function (Chen *et al.* 2000), since abnormalities in liver regeneration may contribute to chronic hepatitis, cirrhosis and cancer (Leevy 1998). Cellular control of activin expression appears to occur at many levels, including the amount of growth factor, dimer combinations formed, the presence of binding proteins, receptor recruitment and presence of other peptides (Phillips 2000). The presence of β -activin subunits, follistatin and activin receptor mRNAs, and the ability of these proteins to affect hepatic cell division, implies that these growth factors may be involved in the liver's regenerative response. In order to examine the ability of the β C-activin subunit to antagonise activin A action *in vivo*, we examined hepatocyte cell death and division after PHx in the liver of male rats and assessed mRNA expression of β A-activin, β C-activin, follistatin, and activin receptor subunits ActRIIA and ActRIIB; and also β A- and β C-activin subunit dimer formation. We hypothesised that β C-activin expression is associated both cellularly and temporally with β A-activin expression, at times of liver growth. If this hypothesis is correct we would predict co-localisation of the two activin subunits in mitotic or resting hepatocytes. Furthermore we predict β A-activin expression to be high, and the expression of antagonists of activin A (β C-activin and follistatin) to be decreased at times of maximal hepatocyte apoptosis.

Methods

Ethical approval

All experiments using animals were performed in accordance with the 1999 Animal Welfare Act regulations of New Zealand after approval was granted

by the University of Otago Medical School Animal Ethics Committee.

Surgery

Male (220–250 g) Sprague–Dawley rats were randomly allocated to a PHx or control group. PHx was performed under ether anaesthesia according to a previously described method (Higgins & Anderson 1932). Control sham operations consisted of opening the abdominal cavity and briefly handling the liver. Two sham-operated control animals were killed at 12, 24, 48, 72, 96, 168 and 240 h post-sham PHx. Five PHx rats were killed at 12, 48 and 168 h; while three PHx rats were killed at 24, 72, 96 and 240 h post-PHx. Samples of caudate lobe from regenerating or sham control liver samples were removed and either immediately stored at -80°C or prepared for histology. The caudate lobe is not affected by the surgery, but does participate in the restoration of liver mass (Higgins & Anderson 1932).

Histology

Samples were fixed in 4% paraformaldehyde and processed to 4 μm thick sections. Serial sections were used for immunohistochemistry, cell death detection, and haematoxylin and eosin staining for histology. Hepatocyte cell death was assessed with an *in situ* cell death detection kit (TUNEL, Roche) and was confirmed by counting apoptotic bodies in serial sections, as previously described (Gold *et al.* 2003). Mitotic hepatocytes were identified based on histology (prophase, metaphase, anaphase or telophase). Within sections, the percentage of mitotic hepatocytes or TUNEL-positive hepatocytes were counted. Five randomly selected fields were assessed in duplicate sections, in three to five animals per time point and the results are presented as means $\pm$ S.D.

Molecular biology

Total RNA was extracted from liver using a RNeasy Mini Kit (Qiagen) according to manufacturer directions. RNA concentration was determined by spectrophotometry at 260 nm. An equal amount of RNA from each tissue was used for generation of first-strand cDNA. Primers for PCR were designed based on published rat sequences, or from consensus sequence of human and mouse β C-activin cDNA (Gold *et al.* 2003). All primers were designed so that the amplicon was derived from two or more exons. Preliminary PCR assays were performed to determine the optimum annealing temperature, MgCl_2 concentration and range of cycles over which the amplified cDNA samples remained in the exponential phase of amplification (Gold *et al.* 2003). Reactions without template, genomic DNA and RNA

controls, were run alongside all experimental samples. PCR products were separated on agarose gel, photographed and semi-quantified by densitometry as previously described (Gold *et al.* 2003). The density of amplified product for each sample was normalised with respect to the density of β -actin mRNA. To confirm amplicon identity, PCR products from two separate reactions were sequenced in both directions using the direct-cycle method, by the University of Otago Centre for Gene Research sequencing facility.

Immunoblotting

The identity of activin dimers present in tissue extracts and serum was determined by western immunoblotting. Protein samples were prepared for electrophoresis by homogenising 50 mg tissue in 500 μ l non-reducing sample buffer (4% (w/v) SDS, 2 mM EDTA, 50 mM Tris-HCl), heating to 100 °C for 5 min and centrifuging at 10 000 *g* for 10 min. Total protein concentration was determined with a Sigma BCA kit (Sigma-Aldrich). Equal amounts of protein (2 mg) were loaded onto 15% polyacrylamide gels and separated by electrophoresis under non-reducing conditions. The proteins were transferred to polyvinylidene difluoride membrane with a reducing transfer buffer (700 mM glycine, 300 mM Tris, 15.6% ethanol, 15 mM dithiothreitol). Antibodies were diluted [β A-activin 1:2000 (R&D Systems, Inc., Minneapolis, MN, USA) and β C-activin 1:3000 (Mellor *et al.* 2000)], incubated overnight at 4 °C in Tris-buffered saline (TBS)–5% milk powder. Biotinylated goat anti-mouse secondary antibody (Amersham) was diluted 1:3000 in TBS and blots incubated for 1 h at room temperature, followed by streptavidin biotinylated horseradish peroxidase (Amersham) at a dilution of 1:1500. Blots were developed with the ECL chemiluminescent detection system (Amersham) according to manufacturer's directions. Three representative liver and serum samples per time point were assessed in duplicate and results from each were consistent. Duplicate samples were run at the same time on two separate gels. Gels were incubated with either β A- or β C-activin antibodies, exposed to X-ray film, stripped and re-probed with the alternative activin antibody. When films were overlaid, each activin antibody was shown to detect different-sized monomer peptides. This also confirmed the presence or absence of a β A β C activin dimer.

Immunohistochemistry and staining indices

Paraffin-embedded tissue blocks were sectioned and deparaffinised by standard techniques. Sections underwent microwave heat antigen retrieval submerged in 0.01 M glycine buffer, pH 4.4 (Mellor *et al.* 2000). After

cooling to room temperature, endogenous peroxidase activity was eliminated with hydrogen peroxidase. β C-activin antibody, diluted to a final concentration of 5 μ g/ml in PBS, was added. Sections were incubated overnight at 4 °C, washed in PBS containing 0.1% (v/v) Tween-20 (PBS-T) and incubated with biotinylated goat anti-mouse secondary antibody (Amersham). Sections were washed with PBS-T and incubated with streptavidin biotinylated horseradish peroxidase (Amersham). Peroxidase activity was detected using 3'3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories Inc, Peterborough, UK), before counter-staining with Gill's haematoxylin (Mellor *et al.* 2000, Gold *et al.* 2003). The β C-activin subunit antibody has previously been shown not to cross-react with β A- or β B-activin subunits or the closely related β E-activin subunit (Mellor *et al.* 2000). β C-activin staining patterns were confirmed using a DAKO autostainer (DAKO Corporation, Carpinteria, CA, USA), with 0.01 M glycine buffer, pH 4.4, for antigen retrieval, a 90 min primary antibody incubation at room temperature, a 20-min secondary antibody incubation and a 15 min tertiary incubation. Antibodies were diluted in TBS containing 1% BSA (Sigma) and sections washed with TBS containing 0.1% Tween-20. Negative controls were included in every experiment: sections were incubated with primary antibody only, secondary antibody only or mouse monoclonal IgG substituted for primary antibody (Sigma-Aldrich). Specificity of staining was also assessed by pre-absorption of the β A- or β C-activin primary antibodies with 500 μ g/ml antigenic peptide as described previously (Mellor *et al.* 2000). The percentages of intensely stained β A- and β C-activin hepatocytes undergoing mitosis or apoptosis were assessed in liver sections. Slides from regenerating liver were analysed for positive β -activin subunit staining in apoptotic (12 h, 7 day and 10 day sections) or mitotic hepatocytes (24 h, 48 h and 72 h sections). Apoptotic hepatocytes in β A- or β C-activin-stained sections were identified by their histological appearance (Gold *et al.* 2003) and by positive TUNEL staining in the adjacent section. Mitotic hepatocytes in prophase, metaphase, anaphase or telophase were identified by histological appearance. These time points were chosen as they contained increased evidence of apoptosis or mitosis compared with the other time points (see Fig. 1). The frequencies of apoptotic or mitotic hepatocytes that exhibited intense activin subunit immunoreactivity were presented as a staining index (stained hepatocytes/total hepatocytes in mitosis or apoptosis $\times$ 100).

Statistics

The normalised densities of PCR products ($n=41$) were log transformed and variation with time assessed by ANOVA (SPSS for Windows 6.1). In figures, normalised

Figure 1 Hepatocyte cell death (%) assessed by *in situ* TUNEL staining (filled columns) and cell division (open columns) – prophase, metaphase, anaphase or telophase – assessed histologically, in rat regenerating liver following PHx. Results are means $\pm$ S.D., $n=3$ male rats per time point. Results were analysed by Mann–Whitney *U*-test: * $P<0.02$, ** $P<0.01$, *** $P<0.001$. Mitosis was evident at 48 and 72 h, whereas increased cell death occurred at 12 h and from 96 h on in regenerating male rat liver.

densities are expressed as a percentage of sham-operated controls at each time point. Gene expression results are presented as means $\pm$ S.D. of 3 independent PCR reactions with different cDNAs, $n=3$ –5 PHx and 2 sham controls per time point.

Mitotic/apoptotic staining percentages were analysed by Mann–Whitney *U*-test (SPSS for Windows 6.1).

Results

Body weight

Rats lost 4% of their body weight post-PHx, returning to pre-operation weight after 5 days (120 h). At 7 days (168 h) post-PHx, by which time liver mass had been restored, rat body weight was steadily increasing (14% increase compared with pre-operation weight). Control rats did not lose weight after the sham operation and their body weight increased throughout the study (25% increase by day 7).

Histology

The percentages of mitotic and apoptotic hepatocytes in regenerating male rat liver are presented in Fig. 1. Mitosis was observed rarely in sham-operated control liver. An increase in the percentage of mitotic hepatocytes was evident in the caudate lobe 48 h ($P<0.001$) and 72 h ($P<0.02$) post-PHx. Cell death was low in sham-operated control liver, but an increased rate

Figure 2 RT-PCR band densities, relative to β -actin densities, in the caudate lobe of livers from sham-operated control rats. Results are means $\pm$ range of triplicate RNA extracts from male control rats ($n=2$ at each time point). Sham operation did not lead to significant differences in expression of mRNA for any of the genes analysed.

of TUNEL staining was evident at 12 h and from 96 h on, in regenerating liver ($P<0.01$).

Gene expression

Concentrations of β -actin mRNA did not vary significantly after control sham operations or during the course of liver regeneration ($P=0.537$, ANOVA). Control sham operations did not lead to significant changes in β A- or β C-activin, ActRIIA, ActRIIB or follistatin mRNA (Fig. 2). Results are mean $\pm$ range in $n=2$ control rats at each time point. Follistatin mRNA expression was very low in control livers. Consistently more β C-activin than β A-activin mRNA and equivalent amounts of ActRIIA and ActRIIB mRNA were detectable.

Results from the caudate lobe of regenerating rat liver are presented graphically as a percentage of sham controls. Significantly lower amounts of β A-activin mRNA were measured in the caudate lobe of regenerating liver at 12 and 24 h following PHx, compared with sham-operated controls ($P<0.05$, Fig. 3A). β A-activin mRNA increased at 168 h, when liver mass appeared fully restored ($P<0.001$). Amounts of β C-activin mRNA decreased significantly compared with controls levels at 12, 24 ($P<0.05$) and 72 h ($P<0.001$) post-PHx (Fig. 3A). Follistatin mRNA increased from very low values to a peak at 24 and 48 h in regenerating liver ($P<0.001$), decreasing to barely measurable values at 72, 168 and 240 h compared with controls ($P<0.001$, Fig. 3B). The expression of ActRIIA mRNA showed no significant changes with restoration of liver mass, whereas ActRIIB receptor mRNA increased markedly at 168 h ($P<0.001$, Fig. 3C), compared with controls.

Activin peptides in liver

Activin subunit immunoreactivity in control and restoring rat liver are shown in Fig. 4. Immunoblotting revealed that the caudate lobe of sham-operated male rat liver contained moderate amounts of activin pro-proteins (above 32 kDa), small amounts of a β A-activin homodimer, no evidence of the β A β C (23 kDa) band and moderate levels of the β C-activin homodimer at 21 kDa. β A-activin immunoreactive bands corresponding to pro-peptides (above 36 kDa), β A β A-activin (26 kDa) and β A β C-activin (23 kDa) were evident in the regenerating caudate lobe (Fig. 4A). There were β C-activin immunoreactive bands above 36 kDa

(likely to be β C-activin subunit pro-peptides), at 23 kDa (β A β C-activin) and at 21 kDa (β C β C-activin) in regenerating liver (Fig. 4B).

Activin dimer combinations appeared to alter in restoring rat liver (Fig. 4), but no attempt was made to quantify amounts of immunoreactivity on the blots. The 12-h time point showed little evidence of dimers of either subunit. The proportion of β A β A (26 kDa) and β A β C (23 kDa) activin dimers appeared to increase at 24 h. There was proportionally more β A β C activin (23 kDa) at 48 h than either β A β A (26 kDa) or β C β C (21 kDa) and at 96 h there appeared to be more β C-activin than β A-activin immunoreactivity compared with other time points. At 168 and 240 h the male rat restoring caudate lobe showed evidence of more β A β A activin (26 kDa) and β A β C activin (23 kDa), than β C β C activin (21 kDa).

Activin peptides in serum

In the serum of PHx rats the anti- β A-activin antibody detected a double band at 23–26 kDa; this is likely to be activin AC (β A β C dimer), activin AB (β A β B dimer) and/or activin A (β A β A, Fig. 5A). The β C-activin antibody also detected two circulating peptides in regenerating and control rat serum (Fig. 5B). These bands may be activin AC (23 kDa), activin BC and/or activin C (β C β C dimer, 21 kDa). The unidentified intensely immunoreactive band evident at around 30 kDa for both β -activin antibodies is likely to be an artefact caused by excess loading of serum onto the gel.

Figure 3 (A) β A-activin (●) and β C-activin (○) mRNA expression after PHx, assessed by RT-PCR, in triplicate RNA extracts from 5–7 male rats (2 controls and 3–5 PHx) at each time point. Data are presented after normalisation against the β -actin density, as the percentage of sham-operated control liver expression at each time point (means $\pm$ s.d.). Data were log transformed and analysed by ANOVA for group differences (sham vs PHx) and time differences (12–240 h). β A- and β C-activin densities were significantly different from sham control values (P values between 0.001 and 0.05), 24 h post-PHx. β C-activin densities were significantly lower than sham control and other time points (P values between 0.001 and 0.05) at 72 h, whereas β A-activin densities were significantly higher than sham control and other time points (P values between 0.001 and 0.05) at 168 h post-PHx. (B) Expression of follistatin mRNA in regenerating rat liver expressed as the percentage of sham-operated control liver expression at each time point (means $\pm$ s.d.). Follistatin densities were significantly higher than sham control values and other time points (P values between 0.001 and 0.05), 24 and 48 h post-PHx and significantly lower than sham control values and other time points ($P < 0.05$) at 72, 168 and 240 h post-PHx. (C) ActRIIA (●) and ActRIIB (○) mRNA expression as a percentage of sham control. ActRIIB densities were significantly higher than sham control values or other time points at 168 h post-PHx (P values between 0.001 and 0.05).

Figure 4 β A-activin (R&D, monoclonal antibody) (A) and β C-activin (clone 1, monoclonal antibody) (B) immunoreactive proteins in the caudate lobe of sham-operated control and regenerating rat liver were assessed by SDS-PAGE and non-reducing Western blot, at various times after PHx. Results are representative of duplicate, independent tissue extracts of caudate lobe samples from 3 rats at each time point. Bands representing pro- β A- or β C-activin were present at 43–46 kDa; activin A, activin AC, 23 kDa; activin C, 21 kDa; β A-activin, 13 kDa; and β C-activin, 12 kDa (Mellor *et al.* 2000). The control sample, run on a different gel to the PHx samples, contained more activin C homodimer (*) and less activin AC than the PHx samples.

Activin subunit immunohistochemistry

Representative views of activin subunit immunoreactivity are displayed in Fig. 6. Mild β A-activin subunit immunoreactivity was present in hepatocyte cytoplasm in control and regenerating male rat liver. At 48 h post-PHx, staining was mainly associated with granular or vesicular structures, rather than hepatocyte cytoplasm (Fig. 6A). In the later stages of restoration of liver mass, there was increased β A-activin subunit immunoreactivity associated with apoptotic hepatocytes (Fig. 6B).

Moderate β C-activin subunit immunoreactivity was observed in the majority of hepatocytes in control male liver. At 48 h after PHx, there was intense β C-activin subunit immunoreactivity associated with mitotic cells (Fig. 6C). β C-activin immunoreactivity was evident in approximately 65% of hepatocyte nuclei, as well as in the cytoplasm, at 168 and 240 h post-PHx (Fig. 6D).

β A-activin subunit staining was assessed in 355 mitotic hepatocytes and 29 were strongly positive.

β C-activin subunit immunoreactivity was assessed in 243 mitotic hepatocytes and 218 were intensely immunoreactive. Of 195 apparently apoptotic hepatocytes assessed for β A-activin subunit staining, 144 were markedly positive, whereas 32 of 191 apoptotic hepatocytes assessed for β C-activin subunit immunoreactivity were positive. These data are shown in Fig. 7. Thus an increase in β A-activin subunit peptide was associated with hepatocyte cell death ($P < 0.01$), whereas an increase in β C-activin subunit immunoreactivity was significantly associated with hepatocyte cell division ($P < 0.001$).

Discussion

Temporal changes in mRNA expression of β A- and β C-activin subunits, follistatin and activin receptor RIIb were observed in the regenerating caudate lobe of male rat liver.

The ratio of β A- and β C-activin subunit dimers appeared to alter in restoring rat liver. An increase in β A-activin subunit protein was associated with

Figure 5 Circulating β A-activin (A) and β C-activin (B) peptide subunit in serum of sham-operated control and PHx male rats during the restoration of liver mass. β A-activin (R&D, monoclonal antibody) and β C-activin (clone 1, monoclonal antibody) subunit peptides were assessed by SDS-PAGE and non-reducing Western blot in the serum of male rats at various times after PHx. Results were repeated in duplicate, independent serum samples (2 mg) from 3 rats at each time point. Immunoblots showed bands representing: activin A, 26 kDa; activin AC, 23 kDa; and activin C, 21 kDa (Mellor *et al.* 2000).

hepatocyte apoptosis, while increased β C-activin subunit protein was associated with hepatocyte mitosis.

Activin A and activin receptor subunit ActRIIA have previously been shown to be associated with apoptosis (Schwall *et al.* 1993, Yasuda *et al.* 1993, Hully *et al.* 1994, Coerver *et al.* 1996, Draper *et al.* 1997). Our results indicate that locally produced activin A acts as an autocrine factor to regulate hepatocyte cell proliferation, however we failed to demonstrate changes in ActRIIA mRNA. Activin-induced apoptosis of hepatoma cells is blocked by dominant negative forms of ActRIIB or Smad 2, whereas over-expression of either ActRIB, ActRIIB, Smad 2 or Smad 4 is sufficient to stimulate apoptosis in the absence of activin A (Chen *et al.* 2000, 2002). This suggests that activin A signals through ActRIB/ActRIIB receptors and the receptor Smad pathway plays a central role in determining cellular fate. Hubner *et al.* (1999) and Rosendahl *et al.* (2001) have examined activin receptor type II mRNA in wound healing and lung inflammation. Neither study demonstrates changes in these mRNAs in their *in vivo* models. To the best of our knowledge the data presented here represent the first report of dynamic regulation of activin type IIB receptor subunit mRNA.

The mechanisms by which activin A regulates hepatocyte growth arrest are largely unknown. In HepG2 cells, retinoblastoma hypophosphorylation – via modulation of p53, p21 and the cyclin-dependent kinase Cdk4 – is involved in activin-mediated cell growth inhibition (Zauberman *et al.* 1997). Activin has also been shown to induce hepatocyte growth arrest through

induction of the cyclin-dependent kinase inhibitor p15 and transcription factor Sp1 (Ho *et al.* 2004). Examining the expression of genes shown to be associated with activin-mediated cell cycle arrest *in vitro* in an *in vivo* model of liver regrowth may provide further evidence as to the mechanisms by which activins control hepatocyte cell growth. Follistatin has previously been shown to increase DNA synthesis and to antagonise the inhibitory effects of activin A in regenerating liver (Kogure *et al.* 1995, 1996, 1998, 2000, Zhang *et al.* 1997a). The increase in follistatin demonstrated in our study may therefore serve to protect proliferating hepatocytes from the apoptotic effects of activin A. Mitosis was evident at 48–72 h when follistatin mRNA expression was high. This may indicate that increased follistatin expression promotes hepatocyte cell division, either independently or by blocking the negative growth effects of activin A.

β C-activin has been shown to be growth inhibitory in liver (Esquela *et al.* 1997, Zhang *et al.* 1997b) or growth promoting (Kobayashi *et al.* 2002, Wada *et al.* 2004). Lau *et al.* (2000) demonstrated, by RNase protection assay with a probe from the 3' untranslated region of C-activin cDNA, an increase in β C-activin mRNA 12–24 h after PHx; this differs both from our results and from those of Esquela *et al.* (1997) and Zhang *et al.* (1997b). Discrepancies may be related to the region of β C-activin cDNA examined or the part of the liver used in this study. In our study, β C-activin mRNA was low in extracts of regenerating liver, but significantly associated histologically with mitotic hepatocytes. Thus the gene expression data presented support the theory that

Figure 6 Selected views of β A-activin and β C-activin subunit peptide immunoreactivity in paraffin-embedded, paraformaldehyde-fixed sections of the caudate lobe of male rat regenerating liver. (A) Granular β A-activin subunit immunoreactivity in hepatocytes. Adjacent hepatocytes undergoing mitosis show no positive staining with the β A-activin antibody. (B) β A-activin subunit immunoreactivity in a cell undergoing apoptosis. (C) Cytoplasmic β C-activin subunit immunoreactivity in association with mitosis. (D) β C-activin subunit immunoreactivity within hepatocyte nuclei, 10 days after PHx. (E and F) β A- and β C-activin control sections incubated with mouse IgG. Final magnifications: A, B and C, $\times 400$; D, $\times 40$; E and F, $\times 200$. No staining was observed in control sections incubated with primary antibody pre-absorbed with 100 times excess β A- or β C-activin subunit synthetic peptide (not shown, see Gold *et al.* 2003).

β C-activin may act as a liver chalone, whereas the immunohistochemical data suggest a local role for β C-activin in hepatocyte proliferation. In addition, our data suggest that activin mRNA may be labile and translated peptide stored in hepatocytes, as mRNA levels did not accurately reflect the amount of activin subunit proteins evident by immunohistochemistry or immunoblotting. These discrepancies highlight the importance of examining activin at both the mRNA and protein level.

It has been proposed that high levels of β C-activin subunit peptide may protect dividing cells from the apoptotic effects of activin A by forming intracellular heterodimers (Mellor *et al.* 2000). There was, however, very little evidence of β A-activin subunit immunoreactivity in mitotic hepatocytes, indicating that the heterotrophic effects of β C-activin subunit protein were independent of β A-activin subunit protein within mitotic hepatocytes. Mellor *et al.* (2000, 2003) and

Figure 7 Mitotic (filled columns) and apoptotic (open columns) labelling indices for β A-activin and β C-activin subunit immunoreactivity. Slides from regenerating liver were assessed for markedly positive β -activin subunit staining in apoptotic (12 h, 7 days and 10 days PHx) or mitotic hepatocytes (24 h, 48 h and 72 h PHx). Hepatocytes undergoing cell death were identified by histological appearance and apoptosis confirmed on an adjacent TUNEL-stained section (not shown). Mitotic hepatocytes were identified based on histological appearance. Apoptotic or mitotic cells that were markedly positive for activin subunit immunoreactivity were counted and divided by the total number of cells assessed; results are presented as a staining index (%).

Vejda *et al.* (2002) reported the formation of β C-activin subunit peptide homodimers and heterodimers *in vitro*. Our study confirms that β C-activin homo- and heterodimers also form *in vivo*. When non-reduced samples were assessed, the β -activin subunit antibodies detected a range of activin dimers. The presence of a β A β C activin dimer was confirmed with both the β A- and β C-activin subunit antibodies. The exact identity of other *in vivo* β C-activin peptides needs to be confirmed by immunoprecipitation studies. To the best of our knowledge our study is the first evidence of *in vivo* β C-activin subunit peptide dimers in regenerating rat liver. Wada *et al.* (2004) propose that activin C homodimer, rather than activin A, is formed in normal liver and PHx may lead to increased activin A, terminating liver regeneration (Wada *et al.* 2004). We show activin homodimers are present at low to moderate levels in control liver while increased activin AC was evident in regenerating liver. Therefore the formation of activin heterodimers may antagonise the formation of homodimers and allow restoration of liver mass. β E-activin is also expressed in the liver and can dimerise with β A- and β C-activin subunits, and is therefore likely to be an additional player in the restoration of liver mass (Lau *et al.* 2000, Vejda *et al.* 2002).

Significant amounts of β C-activin subunit peptide were detected in the serum of rats by western blotting. During regrowth the liver is able to carry out most

normal physiological functions; however, functional reserve is very low. Therefore some peptides, rather than being processed in the liver, are secreted (Fausto 1990). The presence of circulating β C-activin subunit dimers in control rat serum suggests these dimers are not merely a by-product of a liver with low functional reserve. However the local effects of liver activins are difficult to reconcile with evidence of secreted activins. These results may indicate that β C-activin subunit proteins, like activin A, have endocrine, autocrine and paracrine roles *in vivo*. These findings also raise the possibility that serum levels of β C-activin subunit peptide may be able to be used as a diagnostic marker of liver health.

The nuclear staining observed with the β C-activin subunit antibody in the later stages of regeneration of male rat liver mass (168 and 240 h) was unexpected. Nuclear localisation of β A-activin subunit in rat seminiferous epithelium was presented by Blauer and colleagues in 1999. This group showed the β A-activin subunit precursor contained a functional nuclear localisation sequence (Blauer *et al.* 1999). A search of protein databases (Psort database) indicates that human β C-activin contains a leucine zipper domain at amino acids 148–169 (<http://psort.nibb.ac.jp>). Despite the specific times post-hepatectomy at which nuclear staining was observed and our observation of nuclear β C-activin staining with a polyclonal antibody, in mouse ovarian cysts (Richardson *et al.* 2005), we cannot entirely discount the possibility that nuclear staining may be a tissue fixation artefact. In order to determine if nuclear localisation of β C-activin subunit in the later stages of regeneration of male rat liver mass indicates a novel signalling pathway, future studies will perform western blotting on nuclear extracts of regenerating rat liver.

Lau *et al.* (2000) proposed that β C-activin performs no essential *in vivo* function, as mice deficient in β C-activin showed no obvious phenotype, even when female mice were subjected to PHx. However, a redundancy of roles, with other TGF- β s substituting for the lack of the liver activins cannot be excluded. Furthermore, we consider it is highly unlikely that a protein with no physiological function in the liver would show *in vivo* temporal and cellular responses to liver insult.

We have demonstrated concurrent changes in the expression of β A-activin, β C-activin, follistatin and activin receptor subunit mRNA in the caudate lobe of regenerating rat liver, over 240 h post-PHx. β C-activin appears to be regulated in parallel to β A-activin *in vivo*, however the two subunits are not always localised in the same cell types.

We conclude that β A- and β C-activin subunit proteins are autocrine growth regulators in regenerating liver and when expressed independently lead to hepatocyte apoptosis (β A-activin) or mitosis (β C-activin) in a subset of hepatocytes. In the liver, apoptosis is an important part of the tightly controlled homeostatic

mechanisms regulating liver function (Chen *et al.* 2000), perhaps indicating the importance of locally produced, autocrine growth regulators such as the activins in the liver. The mechanism by which β C-activin promotes hepatocyte mitosis and the role of the closely related β E-activin subunit in liver regeneration will form the basis of future studies.

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