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Transforming growth factor β_1 regulates production of acute-phase proteins

(α_1-protease inhibitor/α_1-antichymotrypsin/fibrinogen/α-fetoprotein/interleukin 6)

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ABSTRACT **We explored the possible role of transforming growth factor β_1 (TGF-β), a cytokine that appears to be an important modulator of inflammation and tissue repair, in regulation of human plasma protein synthesis during the acute-phase response. In Hep 3B cells, TGF-β led to increased secretion of the positive acute-phase proteins α_1-protease inhibitor and α_1-antichymotrypsin and decreased secretion of the negative acute-phase protein albumin. In Hep G2 cells, after incubation with TGF-β, the same changes in secretion of α_1-protease inhibitor, α_1-antichymotrypsin, and albumin were observed, as well as decreased secretion of both the negative acute-phase protein α-fetoprotein and the positive acute-phase protein fibrinogen. In addition, TGF-β modulated the effects of interleukin 6; these cytokines, in combination, were additive in inducing synthesis and secretion of α_1-protease inhibitor and α_1-antichymotrypsin and in decreasing secretion of albumin and α-fetoprotein. TGF-β inhibited the induction of fibrinogen caused by interleukin 6. The effects on α_1-protease inhibitor were confirmed by metabolic labeling in Hep 3B cells and by demonstrating increased accumulation of specific mRNA in Hep G2 cells, and the effects on fibrinogen were confirmed in Hep 3B cells by studies of mRNA for the α chain of fibrinogen. TGF-β had no effect on haptoglobin or α_1-acid glycoprotein secretion, either directly or in the presence of interleukin 6, which is capable of inducing these proteins. These studies demonstrate that TGF-β can affect hepatic synthesis and secretion of a subset of acute-phase proteins, both directly and by modulating the effect of interleukin 6. The affected group of plasma proteins is distinct from those affected by other recognized acute-phase protein-inducing cytokines. These findings support the view that combinations of cytokines mediate the response of the hepatocyte to inflammatory stimuli.**

Tissue injury and infection lead to a broad array of systemic and metabolic alterations, collectively termed the acute-phase response (1, 2). Among these alterations are changes in hepatic synthesis of a number of plasma proteins, referred to as acute-phase proteins; synthesis of some proteins, the positive acute-phase proteins, increases, while synthesis of others, the negative acute-phase proteins, decreases. Studies in primary hepatocyte cultures and hepatoma cell lines have shown that hepatic synthesis of human acute-phase proteins can be influenced by several cytokines including interleukin 6 (IL-6), a major inducer of acute-phase changes, interleukins 1α and -β (IL-1), cachectin/tumor necrosis factor α (TNF), interferon γ, and leukemia inhibitory factor (3–18). One cytokine can modulate the effect of other cytokines in human model systems—e.g., IL-1 diminishes the inducing effect of IL-6 on fibrinogen synthesis (7, 12, 18) and interferon γ blocks induction of α_1-antichymotrypsin (ACT) and haptoglobin by IL-6 (16).

Transforming growth factor β_1 (TGF-β) is a multifunctional peptide, which appears to play an important role as a modulator of inflammation and tissue repair (19). TGF-β induces synthesis of plasminogen activator inhibitors, antagonizes many of the effects of IL-2, is a potent chemotactic factor for monocytes, and increases mRNA for IL-1. TGF-β is also capable of inhibiting or stimulating growth of many cell types and regulating synthesis of numerous components of extracellular matrix and of several hormones (20). Although many cells synthesize TGF-β, cells that play an essential role in wound healing, such as platelets, monocytes–macrophages, and lymphocytes, release significant amounts of this cytokine when activated (21–23). TGF-β receptors are present on essentially all cells (20). TGF-β has been shown to inhibit growth of normal rat hepatocytes *in vitro*, but proliferation of transformed hepatocytes cultured under similar conditions is ordinarily not affected (24). In the present paper, we demonstrate that TGF-β regulates expression of a number of plasma protein genes in two human hepatoma cell lines.

MATERIALS AND METHODS

Induction of Acute-Phase Protein Changes in Hep 3B and Hep G2 Cells. Hep 3B and Hep G2 human hepatoma cell lines were generous gifts of G. Darlington (Baylor College of Medicine, Houston) and B. Knowles (Wistar Institute, Philadelphia), respectively. Induction of acute-phase protein changes was assessed in these cell lines by determining rate of secretion of all proteins studied by electroimmunoassay, by metabolic labeling of α_1-protease inhibitor (PI), and by analysis of mRNA concentrations of α-fibrinogen and PI as described (25–27). Briefly, after subculture, cells (1–1.5 $\times$ 10^6) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, for 5 days, after which medium was replaced by serum-free RPMI 1640 medium containing dexamethasone (1 μM), insulin (0.02 unit/ml), tobramycin (50 μg/ml), and, in experimental cultures, various cytokines, to a total vol of 1 ml. Cytokines used included platelet-derived purified human TGF-β_1 (R & D Systems Inc., Minneapolis), recombinant human IL-6 obtained from Chinese hamster ovary (CHO) cells (specific activity, 2.5 $\times$ 10^6 units/mg; generous gift of G. Wong, Genetics Inst., Cambridge, MA), recombinant human IL-1α (specific activity, 2.5 $\times$ 10^9 units/mg; generous gift of P. Lomedico, Hoffman–La

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Abbreviations: TGF-β, transforming growth factor β_1; IL, interleukin; PI, α_1-protease inhibitor; ACT, α_1-antichymotrypsin; AGP, α_1-acid glycoprotein; TNF, cachectin/tumor necrosis factor α; AFP, α-fetoprotein.

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Roche), and recombinant human TNF (specific activity, 2×10^7 units/mg; Genzyme). For secretion and mRNA studies, cells were incubated for 48 hr with a change to fresh medium after 24 hr. Analyses were carried out in medium collected over the final 24 hr. For metabolic labeling studies, monolayers were incubated for 18–24 hr, then washed with Hanks' balanced salt solution, and further incubated for 4 hr in 1.0 ml of methionine-free RPMI medium containing 100 μCi of L-[^{35}S]methionine (1 Ci = 37 GBq). All experiments were carried out in duplicate or triplicate.

Analysis of Acute-Phase Proteins Secreted by Hep 3B and Hep G2 Cells into Culture Medium. Concentrations of PI, ACT, α_1-acid glycoprotein (AGP), fibrinogen, haptoglobin, albumin, and α-fetoprotein (AFP) secreted into culture medium were determined by quantitative electroimmunoassay (25, 26). A human serum calibrator kit (Atlantic Antibodies, Scarborough, ME) was used as a standard for PI, ACT, AGP, haptoglobin, and albumin estimation and NormTrol coagulation control plasma (Helena Laboratories) was used for fibrinogen concentration determination. For estimation of AFP levels, serial dilutions of concentrated medium collected from Hep G2 cells were used as standards and the results were expressed as percentages of control.

Analysis of Radiolabeled PI. Radiolabeled PI was immunoprecipitated from 25 μl of medium (in the presence of 20 mM Tris·HCl/0.15 M NaCl/1% Triton X-100/1% sodium deoxycholate/0.1 mg of bovine serum albumin per ml) with goat anti-human PI (Atlantic Antibodies, Scarborough, ME), and antigen–antibody complexes were then isolated with immobilized protein A (IgGsorb; The Enzyme Center, Malden, MA). The immunoprecipitates were washed and subjected to electrophoresis on SDS/polyacrylamide gels, which were stained, fluorographed in 1 M sodium salicylate/0.1% glycerol, dried, and autoradiographed. The radioactivity in PI was quantitated by dissolving the excised band in 30% hydrogen peroxide, adding scintillation fluid, and counting in a Packard Tri-Carb β-counter.

Analysis of mRNA for PI and Fibrinogen. After 48 hr of incubation, cells from duplicate or triplicate dishes were pooled, washed in phosphate-buffered saline (PBS), and total cellular RNA was extracted by the acid guanidinium thiocyanate/phenol/chloroform method (28). RNA (10 μg per lane) was separated electrophoretically through 1% agarose/formaldehyde denaturing slab gels and transferred to nitrocellulose before hybridization. We used a human cDNA probe for PI, kindly provided by S. Woo (Houston) and a cDNA probe for the α chain of fibrinogen, the gift of G. Crabtree (Palo Alto, CA). The probes, whose sequences corresponded to single mRNA species, were first labeled with [^{32}P]dCTP by the random-primer technique described by Feinberg and Vogelstein (29) and were then used for hybridization with subsequent autoradiography.

Determination of DNA Concentration. DNA was determined according to the procedure of Labarca and Paigen (30) using the fluorochrome compound Hoechst 33258. Cells (Hep 3B; two experiments) in 35-mm tissue culture dishes were scraped and washed with PBS and sonicated in 1 ml of 0.05 M sodium phosphate/2.0 M NaCl/0.002 M EDTA/0.0002% Triton X-100, pH 7.4. DNA concentration was determined from a standard curve with calf thymus DNA.

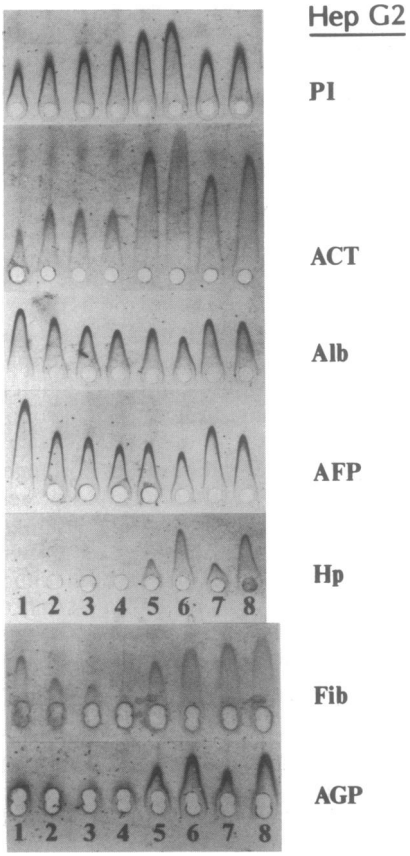

FIG. 1. Effect of TGF-β and IL-6 on the secretion of acute-phase proteins by Hep G2 cells. Electroimmunoassay of PI, ACT, albumin (Alb), AFP, haptoglobin (Hp), fibrinogen (Fib), and AGP. Lanes: 1, control; 2, TGF-β (5 ng/ml); 3, TGF-β (10 ng/ml); 4, TGF-β (20 ng/ml); 5, TGF-β (10 ng/ml) and IL-6 (10 units/ml); 6, TGF-β (10 ng/ml) and IL-6 (50 units/ml); 7, IL-6 (10 units/ml); 8, IL-6 (50 units/ml).

RESULTS

Incubation of both Hep 3B and Hep G2 cells with TGF-β caused a significant increase in accumulation of PI and ACT in medium and a decrease of albumin secretion (Figs. 1 and

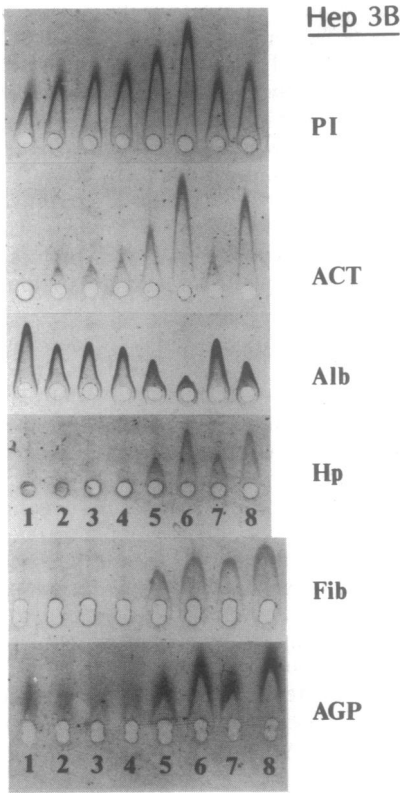

FIG. 2. Effect of TGF-β and IL-6 on secretion of acute-phase proteins by Hep 3B cells. Details are the same as in Fig. 1.

Table 1. Effect of TGF-β on acute-phase protein secretion in Hep G2 cells

Cytokine	Protein secretion, μg per 10^6 cells per 24 hr (mean ± SD)			
	PI	ACT	Fibrinogen	Albumin
None	8.3 ± 1.0	3.5 ± 0.8	3.0 ± 0.3	11.1 ± 0.9
TGF-β (5 ng/ml)	10.6 ± 2.2*	8.2 ± 1.9*	1.8 ± 0.3*	9.0 ± 1.4*
TGF-β (10 ng/ml)	12.1 ± 0.8*	8.3 ± 0.5*	0.9 ± 0.5*	8.1 ± 1.2*
TGF-β (20 ng/ml)	13.0 ± 1.5*	7.4 ± 0.5*	0.5 ± 0.4*	7.5 ± 1.4*
IL-6 (10 units/ml)	11.9 ± 2.1*	13.8 ± 3.9*	5.3 ± 1.0*	8.9 ± 1.2*
IL-6 (50 units/ml)	12.2 ± 0.9*	18.5 ± 5.0*	7.5 ± 1.8*	6.9 ± 1.4*
TGF-β (10 ng/ml) + IL-6 (10 units/ml)	13.3 ± 2.4*	20.2 ± 5.0*†‡	4.0 ± 1.1†	6.6 ± 1.0*†‡
TGF-β (10 ng/ml) + IL-6 (50 units/ml)	15.8 ± 1.3*†§	31.7 ± 6.4*†§	5.2 ± 0.8*†	5.1 ± 0.8*†

*$P < 0.05$ vs. control.
†$P < 0.05$ vs. TGF-β (10 ng).
‡$P < 0.05$ vs. IL-6 (10 units).
§$P < 0.05$ vs. IL-6 (50 units).

2; Tables 1 and 2) but had no effect on secretion of AGP or haptoglobin. TGF-β caused a dose-dependent decrease of fibrinogen and AFP secretion in Hep G2 cells (Fig. 1 and Table 1) in which these proteins are constitutively expressed. Addition of TGF-β to Hep 3B cells did not cause an increase in synthesis of these two proteins; it was not possible to determine whether a decrease occurred, because baseline levels of synthesis of fibrinogen and AFP were too low to permit evaluation.

In both cell lines, the combination of TGF-β and IL-6 produced a greater increase in secretion of PI and ACT and a greater decrease in synthesis of albumin than did either cytokine alone (Figs. 1 and 2; Tables 1 and 2). Similarly, in Hep G2 cells, TGF-β and IL-6 were additive in their effect on decreasing AFP synthesis (Fig. 1). In contrast, TGF-β appeared to inhibit induction of fibrinogen by IL-6 in both lines (Figs. 1 and 2; Tables 1 and 2). Synthesis of haptoglobin and AGP was increased by treatment with IL-6 in both lines but was not affected by addition of TGF-β to IL-6 (Figs. 1 and 2).

Metabolic labeling of PI with [^{35}S]methionine in Hep 3B cells, followed by immunoprecipitation and electrophoretic separation, demonstrated that TGF-β (4 ng/ml) led to approximately a 50% increase in synthesis of this protein. The effects of TGF-β and IL-6 were additive (Fig. 3), leading to a >3-fold increase in synthesis, as determined by directly counting radioactivity in the PI band.

Studies of mRNA concentration of PI in Hep G2 cells and of α-fibrinogen in Hep 3B cells (Fig. 4) showed changes paralleling those seen in studies of secretion of these proteins.

DNA concentrations of Hep 3B cells after 48 hr of exposure to TGF-β (26.7 ± 0.6 μg of DNA per dish) or IL-6 (29.2 ± 1.5 μg of DNA per dish) were approximately the same as in control cultures (28.6 ± 1.05 μg of DNA per dish).

DISCUSSION

The acute-phase response may be defined as the reorchestration of the pattern of gene expression of plasma proteins in hepatocytes (31). Previously, five defined cytokines have been reported capable of influencing human plasma protein synthesis in liver cell cultures. In general, each cytokine has its effect on a specific set of genes (12, 18, 32). IL-6 induces a broad range of changes approaching those seen in plasma after *in vivo* stimuli (5, 12, 13, 27), and the spectrum of changes induced by leukemia inhibitory factor is similar (17, 33). IL-1 and TNF induce more restricted patterns of change (6–8, 15, 18), which resemble one another. Interferon γ has been found to down-regulate synthesis of human ACT and haptoglobin and to enhance synthesis of α_2-macroglobulin (16) and several complement components (15).

It is becoming apparent that regulation of many acute-phase protein genes is not an effect of a single cytokine, but rather may be mediated by combinations of cytokines (16, 18, 42) and, in some cases, by cofactors such as glucocorticoids (18). One cytokine may modulate the effect of other cytokines. IL-1 and IL-6 have been found to have an additive,

Table 2. Effect of TGF-β on acute-phase protein secretion in Hep 3B cells

Cytokine	Protein secretion, μg per 10^6 cells per 24 hr (mean ± SD)			
	PI	ACT	Fibrinogen	Albumin
None	7.5 ± 0.9	*	*	17.5 ± 2.2
TGF-β (5 ng/ml)	11.4 ± 2.0†	0.5 ± 0.2	*	12.0 ± 1.1†
TGF-β (10 ng/ml	11.7 ± 2.2	0.9 ± 0.5	*	11.2 ± 0.1†
TGF-β (20 ng/ml)	12.0 ± 2.2†	0.9 ± 0.9	*	11.2 ± 0.1†
IL-6 (10 units/ml)	10.6 ± 2.1†	1.9 ± 0.7	4.4 ± 1.2	14.1 ± 1.1†
IL-6 (50 units/ml)	12.7 ± 1.9†	12.2 ± 2.6	8.0 ± 1.3	8.0 ± 0.9†
TGF-β (10 ng/ml) + IL-6 (10 units/ml)	15.6 ± 1.8†‡	6.6 ± 1.7‡§	2.2 ± 1.3	9.0 ± 3.1†§
TGF-β (10 ng/ml) + IL-6 (50 units/ml)	18.6 ± 1.5†‡¶	20.5 ± 4.7‡¶	6.2 ± 1.1	5.2 ± 2.0†‡¶

*Undetectable by our assay.
†$P < 0.05$ vs. control.
‡$P < 0.05$ vs. TGF-β (10 ng).
§$P < 0.05$ vs. IL-6 (10 units).
¶$P < 0.05$ vs. IL-6 (50 units).

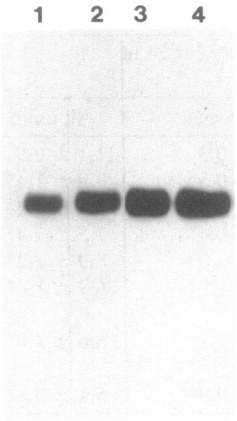

FIG. 3. Effect of TGF-β and IL-6 on the synthesis of PI. Autoradiogram of immunoprecipitated, [^{35}S]methionine-labeled PI synthesized by Hep 3B cells and analyzed by SDS/PAGE. Lanes: 1, control; 2, TGF-β (4 ng/ml); 3, IL-6 (50 units/ml); 4, TGF-β (4 ng/ml) and IL-6 (50 units/ml).

synergistic, or cooperative effect on synthesis of several human acute-phase proteins, including C-reactive protein, serum amyloid A, AGP, and C3 (18, 27, 42). In contrast, an inhibitory effect may be seen—e.g., IL-1, TNF, and interferon γ are reported to diminish the inducing effect of IL-6 on human fibrinogen synthesis (7, 12, 16, 18). These findings suggest that the response of the hepatocyte to various inflammatory stimuli *in vivo* is mediated by specific combinations of cytokines. The results presented in this study, in which additive effects (both positive and negative) of IL-6 and TGF-β were seen, lend further support to the growing recognition that informational content does not reside in individual cytokines, but rather in the combinations or sets of cytokines that affect target cells (20, 34–36).

We found that the cytokine TGF-β is capable of playing a role in regulating acute-phase protein production in liver cells, both directly and by modulating the effect of IL-6. TGF-β affects a unique subset of acute phase proteins different from those reported to be affected by IL-1, TNF, IL-6, or leukemia inhibitory factor. Under the conditions used in this study, in the presence of dexamethasone and insulin, TGF-β was found capable of inducing two positive acute-phase proteins, PI and ACT, in two human hepatoma cell lines. This finding is of particular interest, since TGF-β has been found to stimulate other types of protease inhibitors such as metalloproteases (37) and inhibitors of plasminogen activators (38). In addition, TGF-β decreased the synthesis of two negative acute-phase proteins, albumin and AFP, and of the positive acute-phase protein fibrinogen.

These results underscore the unique features of TGF-β in regulating acute-phase protein synthesis. Some of the activities of TGF-β, such as the ability to increase synthesis of PI, are shared with IL-6 alone. Others, such as inhibition of fibrinogen synthesis, are shared with IL-1, TNF, and interferon γ. Still other activities, such as suppression of albumin and AFP synthesis and stimulation of ACT synthesis, have been found to be shared with IL-6, IL-1, and TNF in our laboratory (8, 27, 31). A role of TGF-β in regulation of acute-phase protein synthesis is supported by the findings of Morrone *et al.* (ref. 39; personal communication), in which porcine TGF-β was found to lead to a decrease of mRNA levels of the negative acute-phase proteins albumin, AFP, and apolipoproteins A-I and A-II in Hep 3B cells. Induction of positive acute-phase proteins, including PI, haptoglobin, and factor B, was not found in their studies.

It is of interest that different laboratories have reported conflicting findings regarding induction of human PI. While several laboratories, including our own (5, 18, 27, 33, 40),

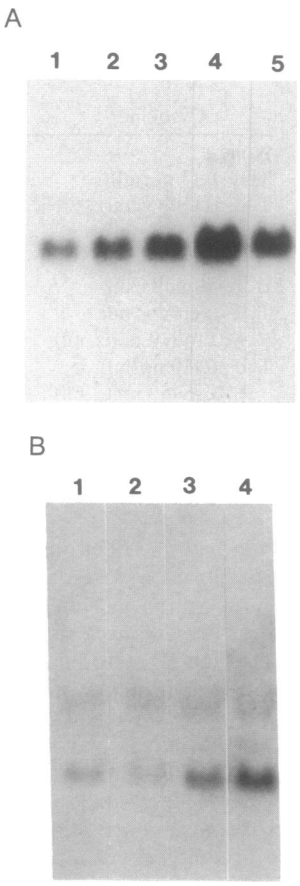

FIG. 4. Northern blot analysis showing effects of TGF-β and IL-6 on PI and α-fibrinogen gene expression. RNA (10 μg per lane), isolated from Hep 3B cells or Hep G2 cells, was analyzed by blot hybridization with cDNA probes to human PI or α-fibrinogen. (*A*) Cell source, Hep G2; probe, human PI cDNA. Lanes: 1, control; 2, TGF-β (2.5 ng/ml); 3, TGF-β (5 ng/ml); 4, TGF-β (5 ng/ml) and IL-6 (50 units/ml); 5, IL-6 (50 units/ml). (*B*) Cell source, Hep 3B; probe, human α-fibrinogen cDNA. Lanes: 1, control; 2, TGF-β (5 ng/ml); 3, TGF-β (5 ng/ml) and IL-6 (10 units/ml); 4, IL-6 (10 units/ml). Ethidium bromide staining of the gels indicated that all lanes had approximately equal amounts of RNA loaded.

have demonstrated induction of this protein by IL-6, others (7, 39, 41) found no induction by either monocyte conditioned medium or IL-6. These and similar differences between laboratories using what are nominally the same cell lines may reflect the phenotypic consequences of genetic drift known to occur in all lines (34). While the PI responses observed previously have generally been modest, it may be that the full response seen *in vivo* may depend on the additive effects of IL-6, TGF-β, and perhaps other cytokines.

Several studies have shown that corticosteroids may play a permissive role or may enhance the effects of cytokines on plasma protein synthesis (18, 31). Our current studies of the effect of TGF-β on plasma protein synthesis were carried out in the presence of dexamethasone and insulin. This system was used since we have previously found these conditions to be optimal for demonstration of the effects of cytokines on plasma protein synthesis by hepatocytes. The current data do not permit us to conclude whether or not TGF-β would manifest activity in the absence of these hormones.

In the current studies, we found changes of the mRNA levels for PI and α-fibrinogen that parallel the effects of TGF-β on protein synthesis and secretion that we observed. These results indicate that TGF-β regulates synthesis of PI and fibrinogen at the pretranslational level, either by enhancing transcription or by mRNA stabilization. It has been

reported that TGF-β inhibits proliferation of freshly isolated rat hepatocytes but does not affect transformed hepatocytes (24); we found no significant differences in DNA content in control Hep 3B cells compared to cells treated with TGF-β, suggesting that cell proliferation in our system was not affected by TGF-β.

Many of the functional capabilities of TGF-β suggest that this cytokine is a major participant in the inflammatory and immunological response to injury and infection and in the consequent process of tissue repair (reviewed in ref. 20). In this paper, we demonstrate another biological activity of TGF-β—that it contributes to the regulation of synthesis of a number of acute-phase proteins in cultured liver cells. When our data are considered in the context of studies of other effects of TGF-β (37, 38), it appears that one of the major roles of this cytokine may be to limit proteolysis by stimulating synthesis of anti-proteases, both locally and systemically. The capacity of TGF-β to diminish fibrinogen synthesis and the fact that platelets synthesize TGF-β in large amounts during clot formation suggest that this cytokine may also play a role in control of the coagulation process. Finally, the observation that TGF-β down-regulates fibrinogen synthesis raises the possibility that circulating TGF-β, and perhaps other cytokines, may participate in physiologic regulation of fibrinogen synthesis.

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