Expression of activins C and E induces apoptosis in human and rat hepatoma cells

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Introduction

Activins belong to the transforming growth factor β (TGFβ) superfamily of growth factors (1). This family includes a number of factors regulating cell proliferation and differentiation. To date, four mammalian activin subunits have been described, activins βA, βB, βC and βE (2–9). Mature proteins are composed of two β subunits. Recently, we and another group demonstrated that homodimers (activins AA, BB, CC and EE) as well as heterodimers (activins AB, AC, AE and CE) are formed from the β subunits (9,10). Activin A has been implicated in liver growth regulation by its capability to inhibit mitogen-induced DNA synthesis (11) and to induce apoptosis in vivo and in vitro (12,13). In addition, the activin-related protein TGFβ1 inhibits DNA synthesis and causes apoptosis in normal and transformed hepatocytes (14–17). Therefore, activin A and TGFβ1 are supposed to act as negative regulators of liver growth.

The activin βC gene was cloned and identified in human (3), mouse (5) and rat (9). The cDNA sequence of the activin βE subunit has been reported for mouse (4), rat (7,9) and human (8). The two subunits share 82 and 61% amino acid sequence similarity of the mature peptides from rat and mouse, respectively, and they are thought to be a subset of related sequences (4,9). We found activin subunits βC and βE expressed almost exclusively in the liver, in hepatocytes (9). The activin βC protein was localized to human liver and prostate (10). As in the rat, activin βE mRNA is predominantly expressed in the liver in humans, with very low levels also detected in heart, testes, peripheral blood leukocytes, placenta and skeletal muscle (8). It has been postulated that activin C may act as a liver chalone, because, following partial hepatectomy, transient down-regulation of activin βC mRNA was observed (18–20). Decreased expression of activin C would reduce its inhibitory effect on cell proliferation to allow liver regeneration.

Lau et al. demonstrated that activin βE mRNA increased rapidly and decreased to near basal levels by 48 h following partial hepatectomy (20). A rapid increase in mRNA was also reported in mice treated with lipopolysaccharide, which correlates with a pattern of acute phase response in the liver (7). Mice deficient in the activin βC gene, activin βE gene or in both genes appeared grossly normal. The animals were viable, survived to adulthood and liver regeneration following partial hepatectomy proceeded similar to that of wild-type mice (20).

Up to now, the biological roles of activins C and E have not been elucidated. Since the two factors display a liver-specific expression pattern and belong to the TGFβ superfamily of growth factors, one may envision that activins C and E could also play roles in the sophisticated regulatory network that maintains a constant liver mass. In line with this hypothesis we have recently shown that overexpression of activin βC or βE in mouse liver can inhibit regenerative DNA synthesis (21).

To investigate potential functions of the liver-specific activins in the context of tumor cell growth, we initially examined the expression of activin subunits in logarithmically growing hepatoma cell lines in comparison with liver and primary hepatocytes. Since the human hepatoblastoma cell line HepG2, the rat hepatoma cell line H4IIEC3 and the human hepatocellular carcinoma cell line Hep3B all had either completely lost or drastically reduced expression of activins, we subsequently investigated the effects of ectopically expressing activin A (as a positive control), C or E in these cell lines.

Abbreviations: EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRES, internal ribosomal entry site; MEM, minimal essential medium; MITT, methylthiazol tetrazolium; MTX, methotrexate; PBS, phosphate-buffered saline; RPI.6, ribosomal protein L6; TGFβ, transforming growth factor β.

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Transient transfection of activin βα, βE and βE CDNA delayed the increase in cell number of all tested cell lines as determined by the methylthiazol tetrazolium (MTT) cell proliferation assay and cell counting. Furthermore, we demonstrated that these activins induce caspase activation and increase the rate of apoptosis in HepG2, H4IIIEC3 and Hep3B cells.

Materials and methods

Cell culture

HepG2 (human, hepatoblastoma) cells were maintained in minimal essential medium (MEM) (Invitrogen) supplemented with 1 mM sodium phosphate, 1% non-essential amino acids (Biochrom KG) and 10% fetal calf serum (FCS). Hep3B (human hepatocellular carcinoma) and H4IIIEC3 (rat hepatoma) were grown in RPMI-1640 (Invitrogen) supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO2. Primary rat hepatocytes were isolated and cultured as previously described (22) in accordance with the Austrian guidelines for animal care and protection.

Plasmids

The complete coding sequences of the rat activin subunits βα (1275 bp) (23), βE (1053 bp) (9) and βE (1056 bp) (9) were cloned into either pTracer-CMV (βα and βE) or pcDNA3 (βE) (Invitrogen). The sequencing performed on the initiation codons were changed to a Kozak consensus sequence (24) by PCR mutagenesis in each case. Plasmid DNA was purified using a plasmid maxi kit (Qiagen). In addition, the complete coding sequences of the rat activin subunits were cloned into the multiple cloning site of pIRESe-EGFP. This vector contains the internal ribosomal entry site (IRES) of encephalomyocarditis virus between the MCS and the enhanced green fluorescent protein (EGFP) coding regions. This permits both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA. Empty vectors as well as a plasmid containing β-galactosidase cDNA (pCMV- lacZ) were used as control plasmids.

Transient transfection of cells

HepG2, Hep3B and H4IIIEC3 cells were seeded into either 96-well (4000 cells/well), 24-well (10 000 cells/well) or 12-well (20 000 cells/well) tissue culture plates (Greiner). Twenty-four hours after seeding DNA transfection using the FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Roche Molecular Biochemicals) was performed according to the instructions of the manufacturer. Twenty-four hours after transfection the medium was renewed. Plasmids containing both the activin cDNA and EGFP, activin cDNA alone, lacZ cDNA or plasmids alone were used for transient transfection of HepG2, Hep3B and H4IIIEC3 cells. The EGFP protein served as a positive control to monitor uptake of plasmids.

Isolation of total RNA and RNase protection assay

HepG2, H4IIIEC3 and Hep3B cells were directly lysed on the dishes using TRIZol Reagent (Life Technologies) and total RNA was isolated according to the instructions of the manufacturer. RNA was dissolved in 3 mM EDTA and the concentration was determined photometrically. RNA probes complementary to the respective cDNA sequences of rat activins βα (235 bp), βE (196 bp) and βE (180 bp) and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (106 bp) were used as previously described (9). To analyze the expression of human activins, RNA probes for human activin βα (367 bp corresponding to nt 159–526 of the sequence with Genbank accession no. NM_002192), activin βE (179 bp corresponding to nt 991–1170 of NM_005538), activin βE (248 bp corresponding to nt 1042–1209 of NM_031479), human ribosomal protein L6 (RPL6) (132 bp corresponding to nt 363–495 of NM_000970) and human GAPDH (106 bp corresponding to nt 714–820 of NM_002406) were used. The housekeeping genes GAPDH and RPL6 were used as a control for sample loading and to compare expression levels. RNase protection assays were performed as previously described (25), with the following minor modifications: hybridizations were carried out at 51°C. RNAse A and RNAse T1 were used at 15 and 1 µg/ml, respectively. Dried gels were analyzed with a PhosphorImager and ImageQuant software (Molecular Dynamics).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Hep3B cells were transfected with either activin βα or empty vector and shifted to serum-free medium 24 h later. Another 72 h later proteins were precipitated from media supernatants with 1 vol of acetone, dissolved in loading buffer (7 M urea, 60 mM Tris-HCl pH 6.8, 2% SDS, 100 mM DTT, 0.01% bromphenol blue) and separated by 15% SDS-PAGE. Proteins were subsequently transferred to Hybond-P membranes (Amersham Pharmacia Biotech). Non-fat dried milk at 5% in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) was used to block non-specific binding. The activin βα antibody (kindly provided by W.Vale; 26) was used at 0.4 µg/ml in TBST containing 1% non-fat dried milk. Visualization was performed with an enhanced chemiluminescence (ECL Plus) detection system (Amersham Pharmacia Biotech).

Determination of cell number

As an indicator of cell number we used the MTT assay, which is based on the reduction of soluble yellow MITT tetrazolium salt to a blue insoluble MITT formazan product by mitochondrial succinic dehydrogenase (27). The amount of formazan product is proportional to the number of viable cells. MTT was added to each well to a final concentration of 0.5 mg/ml and incubation continued for an additional 2 h. Reduced MTT was dissolved in dimethyl sulfoxide and measured spectrophotometrically in a dual beam microtitrator plate reader at 562 nm with a 620 nm reference. Experiments were performed in quintuplicate wells, repeated at least four times and the values are expressed as fold of control (cultures transfected with plasmid alone).

For counting of H4IIIEC3 cells, they were detached with trypsin and their number determined in an automatic cell counter (CASY1-Model TTC; Schaerfe System).

Generation of activin C expressing CHO cells

The full-length cDNA of human activin βE (3) was cloned into pABStopXS (28) containing a SV40 promoter, as well as a CMV enhancer, resulting in a plasmid named pAB 121. Plasmid pAB 121, as well as a plasmid pSVO dhfr- have been co-transfected into CHO dhfr- cells (29) at a ratio of 5:1 using a commercially available lipofection kit (Gibco). Upon transfection, cells were cultivated in the presence of increasing concentrations of methotrexate (MTX), ranging from 0.0005 to 6.0 µM MTX after 4 months, in order to amplify the transgene and to increase expression (30). Expression of activin C was verified by western blot. As a negative control, the empty plasmid pAB StopXS has been used instead of pAB 121.

HepG2-CHO co-culture

Tissue culture islands (0.02 µm anopore membrane; Nunc) with either 150 000 activin C expressing or mock transfected CHO cells were placed in 6-well plates containing 40 000 HepG2 cells/well in MEM supplemented with 1 mM pyruvate, 1% non-essential amino acids and 10% FCS. Three days later part of the growth medium was renewed and on day 7 MTT assays were performed as described above. The experiment was repeated three times and the values are expressed as fold of control (co-culture with mock transfected CHO cells).

Scoring of apoptosis and detection of caspase activation

Twenty-four hours after transfection of the cells with either activin βα, βE or βE cDNA or plasmid alone in 24-well plates, they were washed twice in phosphate-buffered saline (PBS), fixed for 15 min in 2% formaldehyde/PBS at room temperature, permeabilized with PBS/0.2% Tween 20 for 1 min and stained in 1 µg/ml Hoechst 33258 (Calbiochem) for 2 min. The percentage of cells displaying typical apoptotic nuclear morphology (crescent shaped condensed chromatin lining nuclear periphery; apoptotic bodies) (16,31), referred to as the apoptotic index, was then assessed using a fluorescence microscope. Four experiments in duplicate (two wells) were performed and between 300 and 800 nuclei per well were counted.

Caspase activity was detected with the CaspACE™ FITC-VAD-FMK in situ marker (Promega). Twenty-four hours after transfection the marker was added to Hep3B cells to a final concentration of 10 µM and cells were incubated for 45 min. Then they were washed twice with fresh medium and assayed for FITC fluorescence under a fluorescence microscope. To compare the pattern of apoptotic chromatin changes with that of caspase activation, Hep3B cells were incubated with 5 µg/ml Hoechst 33258 and 10 µM FITC-VAD-FMK marker simultaneously and the same microscope frame was photographed once with filters for FITC and then with filters for Hoechst 33258.

Measurement of DNA synthesis rates

HepG2 cells (20 000 cells/dish) were seeded into 2.5 cm Nunc dishes and transfected as described above. Twenty-four hours later [3H]thymidine solution (0.5 µC/ml) was added for 2 h. Fixation, processing for autoradiography and staining were done as described (32). Experiments were performed in duplicate, repeated three times and 1000 Hoechst stained nuclei per sample were counted.
Statistical analysis
All values are expressed as means ± SD. Student’s t-test was used to evaluate differences between samples transfected with vector alone and samples transfected with activin cDNA.

Results

Endogenous activin \( \beta_A \), \( \beta_C \) and \( \beta_E \) subunits in human and rat hepatoma cells

In sharp contrast to normal human liver and primary rat hepatocytes, human and rat hepatoma and hepatocellular carcinoma cell lines express very little or no endogenous activin \( \beta \) subunits (Figure 1). In the human hepatoma cell line HepG2 small amounts of activin \( \beta_E \) and \( \beta_C \) were found, whereas activin \( \beta_A \) expression was undetectable. In the hepatocellular carcinoma cell line Hep3B none of the activin subunits could be detected. The rat hepatoma cell line H4IIEC3 expresses activin \( \beta_E \) mRNA, albeit at several-fold lower levels than primary rat hepatocytes. Activin \( \beta_A \) and \( \beta_C \) were at undetectable levels. We have previously reported the absence of activin \( \beta_B \), the fourth mammalian activin \( \beta \) subunit hitherto described, from the rat liver (9).

Transient transfection of activin \( \beta_A \), \( \beta_C \) and \( \beta_E \) cDNA in cultured human and rat hepatoma cells

In order to gain insight into the function of the activin subunits \( \beta_C \) and \( \beta_E \), transfection studies with HepG2, H4IIEC3 and Hep3B cells were performed. Cells were transiently transfected with plasmids containing rat activins \( \beta_A \), \( \beta_C \) and \( \beta_E \) cDNA as described in Materials and methods. In order to monitor activin expression after transfection of cDNA, total RNA was isolated from transfected HepG2, Hep3B and H4IIEC3 cells 24 h after transfection and RNase protection assays were performed. As shown in Figure 2, activin subunits \( \beta_A \), \( \beta_C \) and \( \beta_E \) were highly expressed at a similar level in HepG2 and Hep3B cells using 0.5 or 5 \( \mu \)g total RNA, respectively. Since plasmids containing rat activin cDNA were used to transfect the cells, only the ectopically expressed activin subunits were detected in the RNase protection assays. However, in the rat cell line H4IIEC3 endogenous activin \( \beta_E \) mRNA was detected in addition to the forced expression of activin subunits, and only moderate levels of ectopic expression could be achieved. Uptake of plasmids in all cell lines was also controlled by examining the expression of EGFP protein. Transfection efficiencies of ~30% were achieved in HepG2 and Hep3B cells and slightly less in H4IIEC3 cells.

To ensure that ectopically expressed activin is translated into protein and subsequently secreted into the medium, we checked for the presence of activin A in media supernatants of Hep3B cells transfected with either activin \( \beta_A \) or a vector control. Western blotting with anti-activin A antibody detected activin A in the supernatants of activin A transfected cells but not in untransfected or mock transfected cells (Figure 3).

Reduced growth of HepG2, Hep3B and H4IIEC3 cells in response to forced expression of activin A, C and E

To study the effects of activin expression on hepatoma cell lines, they were transfected with activin cDNA and growth properties of these cells were determined using the MTT assay. In this assay the metabolic activity of cells is used as a parameter for cell number. First, growth of transfected cells was analyzed 24, 48, 72 and 96 h after transfection (data not shown). As the most pronounced effect on cell growth was observed 72 h after transfection, this time point was selected for further MTT assays. The number of HepG2, H4IIEC3 and Hep3B cells was significantly decreased after transfection with activin \( \beta_C \) and \( \beta_E \) cDNA compared with cultures transfected.

Fig. 1. (A) RNA expression of endogenous human activin subunits in HepG2 human hepatoblastoma and Hep3B human hepatocellular carcinoma cells as compared with two samples of human liver. Aliquots of 50 \( \mu \)g of total RNA were hybridized to riboprobes for human activin \( \beta_A \), \( \beta_C \) and \( \beta_E \) and human ribosomal protein L6. (B) RNA expression of endogenous rat activin subunits in H4IIEC3 rat hepatoma cells and in primary rat hepatocytes. Aliquots of 10 \( \mu \)g of total RNA were hybridized to riboprobes for rat activin \( \beta_A \), \( \beta_C \) and \( \beta_E \) and rat GAPDH.
with the empty vector, indicating a reduced growth rate in the presence of either activin C or E (Figure 4). The loss of MTT reducing activity was ~30–40% when cells were transfected with either activin \( \beta \)C or \( \beta \)E cDNA. Longer incubation times did not increase the effect (data not shown). In HepG2 and Hep3B cells transfection with activin \( \beta \)A cDNA served as a positive control, because it is well known that activin A negatively affects the growth rate of these cells (33,34). Indeed, transfection of activin \( \beta \)A cDNA resulted in growth inhibition in HepG2 and Hep3B cells. Furthermore, transfection with activin \( \beta \)A was also inhibitory in H4IIEC3 cells. Of the three activins studied, activin \( \beta \)A transfection induced the most conspicuous effect. Loss of MTT reducing activity of HepG2 and Hep3B cells was 30–50%, while that of H4IIEC3 cells reached as high as 75%. To demonstrate the specificity of the effects, the unrelated \( \beta \)-galactosidase protein was expressed in HepG2 cells, but had no effect on HepG2 cell number (Figure 4A). In order to determine whether the effect seen in the MTT assay was indeed due to reduced cell numbers (and not to reduced metabolic activity of equal numbers of cells), H4IIEC3 cells were counted using an automatic cell counter. As shown in Figure 4D, these experiments confirmed the results obtained by the MTT assays. Cell number was significantly reduced when cells were transfected with activin \( \beta \)C cDNA. Again, the effect on cell growth was weaker in the presence of activin \( \beta \)C and \( \beta \)E cDNA than after transfection with activin \( \beta \)A cDNA.

**Reduced HepG2 cell number caused by co-culture with activin C expressing CHO cells**

To rule out potential artefacts caused by transient overexpression, HepG2 cells were maintained for 1 week in co-culture with a CHO cell clone stably overexpressing human activin \( \beta \)C. CHO cells were kept in inserts in the medium of HepG2 cells, separated by a porous membrane. Under these conditions, activin C expressing CHO cells reduced HepG2 cell number by 25% compared with the control, i.e. mock transfected CHO cells (Figure 4E). These results strongly suggest that secreted activin C protein inhibits cell multiplication of HepG2 cells.
Enhanced rate of apoptosis caused by transfection of hepatoma cells with activin subunits

Since activin A and TGFβ1 are well known inducers of apoptosis in hepatic cells, we investigated whether the reduced cell numbers after transfection of HepG2, H4IIEC3 and Hep3B cells with activin βA, βC and βE cDNA are a consequence of an increased rate of cell death. Therefore, cells were transfected with activin cDNA and 24 h later they were fixed and the cell nuclei were stained with Hoechst 33258. The 24 h time point was chosen for apoptosis detection because in initial studies at later time points the analysis was impeded by the debris from apoptotic cells, which had subsequently undergone secondary necrosis. As shown in Figure 5D, H4IIEC3 cells displayed typical features of apoptotic cell death. These pictures of nuclei were similar in HepG2 (not shown) and Hep3B (Figure 6B) cells. The background level of apoptotic cell death due to the transfection of empty plasmid was low (between 1 and 4% on average). Compared with control cells, transfection of activin βA, βC and βE cDNA resulted in significantly higher percentages of condensed and fragmented nuclei in all three tested cell lines, as shown in Figure 5A–C. Again, activin βA transfection resulted in the most pronounced effect in HepG2 and H4IIEC3 cells. Hep3B cells were the cell line most sensitive to apoptotic cell death after transfection with activin βE and βC.

To further confirm the induction of apoptosis, we investigated the activation of caspases in Hep3B cells, which had shown the most pronounced effect with regard to the appearance of apoptotic nuclei following activin transfection. Activated caspases have been shown to be important executioners of apoptosis and are responsible for many of the morphological and biochemical changes associated with this form of cell death (35). Binding of the FITC-VAD-FMK marker to the catalytic center of activated caspases allows the identification of caspase activation in single cells (36). Transfection with activin βA, βE or βE resulted in an increase in FITC labeled apoptotic cells, suggesting that activin expression induces caspase activation and consequently apoptosis (Figure 6). Moreover, double labeling of cells with FITC and Hoechst 33258 showed good agreement between caspase activation and apoptotic chromatin changes.

DNA synthesis rates in HepG2 cells after transfection with activin subunits

Activin A has been shown to inhibit DNA synthesis rates in rat hepatocytes (11). Therefore, we determined whether transfection of HepG2 cells with activin cDNAs results in decreased rates of cells undergoing DNA synthesis. For this purpose we used the same conditions under which pro-apoptotic activity was demonstrated. To eliminate a potential bias due to DNA of apoptotic cells, autoradiography was chosen and apoptotic nuclei were not included in the count. Under these conditions supplementation with [3H]thymidine revealed no decrease in labeled nuclei in activin transfected compared with mock transfected cells (Table I).

Discussion

Up to now, the biological functions of activins C and E have not been elucidated. Activin C was suggested to act as a liver chalone (18). It has been proposed that liver mass is regulated by an inhibitory factor produced within the liver. This putative factor, designated liver chalone (37), inhibits proliferation of hepatocytes. Expression studies in humans, rats and mice revealed the liver as the major source of activin βC and βE transcripts (3–9,18). On the basis of structural homology, activins belong to the TGFβ superfamily of cytokines. Among these, TGFβ1, activin A inhibit mitogen-induced DNA synthesis (17) and induce apoptosis in hepatocytes in vivo and in vitro (12–15). Despite their high structural similarity (9), it has not been clarified whether activins C and E have similar potential.

We examined the expression of activins A, C and E in hepatoma cell lines and found that in contrast to normal human liver or primary rat hepatocytes (and normal rat liver; not shown), hepatoma cells have partially or completely lost activin expression. Moreover, we could demonstrate that transient transfection with activin cDNA significantly decreased the number of HepG2, Hep3B and H4IIEC3 cells compared with control cultures. This effect was observed using two different detection systems in three different cell lines. Cell number and metabolic activity of cells were determined.

Table I. Determination of the DNA synthesis rates of HepG2 cells 24 h after transfection with the respective activin β subunits, the lacZ gene or empty vector

<table>
<thead>
<tr>
<th>Transfection agent</th>
<th>Labeling index (%)</th>
<th>SD</th>
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<tbody>
<tr>
<td>Activin A</td>
<td>36.0</td>
<td>6</td>
</tr>
<tr>
<td>Activin C</td>
<td>37.5</td>
<td>6</td>
</tr>
<tr>
<td>Activin E</td>
<td>36.3</td>
<td>5</td>
</tr>
<tr>
<td>pTracer-CMV</td>
<td>36.0</td>
<td>5</td>
</tr>
<tr>
<td>LacZ-CMV</td>
<td>37.0</td>
<td>3</td>
</tr>
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Data represent means ± SD of the percentage of labeled nuclei.
MTT assay is based on metabolic conversion of a defined substrate and the amount of the product is proportional to the number of viable cells. Therefore, this assay can be employed to measure a differential increase in cell number. An inhibitory effect of activin A on hepatic cell proliferation has been described by several authors (33,38,39). We demonstrate for the first time that, like activin A, activins C and E also reduce the increase in hepatoma cells and enhance the rate of apoptosis. This was demonstrated by the classical method of observing apoptotic changes in nuclear morphology and confirmed by detection of activated caspases.

When both detection methods were compared we observed that the great majority of cells were either positive or negative in both assays. Nevertheless, some cells displayed activated caspases but not apoptotic nuclei and vice versa. While the first case can easily be explained by the fact that changes in

![Fig. 4. Determination of the number of viable (A) HepG2, (B) H4IEC3 and (C) Hep3B cells after transfection of activin βA, βC and βE cDNAs, lacZ cDNA or plasmid alone by MTT assay 72 h after transfection. Data are expressed relative to cells transfected with plasmid alone. Bars represent the mean ± SD of 4–10 independent experiments (n). Significance levels: * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test). (D) Number of H4IEC3 cells was counted 72 h after transfection of activin βA, βC and βE cDNA or plasmid alone using an automatic cell counter. A representative experiment is shown. (E) Determination of the number of viable HepG2 cells by MTT assay after 7 days co-culture with activin βE transfected CHO cell clones versus mock transfected clones as controls.](https://academic.oup.com/carcin/article-abstract/24/11/1801/2390383)
nuclear morphology occur at least in part as a consequence of caspase activation, the latter case probably represents cells in a late stage of cell death when activated caspases are no longer detectable.

The increase in apoptosis rates following expression of activins C and E, although weaker than with activin A in two of the three tested cell lines, was highly significant when compared with mock transfected controls. In HepG2 and Hep3B cells all three activin subunits were expressed to high levels, indicating that the transfection efficiency of the plasmids was similar. Transfection efficiency in the rat hepatoma cells was generally lower than that in the other tested cell lines, yet they showed the most dramatic effect in response to forced expression of activin bA. Since H4IIEC3 cells produce considerable amounts of endogenous activin bE RNA, we achieved only a moderate increase in total activin bE RNA levels after transfection. Nevertheless, activin bE transfected H4IIEC3 cells reproducibly displayed an increase in apoptosis rates compared with vector controls. A possible explanation may be that although the overall increase in activin production was low due to a lower transfection efficiency than in the other two cell lines, the local increase in the vicinity of transfected cells may still have sufficed for apoptosis induction. Due to the lack of an appropriate quantitative assay, the amounts of activins C and E produced by cells transfected with activin cDNA could not be determined. A comparison of the mRNA expression levels of activins with that of the housekeeping gene GAPDH suggests, however, that the expression levels achieved by forced expression are within a similar range to those found in primary rat hepatocytes. By precipitating activin A from the medium supernatant of transfected cells we have demonstrated that activin A protein is produced and secreted by transfected cells. Due to the lack of equally sensitive antibodies for activins C and E we could not detect activin E in media supernatants of transfected hepatoma cells and could detect activin C only in stably transfected CHO cells (not shown). However, we have previously demonstrated, with a 2-dimensional polyacrylamide gel electrophoresis approach, that all

Fig. 5. Effect of ectopic expression of activins on rate of apoptosis in (A) HepG2, (B) H4IIEC3 and (C) Hep3B cells. Twenty-four hours after transfection with activin cDNA or plasmid alone, cells were fixed, stained with Hoechst 33258 and normal and apoptotic nuclei were counted. Four independent experiments (n = 4) were performed in duplicate for each transfection using activin bA, bC or bE cDNA or plasmid alone and counting at least 300 cells per well. The percentage of apoptotic nuclei is shown as mean ± SD of four experiments. (D) Example of Hoechst stained nuclei of H4IIEC3 cells. Cells were transfected and stained as described above. Arrows indicate apoptotic nuclei.
three activins are produced and secreted following transfection of the same plasmids as were used in this study into 293T embryonic kidney cells, which show 2- to 3-fold higher transfection efficiencies (9). While the lack of effect of the unrelated protein β-galactosidase and the effectiveness of activin C in the co-culture experiment demonstrate the specificity of the activin effects, quantitative dose-effect determination will have to await the availability of bioactive recombinant activins C and E.

As mentioned above, several members of the TGFβ superfamily of growth factors have been implicated in the control of the birth–death balance of liver cells. TGFβ1 is a potent inhibitor of hepatocyte proliferation and inducer of apoptosis. The concentration of activin A required to induce apoptosis to the same extent was reported to be 10-fold higher compared with the concentration of activin C. A pro-apoptotic effect of activins C and E would be consistent with the reduced expression of activins A- and B-type inhibitor follistatin. In conclusion, activins may be important factors for the maintenance of homeostasis of cell number in the liver.

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