Journal of Cellular Biochemistry 39:477–487 (1989)
Growth Inhibitory and Cytotoxic Polypeptides 119–129

TGFβ Gene Transcription in Normal and Neoplastic Liver Growth

Brian I. Carr, Ting H. Huang, Keiichi Itakura, Micheline Noël, and Normand Marceau

Departments of Medical Oncology (B.I.C.) and Molecular Genetics (T.H.H., K.I.), City of Hope National Medical Center, Duarte, California 91010; Laval University Cancer Center (M.N., N.M.) Quebec, Canada G1R 2J6

TGFβ is a potent, nontoxic inhibitor of mitogen-induced DNA synthesis in primary cultures of adult rat hepatocytes. Using a cDNA probe, we investigated TGFβ gene expression in quiescent, regenerating, and neoplastic liver, and several hepatoma lines by Northern gel analysis. We found that regenerating liver had increased TGFβ gene transcripts beginning at about 8 h, with a broad peak of 48–120 h and return to normal after 9 days. Separation of the regenerating liver into its constituent cell types, followed by RNA extraction and reprobing, revealed that increased TGFβ gene transcripts were confined to the enriched endothelial-cell population and not the hepatocytes. Increased hepatic TGFβ expression was also found in fetal liver and in rats immediately after birth. Elevated TGFβ mRNA levels were also found in primary cultures of oval cells and an established bile ductular cell line, as well as in carcinogen-altered liver epithelial cell lines. Transcripts were undetectable in normal human liver but were abundant in the human hepatoma lines Hep G2, Hep 3B, PLC/PRF/5, and SK-Hep-1. Elevated levels were also found in the normal rat liver–derived lines BRL-3A and clone 9 and the H4IIE rat hepatoma, but not in the HTC, MH_1C_1, and MH7777 rat hepatomas. The hepatocarcinogen diethylnitrosamine induced high transcript levels after single injections in a time- and dose-dependent manner. These results suggest that the liver may be a paracrine organ with respect to TGFβ gene expression, which can be induced by carcinogens and by growth stimulation.

Key words: TGFβ gene, hepatic regeneration, hepatomas, carcinogens, growth inhibitor

Primary cultures of adult rat hepatocytes can be induced to undergo DNA synthesis by several mitogens, including epidermal growth factor (EGF), glucagon, insulin, and norepinephrine. We have previously shown that a variety of mammalian sera exerted a dose-dependent inhibition of mitogen-induced DNA synthesis in cultured rat hepatocytes [1]. The inhibitory activity was confined to the platelet fraction of blood, and human platelet–derived TGFβ was found to be a potent yet nontoxic

Received March 24, 1988; accepted November 2, 1988.

inhibitor of EGF-induced DNA synthesis [2]. We presumed that TGFβ was a major contributor to the inhibitory activity in serum. In order to investigate the physiological significance of this inhibitory effect of TGFβ on normal hepatocyte DNA synthesis further, we asked two questions. First, since hepatocytes undergo DNA synthesis and proliferation in vivo as a consequence of a two-thirds partial hepatectomy (PH), it was important to determine whether the stimulated cells from a PH liver were still sensitive to the inhibitory actions of TGFβ. If such cells were also inhibited by TGFβ, then the physiological significance of TGFβ in the control of normal liver growth might be uncertain, unless the levels of hepatic TGFβ were to decline during hepatic regeneration. Second, we wished to determine whether hepatic TGFβ levels, determined initially as TGFβ gene transcripts, were altered during regenerative growth of normal liver or in neoplastic growth.

Our initial working hypothesis was that if TGFβ were an important hepatic negative growth regulator, then it would be expected that under conditions of growth there would be decreased levels of TGFβ or a decreased sensitivity of the hepatocytes to the inhibitory action of TGFβ protein. The findings reported here made this initial hypothesis untenable in part, since in both regenerative and neoplastic growth the gene transcript levels of TGFβ were much higher than in the resting liver. Furthermore, we found that the increased expression of TGFβ in the liver, which was developmentally controlled, was not in the hepatocytes but in the nonparenchymal fraction, probably the endothelial cells. This suggests the possibility that the liver may be a paracrine organ with respect to the action of TGFβ.

MATERIALS AND METHODS

Animals

Young adult male F344 rats weighing 150–200 g (Charles River Labs, Inc., Wilmington, MA) were fed Purina lab chow except for the production of primary hepatocellular carcinomas (HCC). For the production of HCC rats were fed 2-acetylaminofluorene 0.02% (w/w) (AAF) for 3 months and then returned to a basal diet for 1 year.

Cell Isolation and Fractionation

Primary hepatocyte cultures were obtained from normal adult, 14-day-old, fetal or regenerating rat livers by the two-step collagenase perfusion technique [3], as previously adapted [4]. The final cell preparations contained at least 95% hepatocytes, the remaining portion being the sinusoidal cells [5]. After an initial cell attachment to tissue culture dishes for 3 h in 10% fetal calf serum, the medium was replaced by serum-free medium containing EGF 10 ng/ml. Initiation of DNA synthesis was measured by the incorporation of ^{3}H-TdR into hepatocyte DNA over a 24-h period [1].

Sinusoidal cells, mainly composed of endothelial cells and Kupffer cells, were isolated from normal and regenerating adult rat livers by a collagenase/pronase digestion procedure and then separated in a Metrizamide gradient, as described by others [6]. The resulting preparations contained 70% endothelial cells, the rest being Kupffer cells. In some experiments, the sinusoidal cell preparations were allowed to adhere to untreated plastic dishes, a procedure that selectively removes the Kupffer cells by pan-

ning [7]. The proportion of Kupffer cells in the endothelial cell preparations was down to 15%. The attached Kupffer cells constituted an enriched preparation, containing less than 10% endothelial cells.

Ductular oval cells were isolated from the liver of a 3'-methyl-dimethyl-aminoazobenzene–treated rat by perfusion/digestion of the organ with collagenase, treatment of the cell suspension with trypsin and DNAse, selective removal of hepatocytes by panning with an anti-cell-surface reacting antibody, and cell separation by isopyknic centrifugation in a Percoll gradient [8]. The procedure yielded a cell preparation containing 8×10^7 cells/liver, of which 95% expressed the bile ductular cell phenotype [8]. Experiments also were performed on an epithelial cell line that grew out from oval cell primary cultures after transfection with a plasmid containing the SV40 large T oncogene. A typical nonparenchymal hepatic epithelial cell line T51B was used, which was established from adult rat liver cell primary cultures [9]. Recent cell typing analyses indicate that the T51B cell line originates from the bile ductular structures. This cell line was cultured in α-MEM containing 5% fetal calf serum.

Quantitation of TGFβ Gene Transcripts

Immediately after sacrifice, RNA was extracted from rat liver with LiCl-urea. Total RNA was electrophoresed through a 1% agarose–6% formaldehyde gel and blotted onto nitrocellulose filters exactly as we previously described [10]. The filters were prehybridized with 60% formamide, 5× Denhardt's solution, 20× SSPE, 0.1% SDS and tRNA (150 μg/ml) at 35°C for 2 h and then hybridized with a TGFβ cDNA probe (kind gift of Dr. Rik Derynk of Genentech, Inc., San Francisco) with the same buffer but with 1× Denhardt's solution. The probe was heated before hybridization as described [11]. The filters were washed twice with 0.1% NaCl, sodium citrate, 0.1% SDS at room temperature for 10 min, dried, and then exposed to Kodak XAR-5 film at −70°C with two intensifying filter screens for 24 h. Quantitation was performed with scanning densitometry with an integrator.

RESULTS

Differential Sensitivity of Hepatocytes From Normal and Regenerating Liver to Inhibition by TGFβ

We investigated whether hepatocytes obtained at various times after a 2/3 PH differed from normal hepatocytes in their sensitivity to the inhibitory action of TGFβ. Normal hepatocyte DNA synthesis, which we have previously found to peak at 72–96 hours [1], was completely inhibited by TGFβ 1 ng/ml (Fig. 1). In contrast, hepatocytes from livers undergoing mitosis in vivo at 48 h, were found to have two peaks of DNA synthesis in vitro. The first peak of DNA synthesis, during the first 24 h in culture, occurred whether EGF was present or not and was not found in normal hepatocytes. This first peak, which was presumed to reflect the DNA synthesis that was occurring in vivo at the time the cultures were prepared, could not be inhibited by TGFβ 10 ng/ml (Fig. 1). A second peak of DNA synthesis occurred at 72–96 h of culture. This second, EGF-induced peak of DNA synthesis in PH-48-h hepatocytes could also not be inhibited by up to 10 ng/ml of TGFβ, unlike the finding in normal hepatocytes. These results indicate that hepatocytes from regenerating liver lose sensi-

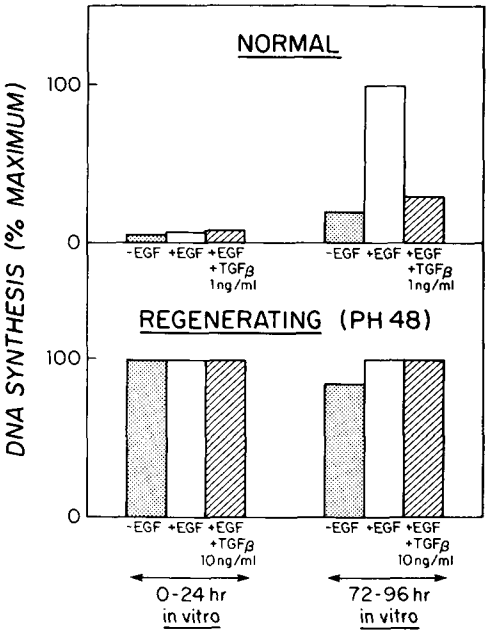

Fig. 1. Effect of TGFβ on maximum EGF-induced DNA synthesis in hepatocytes from resting and regenerating liver. DNA synthesis is expressed as maximum percentage of EGF-induced DNA synthesis at 72–96 h of culture. Although absolute counts vary with each experiment, typically EGF-induced DNA synthesis in normal hepatocytes of 30,000–40,000 cpm ^{3}H-TdR/35-mm dish or 30–40 cpm/μg protein. Results are duplicates of two experiments, with each experimental point representing three dishes.

tivity to the inhibitory actions of TGFβ. This loss is transient, since hepatocytes obtained 72 h after a PH behave like normal hepatocytes, in that the EGF-induced peak of DNA synthesis at 72–96 h of culture regained sensitivity to inhibition by TGFβ.

TGFβ Gene Expression During Hepatic Regeneration

We next investigated whether there was an alteration in the expression of the TGFβ gene during regenerative growth. We found an increase of TGFβ transcript levels beginning approximately 8 h after PH, with a broad peak from 48–120 h reflecting an approximately threefold increase over normal, followed by a gradual return to normal baseline levels by 10 days post PH. We had initially supposed this to reflect an increase in the hepatocyte TGFβ gene expression. However results (below), in which we found a discordance between the gene expression in primary hepatomas and hepatoma cell lines, made us consider the possibility that another cell type was involved. Hepatocytes and nonhepatocytes were prepared from regenerating livers 72 h after a PH. When hepatocytes and sinusoidal cells from regenerating liver were probed separately (Fig. 2) the increased TGFβ mRNA signal was not seen in the hepatocytes but was seen exclusively in the nonhepatocyte cell population. A strong signal was detected in the enriched endothelial cell fraction as well as in an endothelial and Kupf-

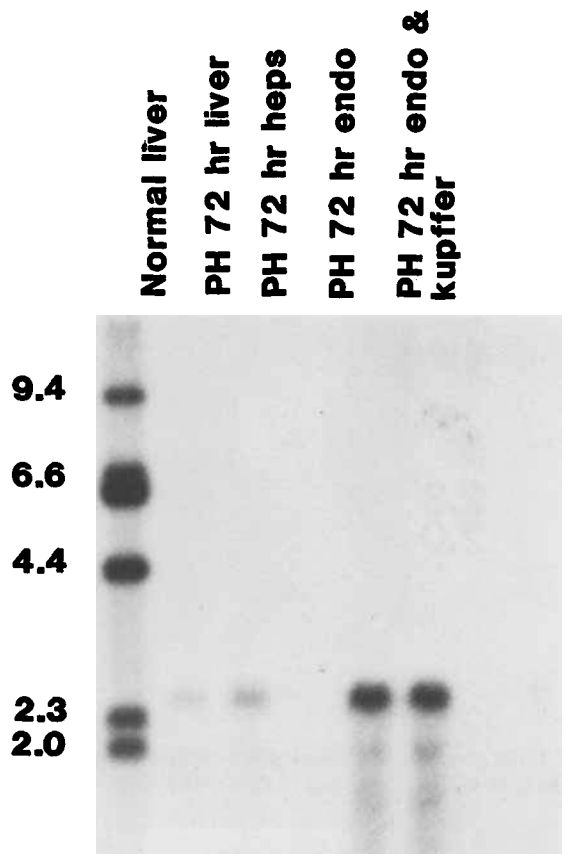

Fig. 2. Northern gel of TGFβ gene transcripts in resting and regenerating liver, regenerating hepatocytes, and sinusoidal cells, with total hepatic or cellular RNA.

fer cell preparation. Although a minor contaminant of the endothelial cell preparation could have contributed to this strong signal, it is more likely that the main signal is generated by the liver endothelial cells. In normal resting liver, TGFβ mRNA can be detected in endothelial cells also, but not in hepatocytes.

TGFβ Gene Expression During Hepatic Development

Since TGFβ transcript levels were increased and then decreased so reproducibly during normal regenerative growth, we considered the possibility that TGFβ gene expression might be developmentally regulated. It is known that the fetal rat liver is a rapidly growing organ and that it continues to grow in the first several days post partum. Livers were therefore obtained from rats at the 14th and 18th days of gestation as well as the second day post partum, and the extracted RNA was probed with TGFβ cDNA (Fig. 3). A high level of expression of TGFβ mRNA was found in midgestation; it decreased with increasing fetal maturity and decreased further in the postnatal period. This finding suggests a developmental regulation of the hepatic TGFβ gene expression.

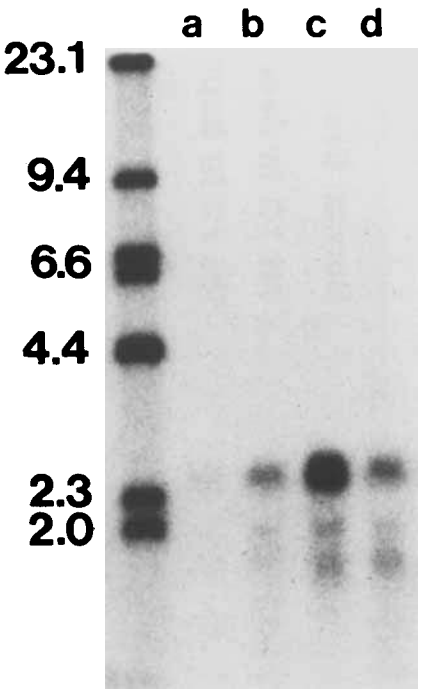

Fig. 3. Northern gel of TGFβ gene transcripts during hepatic development. **Lane a,** adult liver; **lane b,** 14-day neonatal liver; **lane c,** 14-day fetal liver; **lane d,** 18-day fetal liver.

TGFβ Gene Expression in Nonparenchymal Hepatic Epithelial Cell Lines

Since increased transcripts were found in proliferating liver, various liver cell lines were probed to examine their TGFβ gene expression (Fig. 4). We found increased levels of expression in ductular oval cells and from rat nonparenchymal hepatic epithelial cell lines (T51B, BRL3A, and clone 9). Thus, several liver-derived epithelial cell lines have increased TGFβ gene transcripts, compared with normal or proliferating hepatocytes.

TGFβ Gene Expression in Primary Rat Hepatomas and Hepatoma Cell Lines

In view of our finding that normal proliferating liver in both regenerative growth and prenatal development contained increased TGFβ transcripts, we examined whether altered levels were also found in neoplastic growth. Primary HCCs were obtained in rat livers by chemical carcinogen administration (Materials and Methods). RNA from HCCs were probed for TGFβ gene expression, which was found to be increased, compared with age-matched normal rat liver. In contrast, highly variable levels were found in four rat hepatoma cell lines (Table I). While a slight increase was found in the H4IIE cell line, essentially undetectable levels were found in the HTC, MH_1C_1, and MH7777 hepatoma cell lines. The reasons for this discordance between

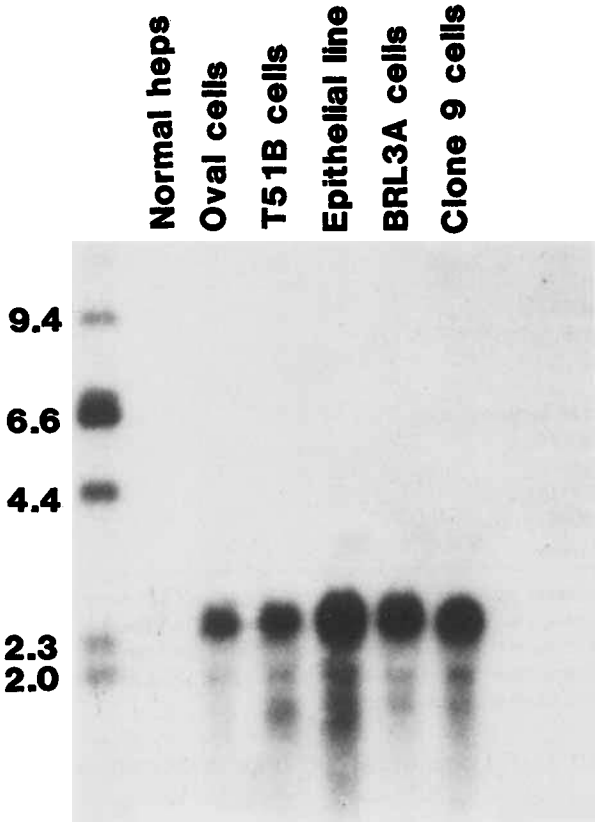

Fig. 4. Northern gel of TGFβ gene transcripts of several nonparenchymal hepatic epithelial cell types, with total cellular RNA.

increased levels in primary HCC but highly variable levels in continuous hepatoma lines are unclear. Variable levels were also found in human hepatoma cell lines with decreasing expression in the following order: Hep 3B, Hep G2, SK-Hep-1, PLC/PRF/5, and HUH-7. TGFβ gene transcripts were almost undetectable in three separate specimens of normal human liver.

Carcinogen-Induced Hepatic TGFβ Gene Expression

The finding that primary HCCs contain increased transcripts of TGFβ mRNA, as reported for hepatoma cell lines elsewhere [12], suggested the possibility that alteration in TGFβ gene regulation might be a consequence of the action of hepatocarcinogens. Normal adult rats were injected with single doses of DEN, a powerful hepatocarcinogen that causes tumor initiation with a single dose and is a complete carcinogen when it is chronically administered. We found a time-dependent increase in TGFβ transcript levels with single 200-mg/kg doses (Fig. 5), with a peak at 72–96 h and a slow return to baseline levels by 14 days after carcinogen administration. An increase in transcript levels also was found with increasing doses of DEN from 10 mg to 200 mg/kg rat weight (Fig. 5 inset). These results suggest that a carcinogen can

TABLE I. TGFβ mRNA in Primary Liver Tumors and Hepatoma Lines*

Normal human livers (3)	−
	−
	−
Rat primary HCCs (2)	+++
	+++
Rat hepatoma lines	
H4IIE	+
HTC	−
MH_1C_1	−
MH7777	−
Human primary HCCs (3)	±
	±
	−
Human hepatoma lines	
Hep 3B	++
Hep G2	++
PLC/PRF/5	+
SK/Hep/1	+
HUH-7	−

*Summary of Northern gel results for primary rat tumors and rat and human hepatoma cell lines. The TGFβ mRNA levels were scored on a relative basis by scanning densitometry and were standardized by comparison to the levels in normal rat liver. Numbers in brackets refer to number of samples tested.

increase the hepatic TGFβ gene transcripts prior to carcinogen-induced liver growth [16].

DISCUSSION

The results reported here show that although quiescent rat liver has very low levels of TGFβ mRNA, normal regenerating and developing liver, neoplastic prolifer-

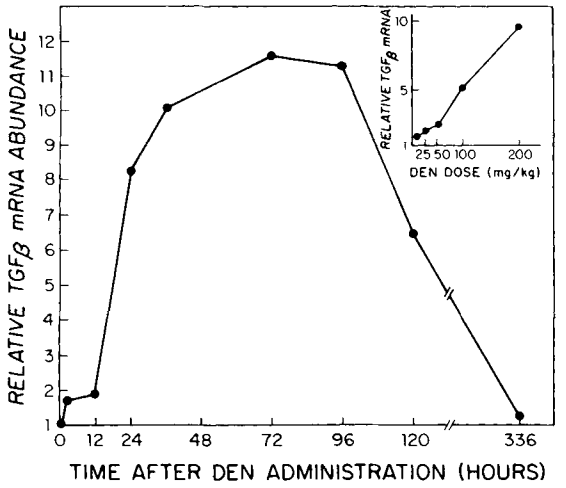

Fig. 5. Time course and dose: response of DEN-induced changes in hepatic TGFβ mRNA levels.

ating liver, carcinogen-treated liver, and several hepatoma cell lines all have increased levels of TGFβ gene transcripts. The finding that TGFβ mRNA is increased in regenerating liver prompts speculation about the function of the TGFβ gene product during hepatic regeneration. Small but reproducible increases are noted as early as 8 h after a PH; a stronger signal was seen at 24 h, which is the peak of DNA synthesis in vivo. If TGFβ protein is produced as an immediate consequence of an increase in mRNA levels and if it acts as an inhibitor in vivo, as it does in vitro, then it becomes difficult to explain why increased levels of mRNA transcripts occur during the peak of DNA synthesis in vivo. One explanation could be that there is a several-hour delay between the increased level of mRNA transcripts and the translation of the increased transcripts into protein. If this explanation is the correct one, then TGFβ protein might be a candidate overgrowth inhibitor or negative growth regulator in vivo. Since the hepatic regenerative response involves a coordinated series of steps that includes DNA synthesis, mitosis, and hypertrophy followed by quiescence, it is likely that there are both growth stimulants and growth inhibitors that act in a coordinated manner. TGFβ is a candidate "stop" signal presumably among a variety of growth-inhibitory factors that remain to be discovered.

If, however, the mRNA levels are translated immediately into mature TGFβ protein, then it is difficult to understand why increased levels of TGFβ protein should be produced at the time of maximal DNA synthesis and growth. However, it is possible that TGFβ protein has a different function in vivo from what has been observed in cultures of hepatocytes and other cell types in vitro. In this regard, we have preliminary evidence that acid/ethanol extracts of regenerating liver contain TGFβ protein (manuscript in preparation) that correlates well with the mRNA levels. However, acid/ethanol extraction converts any latent TGFβ to active TGFβ. In most cell types so far examined in which there are elevated levels of TGFβ mRNA, mature TGFβ protein is not produced; rather, a latent TGFβ form is produced that does not seem to have growth inhibitory activity. (An exception is MCF-7 human breast cancer cells [13]. Since it is not yet clear whether the protein product of the increased TGFβ mRNA levels in rodent liver in vivo is latent or mature TGFβ, it is possible either that only the latent form is produced in vivo, as it is in most cell lines in vitro, or that there is a delay between synthesis of the latent form of TGFβ and its conversion to active TGFβ. In this context, another function might be attributable to the latent form, which could involve either positive or negative regulation of cell growth, or a regulatory role in differentiation. The increased TGFβ gene mRNA levels during normal development lend support to the idea that TGFβ gene product (latent or mature form) may be involved in developmental regulation, either as a maturation promoting factor or as a selective cell-growth inhibitor in the maturation process. The mechanisms by which the latter process might occur remain speculative.

The finding that increased hepatic TGFβ gene transcripts are produced not by hepatocytes but by the sinusoidal cells is of major interest. Whether the increased signal in regenerating liver is attributable to the endothelial cells as such or to a specific subfraction within the endothelial cell population remains to be clearly established. Moreover, on the presumption that the increased TGFβ mRNA levels reflect an increased synthesis of the mature TGFβ protein product, the identity of the target cells becomes a major issue. Two possibilities are considered here. The first is that TGFβ acts in an autocrine fashion on the producing sinusoidal cells, perhaps in some growth stimulatory or growth inhibitory fashion. The other is that TGFβ acts in a paracrine fashion, being produced by the sinusoidal cells but actually acting on the

hepatocytes to inhibit liver overgrowth. If this is the case, then the study of liver regeneration with the use of isolated cultures of purified hepatocytes in culture may be an oversimplification of the events occurring in vivo, because a major regulatory factor (TGFβ) is produced by the nonhepatocytes that are routinely discarded in hepatocyte culture studies. Our finding that regenerating liver hepatocytes have lost sensitivity to inhibition by TGFβ is consistent with the idea that TGFβ may be important in normal hepatocyte growth control and may explain the proliferative response to PH despite altered TGFβ transcript levels. The mechanisms for loss of sensitivity remain to be elucidated.

The significance of the increased levels of TGFβ gene transcripts in hepatomas remains unclear. Why hepatomas, which are characterized by an increased growth rate, overproduce an inhibitor is puzzling. One possible explanation is that the TGFβ that they produce preferentially inhibits the surrounding cells, thereby giving the hepatoma cells a growth advantage. This idea is consistent with our preliminary data, which suggest that various hepatoma cell lines are less sensitive than normal hepatocytes to the growth inhibitory actions of TGFβ. However, it is possible that hepatomas produce only latent, or "pre-TGFβ." Our initial studies on hepatoma-conditioned media do not provide evidence for the presence of mature TGFβ. This suggests that hepatoma cell lines, like many other cell lines, may produce a latent form of TGFβ. One of the more important questions currently in TGFβ biology concerns the function, if any, of the latent form of TGFβ and the physiological determinants responsible for its conversion to active, mature TGFβ. These questions will be studied with the availability of purified latent TGFβ [14]. We have not yet found physiological conditions under which latent TGFβ in a hepatoma-conditioned medium is activated to mature TGFβ. It is also interesting that hepatomas produce TGFβ mRNA but that in regenerating liver only nonhepatocytes do so. This finding could be interpreted either to mean that otherwise silent genes are expressed in hepatocyte-derived tumors or to suggest a nonhepatocyte origin for the tumors.

Our data also provide evidence that the application of a single dose of a carcinogen can lead to elevated levels of TGFβ gene transcripts before any liver cell growth, since DEN-induced proliferation does not appear to occur within 24 h of the carcinogen injection. These data may prove to be a convenient handle for studying the control of TGFβ mRNA levels independent of the many cell alterations that accompany normal growth. Increased levels of TGFβ gene transcripts in the liver are thus associated with hepatic proliferation, as has been recently reported elsewhere [17], as well as with the action of a carcinogen independent of hepatic proliferation; similar increases have been found for the levels of c-*myc*, Ha-*ras*, c-*fos* and heat-shock gene expression in addition to TGFβ [10,15]. TGFβ therefore may be part of a family of genes that have altered transcript levels in liver growth.

ACKNOWLEDGMENTS

We thank Dr. O. Morin for her advice in the isolation procedure for the sinusoidal cells. This work was supported in part by the NCI of Canada and NIH grant CA 44602 (B.C.) and by a gift from Otto and Marianne Wolman.

REFERENCES

1. Hayashi I, Carr BI: J Cell Physiol 125:82–90, 1985.
2. Carr BI, Hayashi I, Branum EL, Moses HL: Cancer Res 46:2330–2334, 1986.

3. Seglen DO: Exp Cell Res 82:391–398, 1973.
4. Deschenes J, Valet JP, Marceau N: In Vitro 16:722–730, 1982.
5. Beribault H, Marceau N: J Cell Physiol 129:77–84, 1986.
6. De Leeuw AM, Bareldo RJ, de Zanger R, Knook DL: Cell Tissue Res 223:201–215, 1982.
7. Morin O, Normand C: J Cell Physiol 129:103–110, 1986.
8. Germain L, Noel M, Gourdeau H, Marceau N: Cancer Res 48:368–378, 1988.
9. Marceau N, Germain L, Goyette R, Noel M, Gordeau H: Biochem Cell Biol 64:788–802, 1986.
10. Carr BI, Huang TH, Buzin CH, Itakura K: Cancer Res 46:5106–5111, 1986.
11. Wahl GM: Proc Natl Acad Sci USA 76:3683–3687, 1979.
12. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH: Cancer Res 47:707–712, 1987.
13. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynk R, Dickson RB: Cell 48:417–428, 1987.
14. Miyazono K, Wernstedt C, Hellman U, Heldin C-H: J Cell Biochem (Suppl) 12A:200; D118, 1988.
15. Fausto N, Mead JE, Braun L, Thompson NL, Panzica M, Goyetti M, Bell GI, Shank PR: In Becker, FF (ed): Symposium on Fundamental Cancer Research 39. University of Texas Press, 1987, pp 69–86.
16. Magee PN, Barnes JM: Adv Cancer Res 10:163–246, 1967.
17. Braun L, Mead JE, Panzica M, Mikumo R, Bell GI, Fausto N: Proc Natl Acad Sci USA 85:1539–1543, 1988.