Cell-specific Expression of Transforming Growth Factor-β in Rat Liver

Evidence for Autocrine Regulation of Hepatocyte Proliferation

D. Montgomery Bissell,* Shao-Shean Wang,* William R. Jarnagin,‡ and F. Joseph Roll*

*The Liver Center Laboratory, San Francisco General Hospital; and the Departments of *Medicine and ‡Surgery, University of California, San Francisco, California 94110*

Abstract

Expression of the group of cytokines known as transforming growth factor-β (TGF-β1, -β2 and -β3) is increased during liver regeneration induced by a 70% partial hepatectomy. The origin of these changes was examined in purified isolates of hepatocytes, sinusoidal endothelial cells, Kupffer cells (liver macrophages), and lipocytes (Ito or stellate cells) from normal and regenerating liver. In normal liver, TGF-β1 and -β2 levels were relatively high in sinusoidal endothelial cells and Kupffer cells. After partial hepatectomy, an early peak of TGF-β2 and -β3 was present in all four cell types, followed by a sustained increase in mRNA for TGF-β1, -β2, and -β3 primarily in the hepatocyte population. The specificity of these changes was established by examining a mechanistically different injury model, fibrosis induced by ligation of the biliary duct. In this model, TGFβ mRNA was increased only in lipocytes and the increase was progressive over a 7-d period of observation. Secretion of TGFβ protein was examined in cell isolates placed in short-term primary culture and generally reflected the corresponding mRNA level. The TGFβ released by hepatocytes was entirely in the latent form, whereas the individual nonparenchymal cell isolates released 50–90% active TGFβ. Hepatocyte-conditioned culture medium, after treatment to activate latent TGFβ, inhibited hepatocellular DNA synthesis as did the authentic factor. The data indicate that after injury TGFβ increases selectively in the cells that are the target of the factor, i.e., in hepatocytes after partial hepatectomy and in lipocytes in inflammation and fibrosis. We conclude that the effects of TGFβ in liver regeneration and fibrogenesis are predominantly, if not exclusively, autocrine. (*J. Clin. Invest.* 1995. 96:447–455.) Key words: partial hepatectomy • regeneration • lipocyte • stellate cells • Ito cell

Introduction

The family of TGFβ comprises three related subtypes, all of which exist in mammals (TGF-β1, -β2, and -β3) (1). Among the numerous and varied effects attributed to these factors, one of the most prominent is growth inhibition (2). This has been documented notably for hepatocytes: TGFβ inhibits hepatocellular DNA synthesis both in culture (3–7) and in vivo (8). Thus, it has been proposed that TGFβ may be the principal counterregulator of hepatocyte proliferation during regeneration (9). Important corroborative evidence for this hypothesis is the finding that TGFβ mRNA increases after a 70% partial hepatectomy (10, 11). To date, studies of isolated hepatocytes or mixed nonparenchymal cells suggest that the increase in mRNA derives from the latter fraction (perhaps endothelial cells), consistent with a paracrine effect on hepatocytes; it has also been stated that the β1 form is quantitatively the most important of the TGFβ subtypes during regeneration (12). However, the cellular source(s) of TGFβ remains unclear: nonparenchymal isolates are a mixture of sinusoidal endothelial cells, Kupffer cells (liver macrophages), and lipocytes (pericyte-like cells also known as Ito or fat-storing cells). Moreover, a role for hepatocytes is not excluded, given the immunohistological evidence that TGFβ protein is present in (or on) these cells in the intact liver (12, 13). Until the question of cellular localization is resolved, the mechanism by which TGFβ regulates growth (autocrine or paracrine) in liver regeneration will remain speculative.

The problem is one that requires isolation and quantitative measurement of TGFβ gene expression in the individual cell types that constitute a tissue—an analysis that has not been performed in any epithelium to date, owing in large part to the difficulty in dispersing and purifying the individual cell types that make up a tissue. In liver, however, the methods for this purpose are well developed; moreover, yields are sufficient for direct mRNA analysis without amplification of cell samples in culture. We have used this approach in the current study. Isolates from normal liver or from the regenerating remnant after partial hepatectomy were prepared, and cell-specific expression of TGFβ was directly examined, using an RNase protection assay to quantitate mRNA for the three TGFβ subtypes. Isolates were also placed in short-term primary culture for evaluating the secretion of TGFβ protein, both the active form and the latent precursor. To document the specificity of the changes after partial hepatectomy, we performed parallel studies of an injury model in which hepatocyte proliferation is minimal, namely, fibrosis induced by ligation of the biliary duct. The findings indicate, contrary to previous conclusions (12), that hepatocytes are a major source of TGFβ during liver regeneration and that they release the latent form.

Methods

Materials. Pronase, collagenase B, and DNase I were purchased from Boehringer Mannheim Biochemicals Biochemicals (Indianapolis, IN). Media and sera were from Flow Laboratories (McLean, VA); Larex (arabinogalactan) was from Consulting Associates (Tacoma, WA). Other enzymes and chemicals were molecular biology grade and were purchased from Fluka Chemical Corp. (Ronkonkoma, NY), Bio-Rad

Address correspondence to Dr. D. M. Bissell, Liver Center Laboratory, Building 40, Room 4102, San Francisco General Hospital, San Francisco, CA 94110. Phone: 415-206-4808; FAX: 415-641-0517.

Received for publication 20 October 1993 and accepted in revised form 22 March 1995.

J. Clin. Invest.

0021-9738/95/07/0447/09 $2.00
Volume 96, July 1995, 447–455

Laboratories (Richmond, CA), GIBCO-BRL (Gaithersburg, MD), or Promega Corp. (Madison, WI). Anti-TGFβ antibodies were purchased from R & D Systems, Inc. (Minneapolis, MN).

Animals. Young, male Sprague-Dawley rats (200–250-g body weight) or mature animals (~ 400 g) were used with similar results. Animals were housed under controlled lighting, with chow and water ad libitum, and were subjected to 70% partial hepatectomy or bile duct ligation under ether anesthesia (14, 15). Sham-operated animals underwent abdominal incision and tissue manipulation, without ligation of the bile duct or liver lobes. All surgery was performed between 08:00 and 09:00 hours. The abdomen was closed with silk sutures, and the animals were maintained postoperatively on food and water ad libitum.

Liver cell isolation and purification. Hepatocytes were prepared by perfusion of the liver in situ with collagenase and purified by centrifugal elutriation, as previously described (16). Lipocytes, Kupffer cells, and sinusoidal endothelial cells were isolated separately from liver dispersed with a mixture of collagenase and Pronase; the initial mixture of cells was fractionated on a discontinuous gradient of Larex and further purified by centrifugal elutriation, as described (17). Each data point represents the cell isolate from a single rat liver, unless otherwise noted.

Established markers were used to assess the homogeneity of specific cell isolates; these have been described previously and can be summarized as follows. Hepatocytes were identified unambiguously under phase contrast microscopy by their size and distinctive cytologic features (18); Kupffer cells were identified by endogenous peroxidase activity, demonstrated histochemically (19). For identifying sinusoidal endothelial cells, human acetoacetylated LDL, prepared with a stable fluorescent lipid, was administered to rats intravenously a few minutes before liver perfusion; within the liver, the lipid accumulates at high concentration only in the sinusoidal endothelium (20). Isolated endothelial cells were identified by their red fluorescence. Lipocytes were identified by fluorescence microscopy under ultraviolet excitation, which reveals pale blue granules representing the retinoid-containing vacuoles that are characteristic of these cells (17). Biliary epithelial and "oval" cells (from liver subjected to bile duct ligation) were identified histochemically by their content of γ-glutamyl transpeptidase (21).

The purity of isolates from normal or regenerating liver was 98% for hepatocytes, > 95% for lipocytes and endothelial cells, and 90–95% for Kupffer cells. Results were similar for isolates from liver after bile duct ligation except for the Kupffer cell fraction, in which 5–10% of the cells were lipocytes and 5–10% were biliary epithelial cells. An isolate enriched in biliary epithelial cells was obtained from the cholestatic liver after fractionation of the initial digest by Larex density gradient centrifugation as used for the isolation of lipocytes (17). The top interface of the Larex gradient, which normally consists of essentially pure lipocytes, contained two bands: a light band of lipocytes and a heavier, brown-colored band that proved to be largely biliary/oval cells (85–90% of the isolate, by cell number) when stained for γ-glutamyl transpeptidase.

Liver cell culture. Hepatocytes were cultured on collagen-coated plastic dishes in a modified Medium 199 (16) containing 5% calf serum. Individual nonparenchymal cell isolates were cultured in a 1:1 mixture of Ham's F-10 medium and Dulbecco's MEM containing 20% serum (1:1, horse/calf) (17). Media were changed daily.

For immunohistological demonstration of TGFβ, hepatocyte cultures were exposed to acidic pH to activate latent TGFβ (see the following section) and then fixed in methanol. Incubation with primary antibody was performed as described previously (18), followed by incubation with biotinylated secondary antibody and streptavidin-conjugated Texas red.

Assay of TGFβ mRNA. cDNAs for mouse TGF-β1, -β2, and -β3 in riboprobe vectors (22) were generously provided by Dr. H. Moses (Vanderbilt University, Nashville, TN). Radiolabeled cRNA (sense or antisense) was prepared by transcription of the plasmid with the appropriate polymerase in the presence of [^{32}P]CTP. RNA from cells or tissue was extracted and quantitated by RNase protection assay, as described previously (23). Negative controls included cellular RNA incubated with radiolabeled sense cRNA and a nonspecific RNA (yeast tRNA) incubated with the antisense probe. Neither yielded detectable specific signals; the controls using tRNA are shown. The hybridization temperature was 58°C for assay three TGFβ mRNAs and 55°C for the S-14 assay. Protected fragments were separated on a 5% polyacrylamide–urea sequencing gel at 50–55°C. Radiolabeled bands were visualized by autoradiography and quantitated by scanning densitometry (Hoefer Scientific, San Francisco, CA). The autoradiographic data were normalized to an internal mRNA, S-14, which encodes a ribosomal protein (24). The expression of S-14 varies minimally among the cell types of the liver or between normal and injured liver (25). In a given experiment, assays for TGFβ using the different cell extracts were performed and analyzed together.

The amount of RNA in extracts from cells or tissues was monitored spectrophotometrically. In preliminary experiments, it was found that the RNase protection assay was linear for input RNA up to at least 100 μg.

Bioassay of TGFβ. The assay was based on a published method (26), in which TGFβ is measured by its ability to inhibit proliferation of mink lung epithelial cells. With pure standards (R & D Systems, Inc.), the assay was found to have a working range of 0.1–2.0 pM. Fresh serum-free medium had no effect on the standard curve generated by pure TGFβ. Hepatocyte-conditioned medium contained growth-stimulating activity, as reported previously (11), causes a shift in the standard curve. The reported values for hepatocytes have been corrected for this effect. For evaluating TGFβ secretion in culture, the various liver cell isolates were plated and maintained for 2 d under standard conditions (see previously described procedure), washed, and incubated further in serum-free medium. In some experiments, cells were isolated, plated directly in serum-free medium, and incubated for 24 h only. The conditioned medium was harvested, cleared of cells by centrifugation, and assayed for TGFβ. Samples were assayed both with and without prior activation of latent TGFβ. The latter was accomplished by acidification of the medium (0.05 N HC, final concentration). After a 10-min incubation at 25°C, the medium was neutralized with 0.05 N NaOH and buffered with Hepes, pH 7.4 (0.025 M, final concentration). An aliquot (10 μl) was diluted into assay medium (200 μl, total volume). Each assay was performed in duplicate; the concentration of TGFβ was determined by comparison with the appropriate standard curve.

Antibody to TGFβ was added to the sample for assay, at a concentration of 20 μg/ml for anti–TGF-β1 and 10 μg/ml for anti–TGF-β2 or -β3. Under these conditions, the antibody to TGF-β1 blocked 70–80% of TGF-β1 activity; the antibodies to TGF-β2 and -β3 were 100% effective; cross-reactivity was negligible. Control assays with nonimmune IgG were included.

Statistical analysis. Data are presented as mean±SD. Differences have been analyzed by *t* test for unpaired groups.

Results

TGFβ mRNA in whole-liver extracts after injury. In extracts of normal liver, mRNA for all three TGFβ isoforms was low but detectable by RNase protection assay of 100 μg of total RNA. The levels were essentially unchanged in sham-operated animals at various times points between 2 h and 7 d, except for a transient increase in TGF-β3 mRNA at 2 h to 2.7-fold above the control level (data not shown).

After partial hepatectomy, TGF-β2 and -β3 exhibited a peak at 6 h; by 48 h, TGF-β2 decreased to a plateau approximately two-fold above the control level, and TGF-β3 returned to baseline. The profile of TGF-β1 lacked the early peak, showing instead a rise over 24–48 h to a plateau that was 2.5-fold above control (Fig. 1 *A* and Table I).

With bile duct ligation, little change from control levels occurred for 24 h; then all three TGFβ mRNAs increased progressively over the entire 7-d period of study (Fig. 1 *B*).

TGFβ mRNA in cell isolates from normal liver, after partial

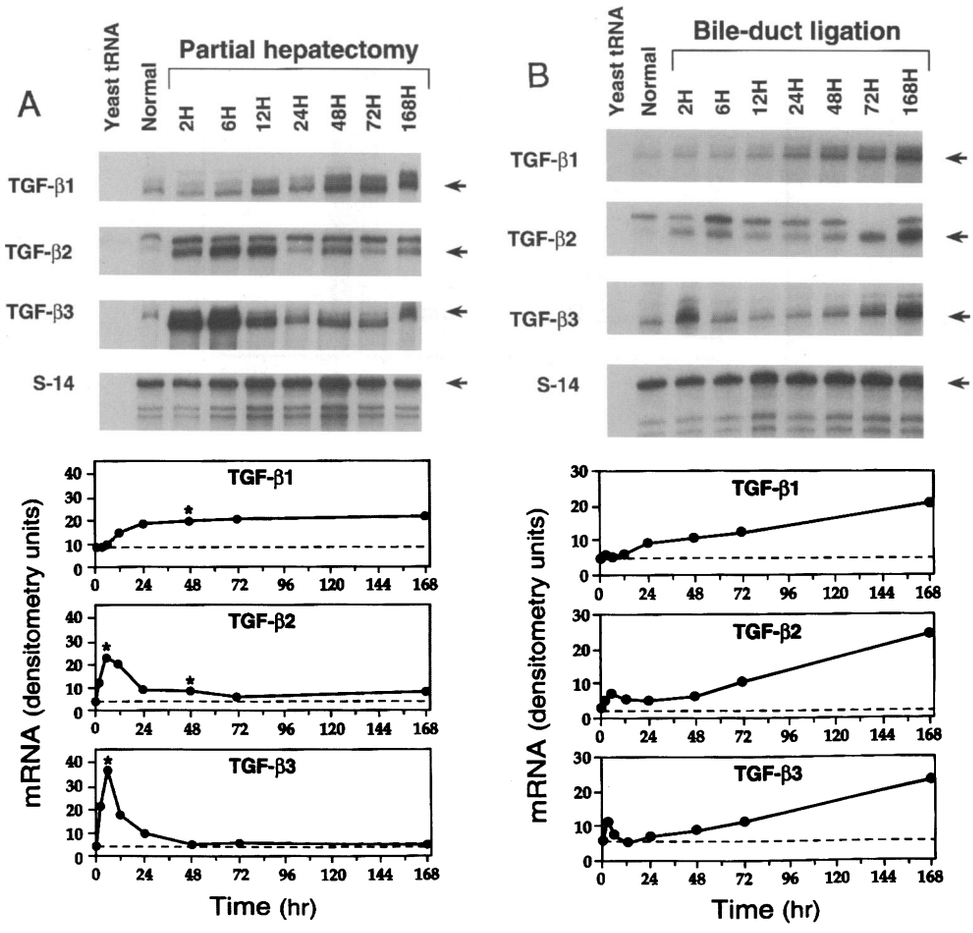

Figure 1. Effect of experimental injury on TGFβ mRNAs in whole-liver extracts. Animals were subjected to either a 70% partial hepatectomy (*A*) or ligation of the biliary duct (*B*). At the indicated times (in hours) after the operation, liver samples were taken and processed for measurement of mRNA by RNase protection assay (see Methods). Yeast tRNA served as a negative control; the specific protected fragment for each assay is indicated by an arrow. The lower portion of the figure presents the data in quantitative form, after correction for differences in the level of S-14 mRNA among the individual samples. The values from sham-operated animals also have been subtracted. The scale is arbitrary. The horizontal dashed line indicates the normal value. Values marked with an asterisk were significantly different from baseline (see Table I). Arrows indicate the position of the specific protected fragment. Extra bands represent either incompletely digested probe (as in the TGF-β2 assay) or points of mismatch between probe and mRNA (as in the S-14 assay, which uses a human probe). The extra bands do not affect the validity of the assay. The entire time course was performed twice, with essentially identical results.

hepatectomy, or after bile duct ligation. In cell isolates from normal liver, the mRNA for TGF-β1 was relatively abundant in sinusoidal endothelial cells and in Kupffer cells, exhibiting values that were about fivefold those for lipocytes (Fig. 2). TGF-β2 was expressed at low but detectable levels in the same cell types, whereas TGF-β3 was present mainly in lipocytes and Kupffer cells. These data are compatible with those of previous studies (10–12), in which expression of TGFβ was localized to mixed nonparenchymal cells.

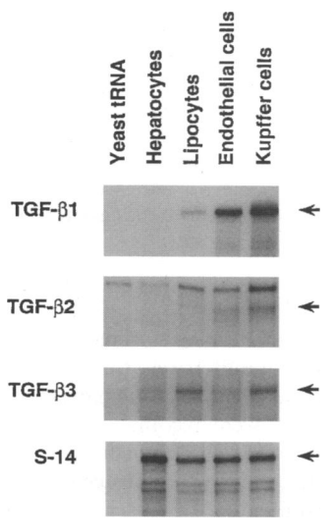

Figure 2. TGFβ mRNAs in specific cell populations isolated from normal liver. Cells were isolated, purified, and extracted as described in Methods. The autoradiogram shown is representative of five independent experiments. Arrows indicate specific bands (see legend to Fig. 1).

Cells were also isolated at 6 and 48 h after partial hepatectomy; these time points represent the early peak and the plateau, respectively, of TGFβ expression in the intact liver (Fig. 1 *A*). Somewhat unexpectedly, the level of baseline expression for an individual cell type did not predict the relative change in expression after partial hepatectomy, particularly at 48 h. For emphasizing this point, the data are presented as relative change in expression rather than as absolute values (Fig. 3). The latter can be inferred from a comparison of Figs. 2 and 3.

At 6 h after partial hepatectomy, expression of TGF-β1 was minimally changed in all cell types, consistent with the study of whole-liver extracts. At 48 h, an increase in this mRNA was seen only in hepatocytes, and quantitatively it was similar to the 2.5-fold change observed in whole-liver extracts. The early peak of mRNA for TGF-β2 involved all four populations; the increases ranged by cell type from 3- to 12-fold. At 48 h, however, the findings from different cell types diverged: whereas the values for lipocytes, endothelial cells, and Kupffer cells decreased toward, or to, the control level, that for hepatocytes underwent a further increase to six-fold above controls. This suggests that the later, sustained increase in TGF-β1 and

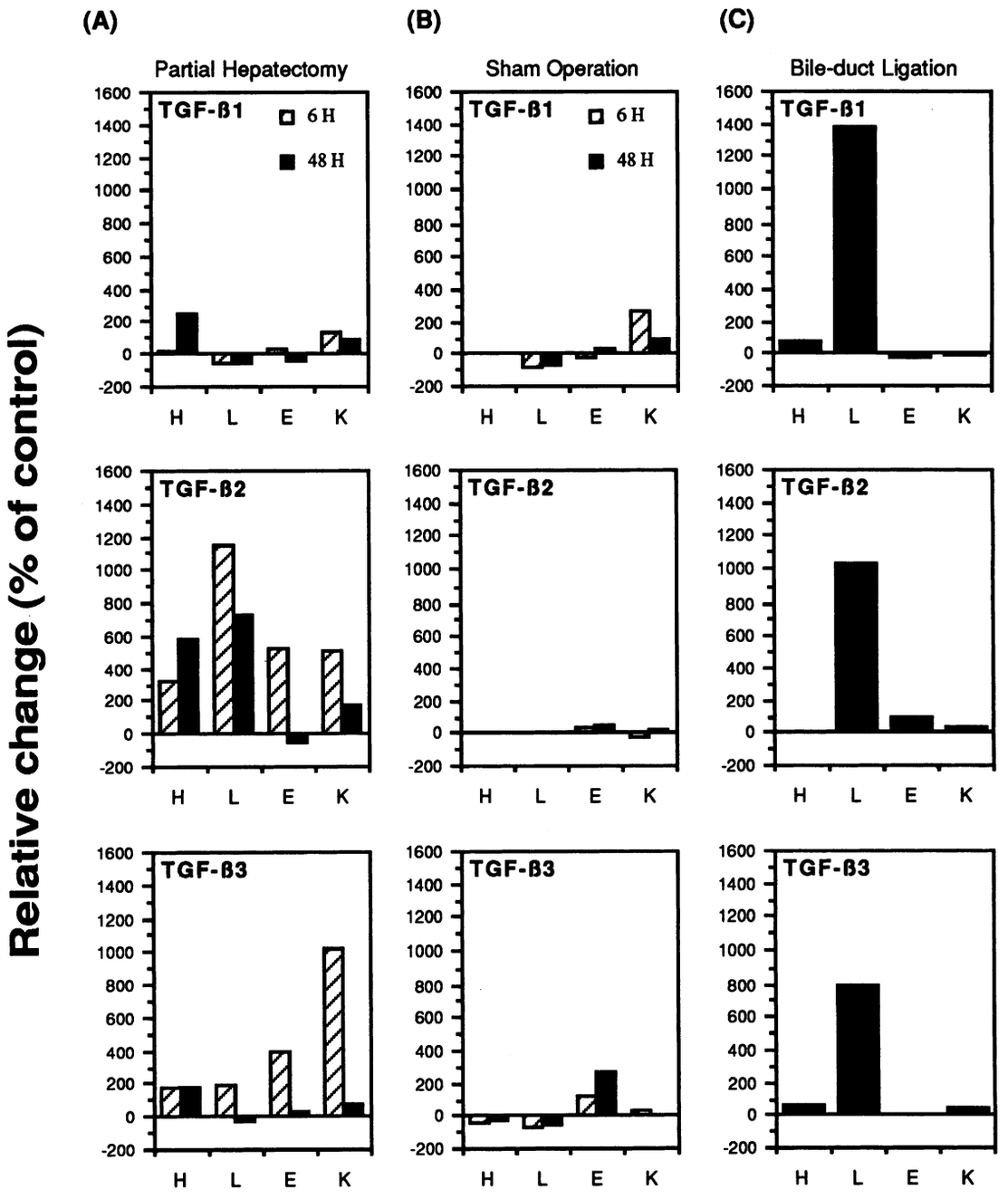

Figure 3. Changes in TGFβ mRNAs after partial hepatectomy or bile duct ligation. (*A*) Partial hepatectomy was performed as in Fig. 1, and cells were isolated at 6 h (*cross-hatched bars*) or at 48 h (*filled bars*), corresponding to the early peak and plateau, respectively, of the whole-liver extracts (Fig. 1 *A*). For obtaining sufficient cells from the liver remnant, six animals were used and the cell isolates were combined at each time point. (*B*) Sham-operated animals. (*C*) Animals subjected to bile duct ligation, performed as in Fig. 1. All extracts were probed for S-14 mRNA (data not shown). The autoradiographic data were quantitated and corrected as in Fig. 1. The data are presented as the change in mRNA expression, relative to normal, unoperated controls (Fig. 2). The entire experiment was performed twice, with similar results. *H,* hepatocytes; *L,* lipocytes; *E,* endothelial cells; *K,* Kupffer cells.

-β2, seen in the whole-liver extracts (Fig. 1 *A*), derives largely from hepatocytes, given that hepatocytes represent 65% of the cells and 90% of the volume of the liver (27). In the case of the mRNA for TGF-β3, though all four cell populations contributed to the early peak, Kupffer cells predominated. By 48 h, all cells except hepatocytes exhibited reduced levels.

In parallel studies, we also evaluated liver injury induced by ligation of the biliary duct, which involves inflammation and fibrosis but minimal hepatocyte proliferation. Cell-specific expression of the TGFβs was measured 7 d after the procedure, when changes in whole-liver extracts were maximal (Fig. 1 *B*). In contrast to the findings in the regeneration model, the increase in TGFβ was limited to lipocytes, among the four cell types examined (Fig. 3 *C*). Because proliferation of biliary epithelial

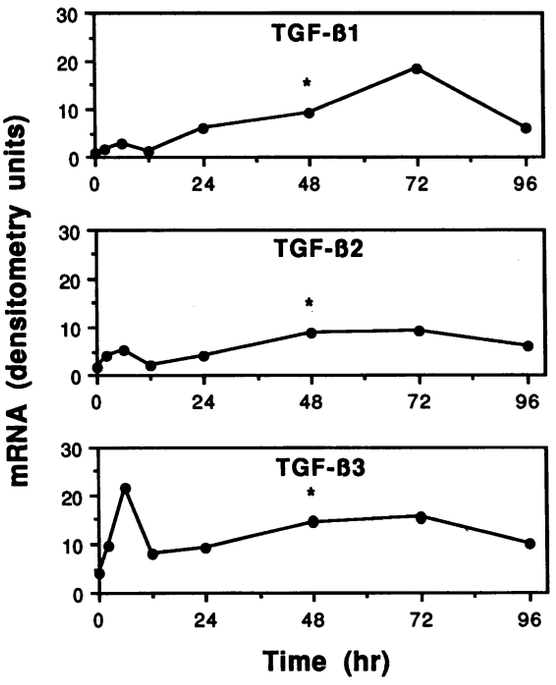

Figure 4. TGFβ mRNAs in hepatocyte isolates during liver regeneration. At the indicated time points after a 70% partial hepatectomy, hepatocytes were isolated, purified by centrifugal elutriation, and extracted for mRNA measurement (see Methods). The resulting autoradiographic data were quantitated and corrected as for Fig. 1. Results at 48 h were reexamined in a separate experiment in which isolates from four different animals were prepared. Asterisks indicate that at 48 h, values for all three subtypes of TGFβ were significantly different from controls ($P < 0.05$).

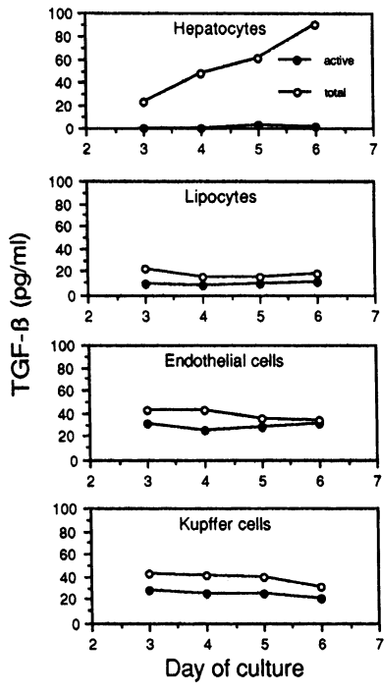

Figure 5. Secretion of TGFβ protein by liver cell populations in primary culture. Cells were isolated from normal liver, and cultured as described (see Methods) for 2 d, and transferred to serum-free medium, which was changed daily. At the indicated time points, samples were taken for bioassay of TGFβ activity, without or with activation of latent TGFβ. *Filled circles,* active TGFβ; *open circles,* total TGFβ.

and oval cells is prominent in this model, this isolate also was evaluated: biliary cells expressed all three TGFβ subtypes at a relatively high level, similar to normal Kupffer cells (data not shown).

Finding that the changes in all three TGFβs after partial hepatectomy involved hepatocytes, we performed a detailed study of this cell type (Fig. 4). The time course of expression for each subtype of TGFβ resembled that for the whole-liver extracts (Fig. 1 *A*) except during the later phase of TGF-β3 expression, in which whole-liver extracts were not different from controls but isolated hepatocytes were significantly increased (at 48 h). This discrepancy may be due to the relatively high constitutive expression in the whole-liver extracts of TGF-β3 by lipocytes and Kupffer cells (Fig. 2), which masked a change in hepatocyte expression.

Cell-specific production of TGFβ protein. Hepatocytes and the individual mesenchymal cell types were placed in mass primary culture, and the culture medium was collected and assayed after 24 h as well as at later time points. The results from the earliest cultures mirrored the mRNA data. TGFβ secretion by hepatocytes from normal liver was at the lower limit of the assay. By contrast, hepatocytes isolated from regenerating liver released readily detectable levels of TGFβ, and the amount increased between and 6 and 48 h after partial hepatectomy. Medium conditioned by lipocytes, sinusoidal endothelial cells, or Kupffer cells also contained measurable TGFβ; the concentration paralleled the respective mRNA level. In contrast to the findings with hepatocytes, active TGFβ was present, at proportions ranging from 50 to 90% (Fig. 5). The isoform of TGFβ released by hepatocytes was investigated by adding type-specific antibodies to the bioassay, and the results are shown in Table II. At 6 h after partial hepatectomy, TGF-β3 was predominant, reflecting the mRNA profile at this time point (Fig. 4); at 48 h, the three isoforms were secreted at similar levels.

Over a longer period of culture (up to 6 d), release of TGFβ by hepatocytes increased progressively, whereas that by individual nonparenchymal cell types either was flat or fell slightly (Fig. 5). The material produced by long-term hepato-

Table I. Expression of TGFβ mRNAs after Partial Hepatectomy: Whole-Liver Extracts

		Partial hepatectomy	
	Normal	6 h	48 h
TGF-β1	8.4±1.2	9.8±1.3 (NS)	20.2±2.0 ($P < 0.01$)
TGF-β2	3.4±0.2	21.7±3.5 ($P < 0.01$)	8.1±0.7 ($P < 0.01$)
TGF-β3	3.8±0.4	36.9±4.7 ($P < 0.01$)	5.1±0.4 (NS)

Data are given as arbitrary densitometry units, with background set at 1. All samples were processed and analyzed together with a single autoradiogram, for purposes of comparison. Values shown are mean±SD, with $n = 4$ experimental animals per group. Sham-operated animals were not significantly different from normal controls (see text). All comparisons are with the corresponding normal animals. *NS,* no significant difference ($P > 0.05$).

Table II. Secretion of TGFβ by Hepatocyte Isolates

Source of cell sample	TGFβ production		Isoform produced
	Total	Active	
	pg/mg protein/24 h		*% of total*
Normal liver	0.5±0.2	0	—
Regenerating, 6 h	12	0	—
β1			16
β2			27
β3			57
Regenerating, 48 h	31	0	—
β1			35
β2			37
β3			28

Hepatocytes were isolated from normal liver or from regenerating liver at the indicated time points, placed in serum-free conditions in primary culture, and incubated for 24 h before assay. One aliquot of each sample was acidified to activate latent TGFβ; these values are recorded as "total" TGFβ. TGFβ bioactivity was recorded as inhibition of [^{3}H]thymidine incorporation by mink lung epithelial cells (see Methods). Baseline incorporation was in the range of 900–1,000 cpm per assay; in cultures incubated with 125 pg/ml standard TGFβ, it was 50–70 cpm. The study of regenerating liver was performed twice with similar results. Cells from sham-operated animals also were evaluated and were indistinguishable from normal.

cyte cultures continued to be latent, but there was a shift in subtype to the β2 isoform only. The localization of this form in hepatocyte culture was examined and found to be pericellular (Fig. 6). The proportion of active material produced by the nonparenchymal cells did not change significantly with time in culture (Fig. 5).

Finally, the bioactivity of TGFβ was examined with fresh hepatocytes exposed to hepatocyte-conditioned medium. The medium was treated to activate latent TGFβ; controls included cells incubated with authentic TGF-β2. As shown (Table III), the activated conditioned medium, containing an estimated 50 pg/ml TGF-β2 (Fig. 5), completely suppressed replicative DNA synthesis in cultured hepatocytes.

Discussion

This is the first quantitative study of TGFβ expression by the principal cell populations of the liver and its modulation by injury. A salient finding is the increased expression of this factor by hepatocytes after partial hepatectomy (Table IV). Although previously published immunohistological studies had shown TGFβ protein in hepatocytes (12, 13), the mRNA was detectable solely in nonparenchymal cells (12, 28, 29). The presence of the protein in hepatocytes was therefore attributed to uptake of circulating TGFβ (30). The current findings indicate, however, that TGFβ in hepatocytes is at least in part endogenous. This conclusion is based on cell isolates that have been carefully characterized as to homogeneity. With processing by centrifugal elutriation (see Methods), the hepatocyte isolate is 98% pure, the only contaminant being lipocytes (18). At the level of 2%, the contribution of lipocyte RNA to the total extract is negligible, given the relatively weak baseline expression of TGFβ mRNA by this cell type (Fig. 2). The profile of TGFβ protein secretion by individual cell types (Fig. 5) provides further evidence for the purity of the hepatocyte preparations. Hepatocytes release TGFβ in increasing amounts over 6 d in culture, all as the latent form. Because this pattern is unique, it effectively excludes significant contamination of the cultures by any nonparenchymal cell population. Secretion by lipocytes, for example, is essentially flat over a similar time period, and 40–60% is in the active form.

Methodological factors may well account for the fact that previous studies, which used in situ hybridization (28, 29) or

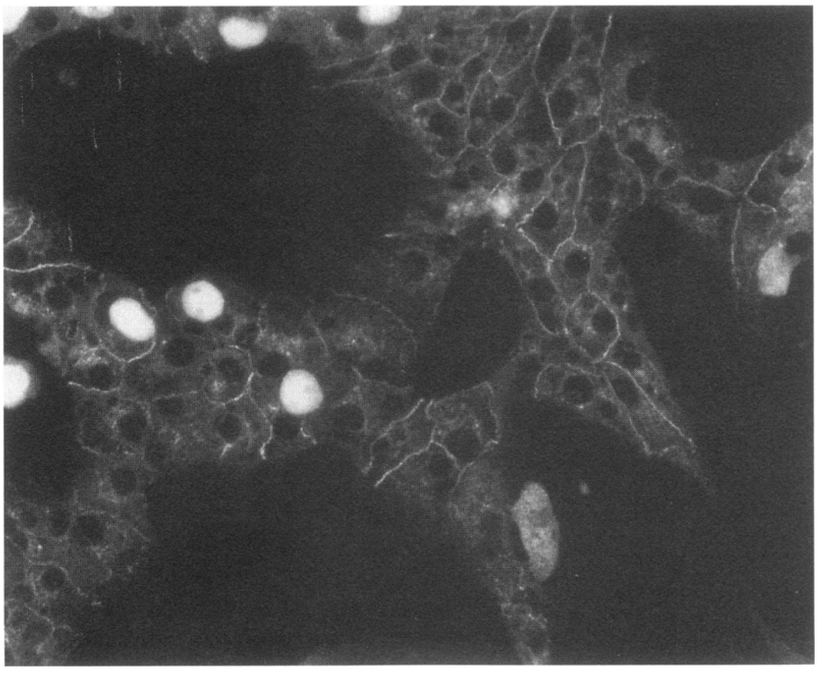

Figure 6. Localization of TGFβ2 in primary culture of normal rat hepatocytes. Hepatocytes were prepared and placed in culture for 4 d and then processed for immunohistology as described in Methods. The specific reaction is limited to intercellular spaces. The brightly fluorescent bodies overlying the hepatocyte monolayer represent autofluorescent nonviable cells.

Table III. Effect of Hepatocyte-conditioned Medium on DNA Synthesis by Hepatocytes in Primary Culture

Culture medium	DNA synthesis	Change
	cpm/well	*% control*
Control	882±136	100
NCM	721±116	82
ACM	297±15*	34
NCM + anti-β2	730±121	83
ACM + anti-β2	1,023±4	116
Control + TGF-β2	288±67*	33
Control + OH-urea	348	39

Conditioned hepatocyte medium was harvested from normal hepatocytes maintained for 4 d in primary culture, filtered, and stored at −20°C. A portion of each sample was used without treatment (NCM); another portion was treated to activate latent TGFβ as described in Methods (ACM). Standard TGF-β2 was added to fresh medium at a concentration of 50 ng/ml. Antibody to TGF-β2 (anti-β2) was added at a final concentration of 0.1 μg/ml. These reagents were tested on freshly isolated normal hepatocytes and plated in 24-well chambers in medium containing 5% calf serum (see Methods) in a total volume of 200 μl. After 24 h, the cells were washed three times in PBS. Further incubation was performed in serum-free medium, with or without the indicated additions. After 18 h, the medium was changed to one containing [^{3}H]thymidine (1 μCi/ml; Sp act 47 Ci/mmol; Amersham Corp., Arlington Heights, IL). Hydroxyurea (10 mM, final concentration) was added to some wells to establish the level of nonreplicative DNA synthesis. After 4 h, DNA was extracted and purified (4). Radioactivity was quantitated by liquid scintillation spectrometry. Assays were performed in quadruplicate. The data represent mean±SD (n = 3), expressed as counts per minute per culture well; where no variation is indicated, the value represents the average of two assays. * $P < 0.05$ versus control. The amount of cellular protein per well as ~0.3 mg and varied by <10% among wells. *NCM,* nonactivated conditioned medium; *ACM,* activated conditioned medium; *anti-β2,* antibody to TGF-β2; *OH-urea,* hydroxyurea.

Northern blot analysis (12), did not detect TGFβ mRNA in hepatocytes. Both methods are relatively insensitive. With the in situ technique, the constitutive signal in endothelial cells and Kupffer cells may have masked the signal in hepatocytes. As for Northern blot analysis, it is at least 10-fold less sensitive than an RNase protection assay. Moreover, its capacity is relatively low with respect to input RNA.

After partial hepatectomy, TGFβ expression is changed in cells other than hepatocytes (Fig. 3). Most of this, however, contributes only to the early peak of TGFβ mRNA from whole-liver extracts (Fig. 2). The significance of the early peak is unclear; it occurs before the onset of DNA synthesis and may play a role in the timing of cell division. With regard to the later peak (at 48 h), only lipocytes, other than hepatocytes, exhibit an elevation of TGFβ mRNA (Fig. 3). Regeneration entails restitution not only of cell mass, but also of extracellular matrix (31). One may speculate that the increase in TGF-β2 in lipocytes is related to this process, as lipocytes are the principal source of extracellular matrix in normal liver (23).

TGFβ figures prominently in two distinct and very different types of liver injury: (*a*) in partial hepatectomy, in which it is implicated in downregulating hepatocyte proliferation stimulated by the injury (3–8); and (*b*) in inflammation, in which it stimulates production of extracellular matrix by lipocytes (13, 28, 29, 32). Interestingly, although the effects of TGFβ are protean, in these injury models they appear to be highly selective. Despite the substantial increase in TGFβ expression in regeneration, there is little evidence of fibrogenesis, apart from that needed for restoration of the liver (31). Similarly, the increase in TGFβ that accompanies the fibrogenic injury induced by bile duct ligation does not appear to be growth inhibitory. On the contrary, there is active proliferation of biliary and mesenchymal cells in this type of injury (31). The present data address, at least indirectly, the basis for selective effects of TGFβ in these two contrasting scenarios.

TGFβ is subject potentially to several different kinds of regulation, both intra- and extracellularly (33). First, at the level of the protein, the conversion of latent precursor to active factor represents a potential point of regulation. In vitro and in cell culture, this is accomplished with proteolysis, possibly mediated by plasmin (34), which itself is subject to multilevel controls (35). Whether plasmin or other proteases serve this function in vivo is speculative. Beyond the activation step, TGFβ receptor expression may govern the effect of TGFβ at the cellular level. Of interest in this regard is the finding that receptors on lipocytes appear to increase in hepatic fibrogenesis, according to studies with a cell culture model of fibrogenesis (36). Finally, as indicated by the current data, the effect of TGFβ in vivo may be linked to its cellular site of secretion.

For the injury models studied, the cell type in which expression of TGFβ is increased is also the apparent target of the factor (hepatocytes and lipocytes in regeneration and inflammation, respectively). From this, we propose that TGFβ is predomi-

Table IV. Summary of TGFβ mRNA Expression by Individual Liver Cell Populations: Baseline Level and Change after Partial Hepatectomy or Bile Duct Ligation

	TGF-β1			TGF-β2			TGF-β3		
	NL	PH	BD	NL	PH	BD	NL	PH	BD
Hepatocytes	+/o	↑	–	o	↑	–	+/o	↑	–
Endothelial cells	++	–	–	+/o	–	–	+/o	↓	–
Kupffer cells	+++	–	–	+	↑	–	+	–	–
Lipocytes	+	–	↑↑	o	↑	↑↑	+	–	↑↑

NL, cell isolates from normal liver; *PH,* cells isolated 48 h after partial hepatectomy; *BD,* cells isolated 7 d after bile duct ligation. *o,* undetectable mRNA signal; *+/o* to +++, trace to marked mRNA signal; –, no significant change after injury; *arrows,* moderate or marked increase (or decrease) in mRNA expression after injury.

nantly autocrine rather than paracrine. Indeed, hepatocytes appear to be isolated from the effects of paracrine TGFβ. Endothelial cells and Kupffer cells express TGFβ at relatively high levels, apparently on a constitutive basis; moreover, they produce the active form of the factor (Fig. 5). If TGFβ from these sources were affecting the hepatocyte population, it presumably would have a tonic inhibitory effect on proliferation, acting as a "chalone" (37). This would require that expression of TGFβ decrease after partial hepatectomy, before the onset of DNA synthesis. According to the current data and previous results (12), however, the mRNA for TGF-β1 is unchanged and that for TGF-β2 and -β3 increases during the first 2–4 h after partial hepatectomy (Fig. 3). Thus, a form of compartmentalization is implied. The basis for this is speculative, given that the physical distance between endothelial or Kupffer cells and hepatocytes is small, estimated at 200–2000 nm (38), and endothelial cells and lipocytes are virtually contiguous. Diffusion of TGFβ, however, may be constrained by extracellular factors, including soluble inhibitors or extracellular matrix (33, 39, 40).

We have used the contrasting injury model of biliary obstruction to demonstrate that increased expression of TGFβ by hepatocytes after partial hepatectomy is specific for regeneration. After biliary ligation, inflammation and fibrogenesis are more prominent than growth, and TGFβ has been widely characterized as a profibrogenic factor in liver (28, 29, 32) with effects specifically on lipocytes in culture (41). We now have evidence that lipocyte expression of this factor is relatively low in normal liver but increases selectively and dramatically in fibrosing injury in vivo (Fig. 3). This is in agreement with a previous study using in situ hybridization, in which the isoform appeared to be TGF-β1 (32). As in the regenerating liver, expression by Kupffer cells and sinusoidal endothelial cells is unchanged (Fig. 3), suggesting that TGFβ from these sources is unrelated to fibrogenesis. Overall, these data provide additional support for the autocrine effects of TGFβ, not only in regeneration, but also in fibrogenesis.

A mixture of TGFβs is secreted by hepatocytes isolated from regenerating liver. It should be noted that the isolate represents a composite of cells in various physiological states. The three isoforms of TGFβ may be present together in all hepatocytes; it is also possible that they are expressed individually in hepatocytes and, moreover, in hepatocytes of a certain type only, e.g., in those at a specific stage of the replicative cycle. In situ studies of mRNA expression in the bile duct ligation model suggest that the β2 isoform is associated with dividing epithelial cells in the periportal region (32). In other tissues also, expression of this isoform is increased selectively in proliferating epithelium. For example, TGF-β2 mRNA is minimal in the virgin mammary gland of the mouse. However, it increases strikingly during pregnancy, when proliferation of the glandular epithelium is prominent; with the onset lactation, it rapidly subsides (42). Another example is the rat intestine, in which renewal of the mucosal epithelium is continuous in the normal state: over a period of ~ 30 h, epithelial cells migrate from the villus crypt to the tip and then slough into the intestinal lumen. In this setting of proliferation, it was found that the β2 subtype was the only form of TGFβ detectable (43). Also, in the mouse embryo, expression of TGF-β2 is notably prominent in the developing foregut (44), which is consistent with a role in the proliferative phase of epithelial morphogenesis. Interestingly, in the current studies, it was found that beyond 3 d of primary culture, hepatocellular production of TGFβ increased notably and was essentially 100% TGF-β2. Cells at this stage of culture exhibit several "fetal" markers (45, 46). Extrapolating to the regenerating liver, one may speculate that the β2 subtype is associated with immature hepatocytes, possibly related to stem cells (47).

A recent addition to the list of potential growth regulators in the liver is activin, which has been implicated in apoptosis of hepatocytes (48) and is a negative regulator of hepatocyte proliferation (49). Activin belongs to the TGFβ superfamily but has limited homology with TGFβ itself (~ 33% at the amino acid level), and its biological effects in general differ from those of TGFβ (50). With regard to the liver, it uses distinct receptors (on hepatocytes) (48) and, as an apoptotic factor, is ~ 10-fold less potent than TGFβ (48). It differs from TGFβ also in being secreted as the active peptide rather than in latent form, and its secretion by hepatocytes occurs only in the presence of a mitogen such as EGF. Also, it has no activity in the mink lung cell assay (Schwall, R., personal communication). Thus, activin and TGFβ are distinct and separable factors, despite their apparently similar effects on hepatocytes in culture.

The fact that TGFβ is released by hepatocytes almost entirely as the latent factor raises the question of where and how it undergoes activation. If a proteinase such as plasmin (34) is required, activation of TGFβ released by hepatocytes could require both sinusoidal endothelial cells and lipocytes, in what might be described as a modified autocrine process. On the other hand, activation of TGFβ may occur on the hepatocyte surface, presumably via a membrane-associated proteinase, providing further support for the autocrine hypothesis. In fact, a recent study suggests that hepatocytes from regenerating liver (but not normal liver) are capable of activating latent TGFβ (12).

In summary, we propose that modulation of TGFβ in liver injury is isoform and cell specific and moreover that the selective effects of the factor in a given type of injury are due predominantly, if not exclusively, to an autocrine mode of action.

Acknowledgments

K. B. Chang and J. Doherty provided skilled assistance with hepatocyte isolations and manuscript preparation, respectively.

The work was supported by National Institutes of Health grants DK31198, DK26743, and T32DK07573.

References

1. Massagué, J. 1990. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* 6:597–641.

2. Moses, H. L., E. Y. Yang, and J. A. Pietenpol. 1990. TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell.* 63:245–247.

3. Nakamura, A., Y. Tomita, R. Hirai, K. Yamaoka, K. Kaji, and A. Ichihara. 1985. Inhibitory effect of transforming growth factor-β on DNA synthesis of adult rat hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* 133:1042–1050.

4. McMahon, J. B., W. L. Richards, A. A. delCampo, M. H. Song, and S. S. Thorgeirsson. 1986. Differential effects of transforming growth factor-β on proliferation of normal and malignant rat epithelial cells in culture. *Cancer Res.* 46:4665–4671.

5. Strain, A. J., D. J. Hill, and R. D. Milner. 1986. Divergent action of transforming growth factor beta on DNA synthesis in human foetal liver cells. *Cell Biol. Int. Rep.* 10:855–860.

6. Carr, B. I., I. Hayashi, E. L. Branum, and H. L. Moses. 1986. Inhibition of DNA synthesis in rat hepatocytes by platelet-derived type β transforming growth factor. *Cancer Res.* 46:2330–2334.

7. Wollenberg, G. K., E. Semple, B. A. Quinn, and M. A. Hayes. 1987.

Inhibition of proliferation of normal, preneoplastic and neoplastic rat hepatocytes by transforming growth factor-β. *Cancer Res.* 47:6595–6599.

8. Russell, W. E., R. J. Coffey, Jr., A. J. Quellette, and H. L. Moses. 1988. Type β transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat. *Proc. Natl. Acad. Sci. USA.* 85:5126–5130.

9. Fausto, N., J. E. Mead, P. A. Gruppuso, and L. Braun. 1990. TGF-β in liver development, regeneration and carcinogenesis. *Ann. NY Acad. Sci.* 593:231–242.

10. Braun, L., J. E. Mead, M. Panzica, R. Mikumo, G. I. Bell, and N. Fausto. 1988. Transforming growth factor β mRNA increases during liver regeneration: a possible paracrine mechanism of growth regulation. *Proc. Natl. Acad. Sci. USA.* 85:1539–1543.

11. Carr, B. I., T. H. Huang, K. Itakura, M. Noel, and N. Marceau. 1989. TGFβ gene transcription in normal and neoplastic liver growth. *J. Cell. Biochem.* 39:477–487.

12. Jakowlew, S. B., J. E. Mead, D. Danielpour, J. Wu, A. B. Roberts, and N. Fausto. 1991. Transforming growth factor-β (TGF-β) isoforms in rat liver regeneration: messenger RNA expression and activation of latent TGF-β. *Cell Regul.* 2:535–548.

13. Czaja, M. J., F. R. Weiner, K. C. Flanders, M.-A. Giambrone, R. Wind, L. Biempica, and M. A. Zern. 1989. In vitro and in vivo association of transforming growth factor-β1 with hepatic fibrosis. *J. Cell Biol.* 108:2477–2482.

14. Higgins, G. M., and R. M. Anderson. 1931. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol.* 12:186–202.

15. Bienkowski, R. S., M. J. Cowan, J. A. McDonald, and R. G. Crystal. 1978. Degradation of newly synthesized collagen. *J. Biol. Chem.* 253:4356–4363.

16. Bissell, D. M., J. M. Caron, L. E. Babiss, and J. M. Friedman. 1990. Transcriptional regulation of the albumin gene in cultured rat hepatocytes: role of basement-membrane matrix. *Mol. Biol. Med.* 7:187–197.

17. Friedman, S. L., and F. J. Roll. 1987. Isolation and culture of hepatic lipocytes, Kupffer cells and sinusoidal endothelial cells by density gradient centrifugation with Stractan. *Anal. Biochem.* 161:207–218.

18. Maher, J. J., D. M. Bissell, S. L. Friedman, and F. J. Roll. 1988. Collagen measured in primary cultures of normal rat hepatocytes derives from lipocytes within the monolayer. *J. Clin. Invest.* 82:450–459.

19. Bissell, D. M., L. E. Hammaker, and R. Schmid. 1972. Liver sinusoidal cells. Identification of a subpopulation for erythrocyte catabolism. *J. Cell Biol.* 54:107–119.

20. Irving, M. G., F. J. Roll, S. Huang, and D. M. Bissell. 1984. Characterization and culture of sinusoidal cells from normal rat liver: lipoprotein uptake and collagen phenotype. *Gastroenterology.* 87:1248–1254.

21. Rutenberg, A. M., H. Kim, J. W. Fischbein, J. S. Hanker, H. L. Wasserkrug, and A. H. Sligman. 1969. Biochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. *J. Histochem. Cytochem.* 17:517–526.

22. Pelton, R. W., B. L. M. Hogan, D. A. Miller, and H. L. Moses. 1990. Differential expression of genes encoding TGFs β1, β2, and β3 during murine palate formation. *Dev. Biol.* 141:456–460.

23. Maher, J. J., and R. F. McGuire. 1990. Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. *J. Clin. Invest.* 86:1641–1648.

24. Rhoads, D. D., A. Dixit, and D. J. Roufa. 1986. Primary structure of human ribosomal protein S14 and the gene that encodes it. *Mol. Cell. Biol.* 6:2774–2783.

25. Jarnagin, W. R., D. C. Rockey, V. E. Koteliansky, S. S. Wang, and D. M. Bissell. 1994. Expression of variant fibronectin in wound-healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. *J. Cell Biol.* 127:2037–2048.

26. Danielpour, D., L. L. Dart, K. C. Flanders, A. B. Roberts, and M. B. Sporn. 1989. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF-β1 and TGF-β2) secreted by cells in culture. *J. Cell. Physiol.* 138:79–86.

27. Weibel, E. R., W. Staubli, H. R. Gnagi, and F. A. Hess. 1969. Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereologic methods, and normal morphometric data for rat liver. *J. Cell Biol.* 42:68–91.

28. Nakatsukasa, H., P. Nagy, R. P. Evarts, Ch. C. Hsia, E. Marsden, and S. S. Thorgeirsson. 1990. Cellular distribution of transforming growth factor-β1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *J. Clin. Invest.* 85:1833–1843.

29. Nakatsukasa, H., R. P. Evarts, C. Hsia, and S. S. Thorgeirsson. 1990. Transforming growth factor-β1 and type I procollagen transcripts during regeneration and early fibrosis of rat liver. *Lab. Invest.* 63:171–180.

30. Coffey, R. J., L. J. Kost, R. M. Lyons, H. L. Moses, and N. F. LaRusso. 1987. Hepatic processing of transforming growth factor-β in the rat. *J. Clin. Invest.* 80:750–757.

31. Yamamoto, H., Y. Murawaki, and H. Kawasaki. 1995. Hepatic collagen synthesis and degradation during liver regeneration after partial hepatectomy. *Hepatology.* 21:155–161.

32. Milano, S., H. Herbst, D. Schuppan, H. Stein, and C. Surrenti. 1991. Transforming growth factors β1 and β2 are differentially expressed in fibrotic liver disease. *Am. J. Pathol.* 139:1221–1229.

33. Sporn, M. B., and A. B. Roberts. 1990. TGF-β: problems and prospects. *Cell Regul.* 1:875–882.

34. Flaumenhauft, R., M. Abe, P. Mignatti, and D. B. Rifkin. 1992. Basic fibroblast growth factor-induced activation of latent transforming growth factor beta in endothelial cells: regulation of plasminogen activator activity. *J. Cell Biol.* 118:901–909.

35. Rieder, H., T. Armbrust, K. H. Meyer zum Buschenfelde, and G. Ramadori. 1993. Contribution of sinusoidal endothelial liver cells to liver fibrosis: expression of transforming growth factor-beta 1 receptors and modulation of plasmin-generating enzymes by transforming growth factor-beta 1. *Hepatology.* 18:937–944.

36. Friedman, S. L., G. Yamasaki, and L. Wong. 1994. Modulation of transforming growth factor beta receptors of rat lipocytes during the hepatic wound healing response. Enhanced binding and reduced gene expression accompany cellular activation in culture and in vivo. *J. Biol. Chem.* 269:10551–10558.

37. Strain, A. J. 1992. Transforming growth factor-beta: the elusive hepatic chalone? *Hepatology.* 16:269–270.

38. Blouin, A., R. P. Bolender, and E. R. Weibel. 1977. Distribution of organelles and membranes between hepatocytes and non-hepatocytes in the rat liver parenchyma. *J. Cell Biol.* 72:441–445.

39. Bissell, D. M., D. M. Arenson, J. J. Maher, and F. J. Roll. 1987. Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. *J. Clin. Invest.* 79:801–812.

40. Paralkar, V. M., S. Vukicevic, and A. H. Reddi. 1991. Transforming growth factor beta type 1 binds to collagen IV of basement membrane matrix: implications for development. *Dev. Biol.* 143:303–308.

41. Matsuoka, M., N. T. Pham, and H. Tsukamoto. 1989. Differential effects of interleukin-1 alpha, tumor necrosis factor alpha, and transforming growth factor beta 1 on cell proliferation and collagen formation by cultured fat-storing cells. *Liver.* 9:71–78.

42. Robinson, S. D., G. B. Silberstein, A. B. Roberts, K. C. Flanders, and C. W. Daniel. 1991. Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development. *Development.* 113:867–878.

43. Glick, A. B., B. K. McCune, N. Abdulkarem, K. C. Flanders, J. A. Lamadue, J. M. Smith, and M. B. Sporn. 1991. Complex regulation of TGFβ expression by retinoic acid in the vitamin A-deficient rat. *Development.* 111:1081–1086.

44. Dickson, M. C., H. G. Slager, E. Duffie, C. L. Mummery, and R. J. Akhurst. 1993. RNA and protein localisations of TGFβ2 in the early mouse embryo suggest an involvement in cardiac development. *Development.* 117:625–639.

45. Walker, P. R., R. J. Bonney, J. E. Becker, and V. R. Potter. 1972. Pyruvate kinase, hexokinase, and aldolase isozymes in rat liver cells in culture. *In Vitro.* 8:107–114.

46. Bissell, D. M., and P. S. Guzelian. 1980. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann. NY Acad. Sci.* 349:85–98.

47. Sell, S. 1990. Is there a stem cell? *Cancer Res.* 50:3811–3815.

48. Schwall, R. H., K. Robbins, P. Jardieu, L. Chang, C. Lai, and T. G. Terrell. 1993. Activin induces cell death in hepatocytes in vivo and in vitro. *Hepatology.* 18:347–356.

49. Yasuda, H., T. Mine, H. Shibata, Y. Eto, Y. Hasegawa, T. Takeuchi, S. Asano, and I. Kojima. 1993. Activin A: an autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *J. Clin. Invest.* 92:1491–1496.

50. Kingsley, D. M. 1994. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* 8:133–146.