Transforming growth factor- β (TGF- β) isoforms in rat liver regeneration: messenger RNA expression and activation of latent TGF- β

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Expression of transforming growth factor- β s (TGF- β s) 1–3 was studied in normal liver and during liver regeneration after partial hepatectomy in the rat to determine whether each of these isoforms might be involved in hepatocyte growth in vivo. Expression of the mRNAs for all three TGF- β isoforms increases in the regenerating liver. In addition, the levels of expression of the mRNAs for several extracellular matrix proteins, including fibronectin, vitronectin, laminin, and collagen, also increase in the regenerating liver. Immunohistochemical staining analysis shows a similar distribution of all three TGF- β s in normal and regenerating liver; however, in both tissues, the level of expression of TGF- β 1 is 8- to 10-fold higher than that of TGF- β 2 as determined by sandwich enzyme-linked immunosorbent assay. Expression of all three TGF- β mRNAs is restricted to liver nonparenchymal cells. Although hepatocytes from normal and regenerating livers do not synthesize TGF- β , they are sensitive to inhibition of growth by all three TGF- β isoforms. Hepatocytes from regenerating livers are capable of activating latent TGF- β 1 complexes in vitro, whereas normal hepatocytes are not. The different TGF- β isoforms may function in an inhibitory paracrine mechanism that is activated during liver regeneration and may also regulate the synthesis of extracellular matrix components in the regenerating liver.

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Introduction

The regenerating rat liver provides a good model system to study the mechanisms of control of cell proliferation in vivo (Fausto, 1990). Adult rat hepatocytes rarely divide under normal physiological conditions; however, hepatocytes undergo a partially synchronous wave of DNA replication followed by cell division after injury to, or surgical removal of, a portion of the liver (partial hepatectomy). The mechanisms responsible for the rapid initiation as well as termination of constitutive DNA synthesis in hepatocytes after hepatectomy are precisely regulated. After partial hepatectomy, DNA synthesis initiates after ~ 14 h and reaches a peak at ~ 24 h. The mass of the remaining liver doubles in 48 h and is restored to its original size after ~ 1 wk (Grisham, 1962; Bucher and Malt, 1971). Although various regulatory pathways have been identified in the regenerating liver, it is still unclear how these pathways may interact.

Among the growth factors that appear to play important roles in the regulation of both normal and pathologic conditions in the liver is transforming growth factor- β (TGF- β). The TGF- β s are polypeptides that act as multifunctional regulators of cell growth and differentiation in nearly all cells examined (for recent reviews see Massagué, 1990; Roberts and Sporn, 1990). Thus far, five distinct, highly conserved, yet functionally similar TGF- β s have been described: TGF- β s 1–3 in mammals and chickens, and TGF- β s 4 and 5 in chickens and amphibians. The TGF- β s show 64–82% identity at the amino acid level, share similar essential structural features, and have analogous biological activities (Cheifetz *et al.*, 1987; Graycar *et al.*, 1989; Roberts and Sporn, 1990).

The liver is a major site of TGF- β 1 metabolism in vivo (Coffey *et al.*, 1987). TGF- β 1 has been shown to be a strong inhibitor of hepatocyte proliferation in vivo and in vitro (Nakamura *et al.*, 1985; Carr *et al.*, 1986; McMahon *et al.*, 1986; Strain *et al.*, 1987; Braun *et al.*, 1988; Russel *et al.*, 1988) and has effects in hepatic fibrogenesis (Czaja *et al.*, 1989; Matsuoka and Tsukamoto, 1990; Nakatsukasa *et al.*, 1990a),

carcinogenesis (Liu *et al.*, 1988; Braun *et al.*, 1990; Houck *et al.*, 1990; Huggett *et al.*, 1990), and liver epithelial cell proliferation (Nagy *et al.*, 1989). Despite these studies, the exact role of the various TGF- β isoforms in liver regeneration is still not well defined.

In this study, we have examined the level of expression of TGF- β s 1–3 mRNAs in different cell populations in normal and regenerating rat liver and the sensitivity of hepatocytes isolated from intact and regenerating liver to DNA synthesis inhibition by these different TGF- β isoforms *in vitro*. We show that the TGF- β isoforms have similar effects and localization in the liver, but that their levels are independently regulated during liver regeneration. We have also examined potential mechanisms of action of TGF- β 1 during liver growth.

Results

Expression of TGF- β mRNAs during liver regeneration

The levels of expression of TGF- β s 1–3 mRNAs in livers of normal, sham-operated, and partially hepatectomized rats at various times after the operations were examined by the use of RNA Northern blot analysis of poly(A)+ RNAs. As shown previously, hybridization with [32 P]-labeled random-primed rat TGF- β 1 cDNA showed expression of a 2.4-kb TGF- β 1 mRNA (Braun *et al.*, 1988; Nakatsukasa *et al.*, 1990a) and, in addition, a minor TGF- β 1 mRNA transcript of 1.9 kb (Fig. 1A). By 4 h after partial hepatectomy, the levels of expression of both TGF- β 1 mRNAs increased significantly and remained elevated until 96 h after the operation. The increase in the levels of expression of the TGF- β 1 mRNAs in regenerating liver compared with normal liver was not merely a consequence of simple surgical stress and anesthesia, as indicated by the constant low levels of expression of both TGF- β 1 mRNAs in livers of rats at various times after sham operation (Fig. 1A). Probing the same blots with mouse TGF- β s 2 and 3 cDNAs revealed five TGF- β 2 mRNAs (3.0, 3.7, 4.0, 5.1, and 5.8 kb) and two TGF- β 3 mRNAs (3.0 and 3.5 kb). The levels of expression of the predominant forms of TGF- β 2 mRNA (4.0, 5.1, and 5.8

kb) as well as that of the major TGF- β 3 mRNA specie (3.5 kb) increased by 4 h after partial hepatectomy (Fig. 1, B and C). In contrast to the TGF- β 1 mRNAs, expression of the TGF- β 2 mRNAs remained elevated for ~8 h after partial hepatectomy before decreasing to about one-half of this level, whereas the TGF- β 3 mRNAs decreased between 4 and 8 h after partial hepatectomy. The levels of expression of all three of the TGF- β mRNAs were very low in the livers of normal or sham-operated rats. As a control, the gels were stained with ethidium bromide and photographed to ensure that approximately equal amounts of RNA had been applied to the gels (Fig. 1D).

Because the liver is composed of several cell types, expression of TGF- β s 1–3 mRNAs was examined in hepatocytes and nonparenchymal cells in both normal and regenerating livers. As shown previously for TGF- β 1 mRNA, expression of all three TGF- β mRNAs was detected in nonparenchymal cells from normal and 24-h regenerating livers, but not in hepatocytes purified from the same livers (Fig. 2).

Expression of extracellular matrix protein mRNAs during liver regeneration

TGF- β has been shown to be of major importance in controlling the formation and digestion of numerous components of the extracellular matrix, including various types of collagen, as well as fibronectin and proteoglycans (see Sporn and Roberts, 1990). Type I collagen mRNA increases during liver growth and toxic injury in rats (Czaja *et al.*, 1989; Nakatsukasa *et al.*, 1990a,b). In patients with chronic liver disease, the levels of Type I collagen mRNA and serum Type III collagen amino-terminal peptide closely correlate with the levels of TGF- β 1 mRNA in the liver (Castilla *et al.*, 1991). Here, we examined the pattern of expression of mRNAs for several extracellular matrix proteins during liver regeneration. Probing RNA Northern blots with fibronectin cDNA showed expression of a 7.7-kb mRNA (Fig. 3A). By 4 h after partial hepatectomy, the level of expression of the fibronectin mRNA increased until 12 h after the operation; expression then decreased gradually until ~72 h. At this time, the

Figure 1. Expression of TGF- β mRNAs in regenerating rat liver. Poly(A)+ RNA was prepared from rat livers at various times (4–96 h) after partial hepatectomy (R), after sham operation (S), or from intact rats (N). Five micrograms of each RNA were electrophoresed in an agarose-formaldehyde gel, transferred to a nitrocellulose filter, and hybridized. The times after partial hepatectomy are labeled above each lane. Hybridization was performed with [32 P]-labeled random-primed (A) plasmid pTGFB-R1 (rat TGF- β 1) cDNA, (B) plasmid pTGFB-M2 (mouse TGF- β 2) cDNA, or (C) plasmid pTGFB-M3 (mouse TGF- β 3) cDNA. The positions of the TGF- β mRNAs are shown to the right in each panel. (D) The ethidium bromide staining profile of the gel showing 18 and 28 S rRNAs.

Figure 2. Expression of TGF- β mRNAs in nonparenchymal cells. Nonparenchymal cells (NPC) and hepatocytes (H) were isolated from normal (N) or regenerating liver 24 h after partial hepatectomy (R). Total cell RNA was extracted and hybridized with [32 P]-labeled random-primed rat TGF- β 1 cDNA, mouse TGF- β 2 cDNA, and mouse TGF- β 3 cDNA. The panel on the lower right shows hybridization of NPC, H, and whole liver (L) RNA with a probe for vitamin D binding protein, used as a positive control for the hepatocyte preparations.

level of expression of fibronectin mRNA in regenerating liver was approximately equal to that of the normal liver. Using vitronectin and laminin B1 cDNAs as probes, we found that the levels of expression of both the 1.6-kb vitronectin mRNA and the 5.7-kb laminin B1 mRNA also increased by 4 h after partial hepatectomy. Similar results were obtained for laminin B2 mRNA (data not shown). However, the levels of expression of both the vitronectin and laminin B1 mRNAs remained elevated for a longer period than that of fibronectin mRNA (Fig. 3, B and C). Expression of vitronectin but not of laminin mRNA was detected in livers of normal and sham-operated rats, and the relative increase in laminin B1 mRNA in regenerating liver compared with normal liver was higher than for vi-

tronectin mRNA. The levels of expression of the 4.7-kb Type I collagen α 2 chain and Type III collagen α 1 chain mRNAs were also elevated by 4 h after partial hepatectomy; and, as in vitronectin and laminin B1, the level of expression of both of these collagen mRNAs remained elevated up to 96 h after the operation (Fig. 3, D and E).

Expression of TGF- β s in regenerating rat livers

To investigate further the roles of the different TGF- β isoforms during liver regeneration, we examined immunohistochemical staining patterns of TGF- β s 1–3 in normal, regenerating, and sham-operated livers. Figure 4A shows the

Figure 3. Expression of mRNAs for matrix proteins in regenerating rat liver. Poly(A)⁺ RNA was prepared from rat livers and electrophoresed as described in Figure 1. Hybridization was performed with [³²P]-labeled random-primed (A) rat fibronectin cDNA, (B) rat vitronectin cDNA, (C) rat laminin B1 cDNA, (D) rat Type I collagen a2 cDNA, or (E) rat Type III collagen a1 cDNA.

immunohistochemical staining pattern of TGF- β 1 in regenerating liver, using antibodies raised against a synthetic peptide consisting of the initial 30 amino acids of the mature region of human TGF- β 1 (identical to that of rat TGF- β 1). In general, staining was seen in bile duct cells and endothelial cells located in portal spaces, in some sinusoidal lining cells, and in scattered hepatocytes throughout the lobules, but fre-

quently localized around central veins (Figure 4B). The staining pattern was similar for TGF- β s 2 and 3 (data not shown). The staining pattern of TGF- β in regenerating liver was similar to that of normal liver. Staining for TGF- β s 1–3 was completely blocked by preincubation of the individual anti-TGF- β IgGs with solutions of the peptides against which they were raised (data not shown).

Figure 4. Immunohistochemical detection of TGF- β . Sections from 48-h regenerating liver were incubated with anti-P 1-30(1) antibody. (A) Positive hepatocytes throughout the lobule. (B) Positive hepatocytes lining a central vein. Bar, 100 μ m.

Figure 5. Effect of TGF- β s 1 and 3 on EGF-induced DNA synthesis of cultured hepatocytes. Hepatocytes were isolated from normal livers. Cells (2×10^5) were allowed to attach for 3 h on collagen-coated 35-mm dishes in MEM containing 5% fetal calf serum. Fresh serum-free MEM supplemented with EGF (20 ng/ml) and TGF- β s 1, 2, or 3 (0–100 pg/ml) was added every 24 h. Cells were labeled for 24 h with [3 H]-thymidine (5 μ Ci; 1 μ Ci = 37 kBq) between 48 and 72 h in culture (peak of DNA synthesis). Incorporated radioactivity (cpm) was measured at 72 h by scintillation counting of trichloroacetic acid precipitates of cell extracts. DNA synthesis was calculated as cpm incorporated per milligram of protein and is expressed as a percentage of DNA synthesis in cells not exposed to TGF- β . Each point represents the average of duplicate plates.

The levels of TGF- β s 1 and 2 in normal and regenerating liver were measured by sandwich enzyme-linked immunosorbent assays (ELISA). The levels of assayed TGF- β s 1 and 2 in normal, regenerating, and sham-operated livers were approximately the same. However, in all samples, the amount of TGF- β 1 was 8- to 10-fold higher than that of TGF- β 2 (TGF- β 1 = 1.5 ± 0.2 [mean $\pm$ SD] ng/ml liver extract vs. TGF- β 2 = 0.19 ± 0.09 ng/ml liver extract). TGF- β 3 cannot yet be quantitated with this type of assay.

Sensitivity of hepatocytes from normal liver to TGF- β : effects on DNA synthesis

TGF- β has been shown to be a strong inhibitor of hepatocyte DNA synthesis in vitro (Nakamura *et al.*, 1985; Carr *et al.*, 1986; McMahon *et al.*, 1986; Braun *et al.*, 1988). To determine whether hepatocytes from normal livers might differ in their sensitivity to inhibition by TGF- β s 1–3, we maintained hepatocytes isolated from normal rats in modified Eagle's minimum essential medium (MEM) in the absence of serum; epidermal growth factor (EGF) and TGF- β s 1–3 were added together at the end of the attachment phase and at every medium change, performed at 24-h intervals. In these hepatocytes, very low concentrations of TGF- β s 1 and 3 inhibited DNA

synthesis induced by EGF measured between 48 and 72 h in vitro (Fig. 5); a similar inhibitory effect was also observed with TGF- β 2 (data not shown). The dose-response curves were very similar; TGF- β s 1–3 at 80 pg/ml inhibited 70–80% of DNA synthesis induced by EGF.

Mechanism of action of TGF- β 1

One of the proposed mechanisms of action of TGF- β 1 involves the inhibition of *c-myc* expression (Moses *et al.*, 1990). We examined whether the TGF- β s would interfere with the expression of certain proto-oncogenes in cultured hepatocytes. Dissociation of liver tissue by collagenase perfusion increased the expression of *c-fos* before the cells were placed in culture (Fig. 6A). *c-fos* expression remained high during the attachment period but then decreased rapidly, as described by Etienne *et al.* (1988). *c-myc* mRNA levels remained high for ≥ 8 h, whereas *p53* mRNA became detectable only after ~ 8 h in culture. Addition of TGF- β 1 at the end of the 2-h attachment period did not alter the expression of *c-myc* and *p53* mRNAs during the first 18 h in culture (Fig. 6B). Similar results were obtained with TGF- β 2 (data not shown). Studies reported by others in hamster fibroblasts have also shown that TGF- β was unable to alter the expression of *c-myc* mRNA (Chambard and Pouyssegur, 1988; Sorrentino and Bandyopadhyay, 1989). This is in contrast to endothelial cells, colon carcinoma cells, keratinocytes, and breast carcinoma cells, in which addition of TGF- β has been shown to inhibit expression of *c-myc* mRNA (Fernandez-Pol *et al.*, 1987; Takehara *et al.*, 1987; Coffey *et al.*, 1988; Mulder *et al.*, 1988).

A potential control point in TGF- β 1 action might involve the activation of latent TGF- β 1 secreted by cells (Lyons *et al.*, 1988; Wakefield *et al.*, 1988; Kamzaki *et al.*, 1990). Because no method is available to measure the amount of latent TGF- β 1 in tissues, we approached this question by examining the capacity of hepatocytes isolated from normal and regenerating liver to activate latent TGF- β 1 added to the culture medium (Fausto *et al.*, 1990). Latent TGF- β 1 was added either 1) as a preparation of nearly pure recombinant TGF- β 1 complexes consisting of molecular species of 75 000 and 100 000 Da (Wakefield *et al.*, 1989; Wallick *et al.*, 1990) or 2) as latent forms present in medium conditioned by A549 human lung carcinoma cells consisting of molecular species of 75 000 and 100 000 Da and, in addition, a 135 000-Da binding protein (A549 cells secrete latent TGF- β 1 but do not have the capacity to activate latent

Figure 6. Expression of proto-oncogenes in cultured hepatocytes. (A) Hepatocytes isolated from normal liver were plated (10×10^6 /150-mm dish) and allowed to attach as described. After a 2-hr attachment period, fresh serum-free medium containing EGF (20 ng/ml) was added and cells were harvested at various times for RNA extraction. Numbers across the bottom indicate the times (h) after the attachment phase. i, hepatocytes that were analyzed immediately after the isolation procedure. (B) Hepatocytes were plated, allowed to attach, and fed fresh serum-free medium containing (20 ng/ml) EGF or EGF (same dose) and TGF- β 1, 2, or 3 (100 pg/ml) as described in Materials and methods. At various times after the attachment phase, cells were harvested, RNA extracted, and examined by RNA Northern blot analysis for the expression of c-myc and p53 using [32 P]-labeled cDNA probes as described in Materials and methods. *, cultures that were treated with TGF- β .

forms; Wakefield *et al.*, 1987). Generation of active TGF- β 1 in either type of experiment was determined by measuring inhibition of DNA synthesis in hepatocyte cultures to which the latent forms were added. For comparison, the effects of heat-activated recombinant complexes or pure TGF- β 1 on hepatocyte DNA synthesis were determined in parallel cultures.

Normal hepatocytes were not able to activate latent recombinant TGF- β 1, because the amount of active growth factor generated in these cultures did not differ from that detected in Mv1Lu mink lung cells, which are incapable of activating the latent complex (Fig. 7A). In contrast, hepa-

toocytes obtained from 24- to 120-h regenerating liver demonstrated activating capacity (Fig. 7, A and B). In several experiments, the percentage of latent complex that was activated varied from 11 to 19% (basal level = 7%). Similar results were obtained in experiments in which medium conditioned by A549 cells (rather than latent TGF- β 1 complexes) was tested (Fig. 8). The two panels in Fig. 8 represent individual experiments using different batches of conditioned medium; although the degree of activation differed significantly between experiments, hepatocytes from regenerating liver showed a significant capacity to generate active TGF- β 1.

Figure 7. Activation of latent recombinant TGF- β 1 by cultured hepatocytes. (A) Hepatocytes isolated from normal (○) or regenerating (●) liver were cultured in MEM supplemented with EGF (20 ng/ml) and various doses (0–200 pM) of latent (—) or activated (---) recombinant TGF- β 1 (latent recombinant complex was activated by heating for 8 min at 80°C followed by rapid cooling). Medium and growth factors were replaced daily and DNA synthesis measured by [3 H]-thymidine incorporated for 24 h (between 48 and 72 h of culture). Incorporation of [3 H]-thymidine in the absence of TGF- β (100% point) corresponded to $3\text{--}3.8 \times 10^6$ cpm. (B) Hepatocytes isolated from normal (NL) and regenerating (RL) liver were cultured as above in the presence of latent or activated recombinant TGF- β 1. Percentage activation of latent TGF- β 1 is expressed as the dose of activated vs. latent TGF- β required to inhibit thymidine incorporation by 50%. Cells in each experiment were plated in duplicate. Mink lung (Mv1Lu) cells grown in MEM with latent or activated recombinant TGF- β , in which [3 H]-thymidine incorporation was similarly determined by a microtitre assay, were used as controls. The numbers across the bottom indicate the times after partial hepatectomy of the regenerating livers. The results of separate experiments are shown (●, ○).

Discussion

The mechanisms involved in hepatocyte replication and liver regeneration are not well understood, but growth factors and their receptors appear to play important roles in this process. The present study demonstrates that expres-

sion of TGF- β s 1–3 mRNAs increases in the liver after partial hepatectomy, beginning before the major wave of DNA synthesis and mitosis have taken place. Various laboratories have shown that TGF- β 1 mRNA levels in the regenerating liver remain high for ≥ 4 d (Braun *et al.*, 1988; Carr *et al.*, 1989; Nakatsukasa *et al.*, 1990a). Although these reports have shown variations in the exact time of the peak of TGF- β 1 mRNA expression, the combined data from these reports indicate that expression of TGF- β 1 mRNA begins in the G1 phase of the cell cycle and continues through the period of DNA synthesis. Expression of TGF- β s 2 and 3 mRNAs also increases early in G1, but, in contrast to TGF- β 1 mRNA, TGF- β s 2 and 3 mRNAs decrease rapidly before DNA synthesis reaches maximum levels.

Although our RNA Northern blot analysis demonstrates that expression of the mRNAs for TGF- β s 1–3 increases in the regenerating liver, no differences could be detected in the levels of protein for TGF- β s 1–3 between normal and regenerating liver by sandwich ELISA or by immunohistochemical staining analysis. Possible explanations for these discrepancies could include 1) lability or masking of epitopes in the regenerating liver (by a binding protein, for example) or 2) failure of the increased TGF- β mRNAs to be translated in regenerating liver. Studies reported by others in lymphocytes (Kehrl *et al.*, 1986b), macrophages (Assoian *et al.*, 1987), and spleen (Thompson *et al.*, 1989) have also shown that levels of TGF- β mRNA and protein production do not always correlate.

The increase in the levels of expression of all three TGF- β mRNAs in regenerating liver, their localization in nonparenchymal cells, the inhibition of hepatocyte DNA synthesis in vitro by all three TGF- β s, and the inhibition of hepatocyte replication in vivo by TGF- β 1 (Russell *et al.*, 1988; Schackert *et al.*, 1990), provide strong evidence that TGF- β regulates hepatocyte proliferation through a paracrine mechanism. The activation of this mechanism during liver regeneration may prevent uncontrolled cell growth. TGF- β 1 reversibly inhibits the growth of a variety of normal cell types (e.g., Moses *et al.*, 1985; Roberts *et al.*, 1985; Frater-Schroder *et al.*, 1986; Kehrl *et al.*, 1986a; Shipley *et al.*, 1986; Cheifetz *et al.*, 1987). These examples, in addition to our results, suggest that the induction of TGF- β by growth stimuli might be part of a general feedback mechanism that is present in nonneoplastic growth processes.

Because most cells, including hepatocytes (Gruppiso *et al.*, 1990), have receptors for TGF- β , selective effects of the TGF- β s must depend on other mechanisms. The TGF- β s differ from

Figure 8. Ability of cultured hepatocytes to activate latent TGF- β 1 in medium conditioned by A549 cells. Hepatocytes obtained from normal (■, □) or 72-h regenerating (▲, △) livers were maintained in medium containing EGF (20 ng/ml) and various doses of 20-fold-concentrated A549 conditioned medium either nontreated (■, ▲) or acidified (□, △). Medium was replaced daily and hepatocytes were labeled with [3 H]-thymidine (5 μ Ci/plate) for 24 h before harvesting after the 3rd d of culture. Incorporation of radioactivity was determined by trichloroacetic acid precipitation; each value represents the average of duplicate plates. Each panel represents individual experiments using different batches of conditioned medium.

most growth factors in that they are usually secreted in biologically latent forms that must be activated before they can exert biological effects on target cells (Lawrence *et al.*, 1985; Wakefield *et al.*, 1987). This latent form of TGF- β appears to be the predominant naturally occurring form of the molecule (Wakefield *et al.*, 1990). Our results suggest that there may be important differences in the ability of the liver to activate latent TGF- β ; thus, hepatocytes from regenerating but not from normal liver are able to activate latent TGF- β complexes *in vitro*. Although these experiments do not mimic the *in vivo* situation, they raise the possibility that replicating hepatocytes *in vivo* might be capable of generating signals that suppress their growth. Neither the time at which latent TGF- β is activated in the regenerating liver nor the mechanisms of activation are known. Nevertheless, it is logical to speculate that activation of TGF- β by hepatocytes may be required to oppose the stimulatory effects of growth factors such as TGF- α (Mead and Fausto, 1989) and might be critical to the regulation of liver regeneration.

The expression of the mRNAs for several extracellular matrix proteins, including fibronectin, vitronectin, laminin, and collagen, is also increased in the regenerating liver. Because one

of the prominent functions of TGF- β 1 is the regulation of extracellular matrix proteins (Ignotz and Massagué, 1986; Roberts *et al.*, 1986), an important function of TGF- β in the liver could be promotion of extracellular matrix protein synthesis, which may be necessary for liver growth. The extracellular matrix provides not only the architecture for hepatocytes' attachment but also the proper medium for their interaction with epithelial cells (Bissel *et al.*, 1987; Collier *et al.*, 1988). Increased expression of the Type I collagen α 1 chain mRNA has been reported in periductal cells and periportal cells during liver regeneration, and this expression has been demonstrated to parallel that of expression of TGF- β 1 mRNA (Nakatsukasa *et al.*, 1990a). The timing of the increase in the expression of all three TGF- β mRNAs and the mRNAs for the several extracellular matrix proteins after partial hepatectomy is thus consistent with a role of TGF- β in regulating extracellular matrix synthesis and turnover in the regenerating liver. These studies indicate that the TGF- β isoforms might be involved in the regulation of hepatocyte proliferation as well as in the induction of extracellular matrix synthesis during liver regeneration after partial hepatectomy. The mechanisms by which the TGF- β iso-

forms block hepatocyte DNA synthesis have not been established. Although it does not appear that inhibition of *c-myc* expression is required for the inhibitory effect of TGF- β in hepatocytes, it is possible that restoration of the extracellular matrix functions as a signal to terminate hepatocyte replication.

Materials and methods

Animals and cells

Male albino rats (CD strain, Charles River Breeding Laboratories, Wilmington, MA; 140–180 g) were used for all experiments, including partial hepatectomy and cell isolation procedures. Partial hepatectomies, sham operations, and maintenance of the animals were as described by Braun *et al.* (1988). A549 human lung carcinoma cells (ATCC #CCL-185) and Mv1Lu mink lung epithelial cells (ATCC #CCL-64) were obtained from the American Type Culture Collection (Rockville, MD).

Cell separation procedures

Hepatocytes and nonparenchymal cells were isolated by a two-step in situ collagenase perfusion of the liver (Seglen, 1976) with minor modifications (Braun *et al.*, 1988). Hepatocytes were collected by repeated centrifugation ($50 \times g$) of the cell suspension. Nonparenchymal cells were isolated by further digestion of the remaining liver with collagenase (0.1%), pronase (0.1%), and DNase (0.004%) (Yaswen *et al.*, 1984).

Hepatocyte cultures

Hepatocytes were plated (2×10^5 cells per 35-mm dish) on dishes coated with rat tail collagen in MEM containing 5% (vol/vol) fetal calf serum, 1 mM pyruvate, 0.2 mM aspartate, 1 mM proline, 0.2 mM serine, and 2 mM glutamine as well as 0.5 μ g of hydrocortisone and 1 μ g of insulin per ml. After a 2-h attachment period, fresh serum-free medium was added along with porcine TGF- β 1, porcine TGF- β 2, or recombinant chicken TGF- β 3 (100 pg/ml). The medium was changed daily, and cells were labeled by the addition of 5 μ Ci (1 μ Ci = 37 GBq) of [3 H]thymidine per well 24 h before harvesting. Thymidine incorporation into DNA was determined after precipitation of cell extracts with trichloroacetic acid (Michalopoulos *et al.*, 1984).

RNA extraction and RNA Northern blot analysis

Total RNA was extracted from normal, regenerating, and sham-operated livers by the method of Chirgwin *et al.* (1979), with minor modifications (Braun *et al.*, 1988). Poly(A)⁺ RNA was obtained by oligod(T)-cellulose affinity chromatography (Collaborative Research, Waltham, MA) according to Aviv and Leder (1972). For RNA Northern blot analysis, equal amounts of RNA (5 μ g for poly(A)⁺ RNA) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH). Ethidium bromide (33 μ g/ml) was included in both the gels and running buffers to visualize the positions of ribosomal RNAs by ultraviolet illumination after electrophoresis. Before blotting, the gels were pretreated with 50 mM NaOH in $1 \times$ SSC (150 mM sodium chloride/15 mM sodium citrate) for 20 min at room temperature and then rinsed two times in $10 \times$ SSC for 20 min each. Blots were hybridized with [32 P]-labeled (3000 Ci/mmol, New England Nuclear, Boston, MA) nick-translated or random-

primed probes at 65°C and washed at 65°C according to Church and Gilbert (1984), then exposed for various times at -70°C using an intensifying screen. Densitometry of autoradiographs was performed using an LKB (Piscataway, NJ) Ultrascan laser densitometer.

cDNA probes

Hybridization was performed using the following cDNA probes: 0.9-kb *Xba*-*Hind*III fragment of rat TGF- β 1; 1.0-kb *Xba*-*Hind*III fragment of mouse TGF- β 2; 0.75-kb *Xba*-*Hind*III fragment of mouse TGF- β 3; 1.5-kb fragment of human vitronectin encompassing nucleotides 1340–2870 (Telios, San Diego, CA); 4.6-kb *Eco*RI-*Hind*III fragment of mouse laminin B1; 1.7-kb *Eco*RI-*Xba*I fragment of mouse laminin B2; 0.5-kb *Eco*RI fragment of rat fibronectin; 0.8-kb *Xho*I fragment of mouse Type I collagen α 2 chain; and 1.8-kb *Eco*RI fragment of rat Type III collagen α 1 chain.

Extraction and quantitation of TGF- β in liver

Normal, regenerating, and sham-operated livers were extracted with acid-ethanol and extensively dialyzed against 4 mM HCl (Roberts *et al.*, 1981). TGF- β s were quantitated by determining the extent of growth inhibition of mink lung epithelial cells (Danielpour *et al.*, 1989a) and were typed based on reactivity in sandwich ELISA (Danielpour *et al.*, 1989b) for TGF- β s 1 and 2.

Preparation of TGF- β -specific antibodies

Synthetic peptides corresponding to unique segments of human TGF- β 1, human TGF- β 2, and chicken TGF- β 3 were purchased from Peninsula Laboratories (Belmont, CA). A summary of the sequences used and the methods for preparation and purification of the antibodies raised to these synthetic peptides have been previously described (Jakowlew *et al.*, 1991).

Preparation of tissues for immunohistochemical staining

Excised tissues were washed in phosphate-buffered saline, fixed for 48–96 h in 10% neutral-buffered formalin, treated in Bouin's solution for 6 h at room temperature, and stored in 70% ethanol before being embedded in paraffin and sectioned at 5 μ m.

Immunohistochemical staining

TGF- β was localized in sections as described by Heine *et al.* (1987), using avidin-biotin-peroxidase kits (Vector Laboratories, Burlingame, CA). After deparaffinization, blocking of endogenous peroxidase in hydrogen peroxide/methanol, and permeabilization with hyaluronidase, the sections were blocked with 1.5% normal goat serum/0.5% bovine serum albumin, incubated overnight at 4°C with affinity-purified antisera at 3–5 μ g/ml, washed extensively, and then incubated with biotinylated goat anti-rabbit IgG and avidin-enzyme complex. Sections were stained with 3,3'-diaminobenzidine (Sigma, St. Louis, MO) and hydrogen peroxide, and counterstained with Mayer's hematoxylin. Controls include 1) replacing primary antisera with normal rabbit IgG and 2) using primary antisera that had been preincubated with a 20-fold molar excess of the appropriate peptide for 2 h at room temperature before this mixture was applied to the section.

Activation of latent TGF- β 1 by cultured hepatocytes

For these experiments, hepatocytes obtained from normal and regenerating livers were maintained in culture for 72 h

in supplemented MEM containing 20 ng/ml EGF, as previously described (Braun *et al.*, 1988). Latent or activated recombinant TGF- β 1 complex (gift of Dr. L. Wakefield) was added to the cultures at concentrations of 5–200 pM. The latent TGF- β 1 complex was activated by heating at 80°C for 8 min, followed by rapid cooling. In other experiments, the source of TGF- β 1 was A549 cells (human lung carcinoma) conditioned medium (Wakefield *et al.*, 1987), concentrated 20-fold by ultrafiltration. The latent TGF- β 1 complex in the conditioned medium was activated by acidification followed by neutralization. Untreated or treated conditioned medium was added to hepatocytes in amounts ranging from 30 to 140 μ l. For experiments involving either recombinant complexes or conditioned medium, the hepatocyte culture medium was replaced daily. [3 H]-thymidine (5 μ Ci/plate) was added at 48 h and the cultures were harvested at 72 h. Duplicate plates with latent or activated TGF- β 1 were included in each experiment.

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