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REGULATION OF ACUTE PHASE REACTION BY TRANSFORMING GROWTH FACTOR β IN CULTURED MURINE HEPATOCYTES

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Transforming growth factor-beta (TGF β_1), a multipotent immunoregulatory peptide produced by human platelets, has been shown to stimulate the synthesis of fibrinogen, contrapsin, complement component C3, and alpha-1-proteinase inhibitor by murine hepatocytes cultured for 2 days in DMEM containing 1 μM insulin and dexamethasone and 0.2% BSA. In the range of 10 pg to 10 ng/ml TGF-β_1 did not elicit any change in albumin secretion. Two main inflammatory cytokines: interleukin-6 (IL-6) and interleukin-1 (IL-1), known to stimulate two different subsets of murine acute phase plasma proteins, failed to increase contrapsin and alpha-1-proteinase inhibitor production. Epidermal growth factor (EGF) in the concentration 1 ng to 10 ng/ml effectively counteracted the stimulatory effect of TGF-β_1 on acute phase protein production. TGF-β_1-induced fibrinogen protein levels were associated with increased β-fibrinogen mRNA content. TGF-β_1 appears to be an additional physiological factor responsible for the direct stimulation of normal mouse hepatocytes to acute phase response.

The liver acute phase response is a complex reaction including drastic changes in the synthesis of certain plasma proteins which represents the adaptive homeostatic reactions to bacterial infection and tissue injury [1].

During the last few years, considerable progress has been made toward the identification of the potential mediators of the liver response to injury, the regulation of the expression of acute phase proteins in cultured hepatocytes, and a partial elucidation of the molecular mechanism of the acute phase genes expression [2, 3]. The stimulation of the acute phase protein synthesis in liver cells is the result of action of several cytokines and hormones such as interleukin 1, interleukin 6, tumor necrosis factor (TNF), interferon gamma (IFN) and glucocorticoids [3, 4].

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In search for other modulators of acute phase protein expression, we have studied the effect of two growth factors TGF-β_1 and EGF. TGF-β_1, a 25 kDa disulfide-linked homodimeric protein, is the prototype of a structurally and functionally related family of proteins that have diverse effects upon growth and development [5-8].

In the present work we have examined the modulation of the acute phase protein production by human platelet TGF-β_1, and we show that TGF-β_1 induces a subset of murine proteins. Furthermore, TGF-β_1 was shown to increase the synthesis of a number of acute phase proteins in both the presence or absence of IL-6. Messenger RNA for the β chain of fibrinogen has accumulated upon TGF-β_1-treatment. Another growth factor, EGF (1-10 ng/ml) slightly reduced the synthesis of the majority of the plasma proteins and counteracted the stimulatory effect of TGF-β_1 on the protein production.

METHODS

Hepatocyte cultures. Primary cultures of mouse hepatocytes were prepared by the collagenase method from CBA mice as described elsewhere [9]. The cells were plated at 10^6 cells per 35 mm well in 6-well Linbro plates, previously coated with rat tail collagen (type III, Flow Lab., U.S.A.) in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 1 μM insulin and dexamethasone and a mixture of antibiotics and antimycotics (100×, Gibco, U.S.A.). After 3-20 h, a fresh serum-free medium containing appropriate concentrations of a cytokine was added and replaced every 24 h. The collected media were used for electroimmunoassay of mouse plasma proteins.

Cytokines and growth factors. Recombinant human IL-6/IFN-β_2 was provided by Dr. W. Fiers (Ghent, Belgium) (8.96×10^8 U/mg). Human recombinant IL-1α was obtained from Dr. P. Lomedico (Hoffmann La Roche, through Dr. A. Falus; 10^6 U/ml).

Human platelet-derived TGF-β_1 has been received from Dr. M. Sporn (NIH, Bethesda) and EGF (isolated from mouse submaxillary glands) was a commercial preparation (Serva, Heidelberg).

Protein determination. The amounts of acute phase proteins secreted into the medium during 2 days in culture were determined daily by rocket immunoelectrophoresis [10]. The collected media were dialyzed against 0.015 M ammonium bicarbonate and concentrated 12.5 times by lyophilization. Monospecific antisera to the various proteins (fibrinogen, complement component C3, alpha-1-proteinase inhibitor and contrapsin) or polyspecific antiserum against mouse plasma proteins (for albumin detection) were used in 1:100 dilution for electroimmunoassay. Concentrations of contrapsin, α-1-proteinase inhibitor and albumin, in the culture media, expressed in μg/24 h × 10^6

cells were calculated using mouse serum as the standard. In most cases, however, the results are expressed as percentage of the control culture taken as 100%.

Isolation and analysis of RNA. The total cellular RNA was isolated and purified from monolayers of hepatocytes by the modification of the urea-LiCl method [11]. The Northern blot analysis was performed as described elsewhere [12]. The relative amount of total RNA in the gel was judged from ethidium bromide staining of 28S and 18S rRNA. β-Fibrinogen mRNA was detected with the rat cDNA probe (in pBR325, a gift from Dr. P. C. Heinrich) labelled with [^{32}P]dCTP by random priming using Amersham kit.

RESULTS

The cultured murine hepatocytes enabled the evaluation of the IL-1 and IL-6 effect on the synthesis of such plasma proteins as albumin (ALB), fibrinogen (FBG), complement component C3 (CC3), alpha-1-proteinase inhibitor (API) and contrapsin (CTN). Two different subsets of murine plasma proteins are affected by the two main inflammatory cytokines (Table 1). Forty

Table 1

Secretion of proteins by mouse hepatocytes

10^6 cells were cultured in 1.25 ml of DNEM containing 0.2% BSA and 1 μM dexamethasone supplemented with a cytokine (IL-6 — 100 ng and IL-1 — 100 U) or a growth factor (TGF-β_1 — 1 ng and EGF — 10 ng) diluted with the medium. The concentrations of ALB, API, and CTN in control culture media were 7.8 ± 0.8; 2.4 ± 0.2 and 0.8 ± 0.1 μg/24 h $\times 10^6$ cells, respectively (mean protein production from 7 cultures ± S.D.). Due to the lack of plasma values for FBG and CC3, the results are calculated only as percentage of the controls. Relative change in the protein production was determined on the second day in culture (24-48 h) and expressed as percentage of control value. The results derive from 2 to 9 experiments (numbers given in brackets). The variability of the results is indicated by minimal and maximal values given in brackets

		ALB	FBG	CC3	API	CTN
Control	(7)	100	100	100	100	100
IL-6	(9)	90 (74—110)	305 (250—360)	203 (146—253)	103 (86—115)	109 (95—129)
IL-1	(6)	100 (94—103)	98 (91—100)	194 (143—240)	94 (75—103)	103 (88—121)
TGF-β_1	(8)	110 (96—121)	216 (150—360)	176 (141—217)	145 (133—195)	169 (144—242)
EGF	(5)	99 (86—106)	92 (75—100)	88 (64—133)	97 (80—106)	103 (100—111)
EGF+TGF	(5)	94 (80—105)	142 (100—225)	78 (68—89)	116 (98—146)	114 (95—136)
IL-6+TGF	(6)	82 (72—95)	396 (215—600)	200 (125—267)	137 (121—154)	141 (126—155)
IL-1+TGF	(2)	137 (133—140)	234 (177—350)	213 (189—236)	141 (140—142)	130 (119—141)

pM TGF-β_1 (1 ng/ml medium) caused an increase in the secretion of all the proteins tested except albumin. The stimulation of synthesis of the two positive acute phase proteins, FBG and CC3, by TGF was smaller than that caused by IL-6. However, the only protein stimulated by IL-1, namely CC3, was enhanced by TGF to the same extent as by IL-1. Two murine proteinase inhibitors, API and CTN, the latter being the murine counterpart of human antichymotrypsin, unaffected by IL-1 or IL-6 treatment, were stimulated by TGF in the range of 10 pg to 100 ng/ml (Table 1). The dose-dependent increase in contrapsin secretion, caused by TGF-β_1, is presented in Fig. 1.

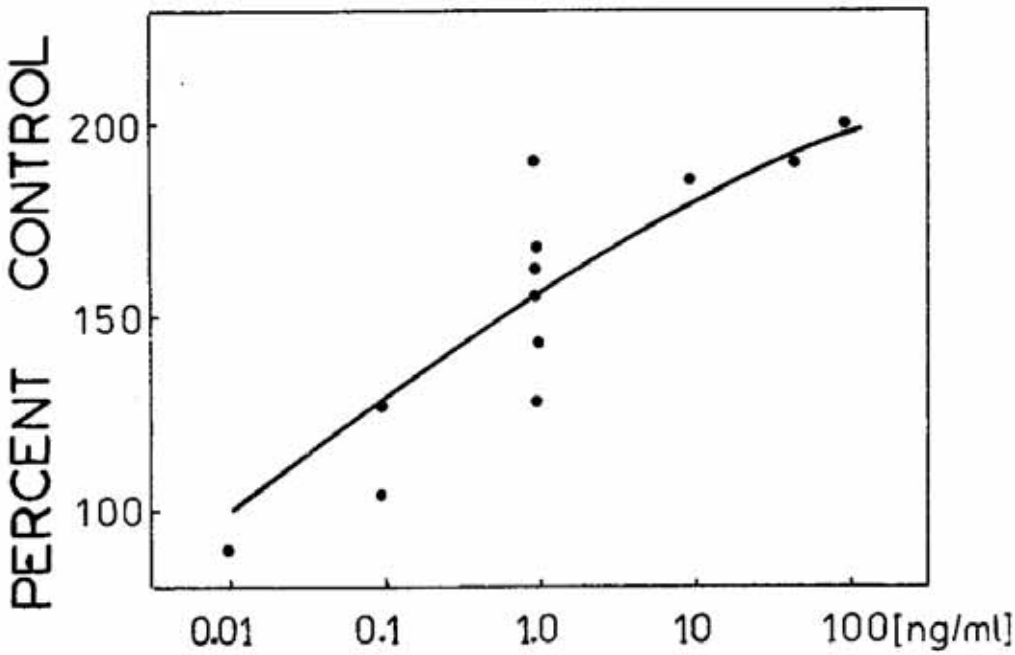

Fig. 1. Dose response for TGF-β of contrapsin secretion by mouse hepatocyte primary cultures. Hepatocytes (10^6 cells per dish) were incubated for 48 h with the daily addition of fresh medium and various concentrations of the growth factor (0.01 - 100 ng/ml). Relative amounts of contrapsin are presented as percentage of the untreated control assumed as 100% in the media from the second day in culture. Each point represents the results of one experiment except the points at the concentration of 1.0 ng/ml where 8 experiments has been done

Forty pM TGF-β_1 stimulated contrapsin secretion to 170% of the control culture, which corresponds to 1.53 μg of the protein/24 h × 10^6 cells. Epidermal growth factor in the dose of 1 or 10 ng/ml (the dose of 0.1 ng/ml was ineffective) reduced the synthesis of positive acute phase proteins: FBG and CC3, not affecting that of ALB, API, and CTN (Table 1). EGF (1.7 nM, 10 ng/ml) added to TGF-β_1-treated cells counteracted the stimulatory effect of TGF-β_1 on all the proteins tested (Table 1).

The level of mRNA in hepatocytes for the β-subunit of fibrinogen increased to 2-3 times the level found in the control cultures at 24 h after TGF-β_1 addition (Fig. 2). EGF (1 and 10 ng/ml) decreased the level of β-fibrinogen mRNA and also reduced TGF-β_1-induced FBG mRNA.

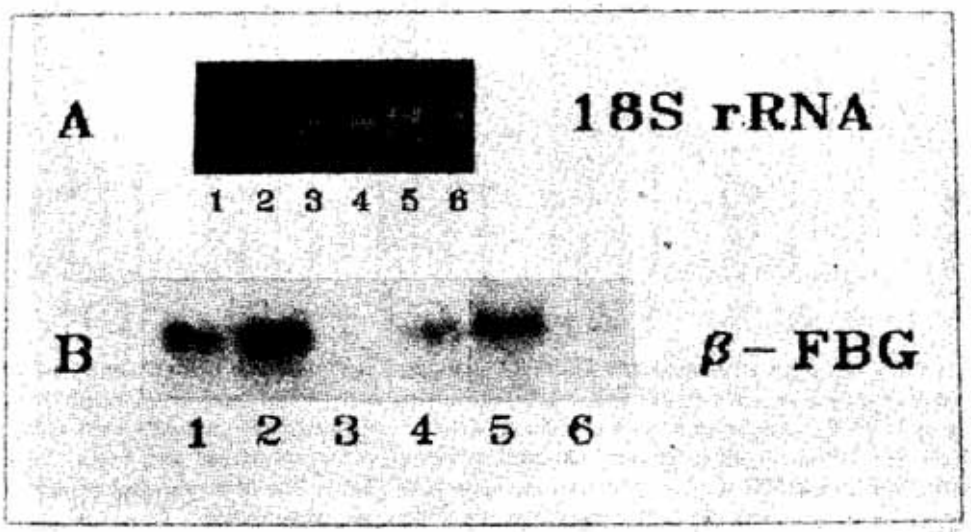

Fig. 2. Northern blot analysis of TGF-β_1 and EGF effect on β-fibrinogen gene expression. A. Ethidium bromide staining of the gels (18 S rRNA) indicated that all lanes had approximately equal amounts of RNA loaded. B. RNA isolated from murine hepatocytes after 24 h of treatment, was analyzed by blot hybridization with cDNA probe to rat β-fibrinogen. Lanes: 1, control; 2, TGF-β_1 (10 ng/ml); 3, EGF (10 ng/ml); 4, EGF (1 ng/ml); 5, TGF-β_1 (10 ng/ml) and EGF (1 ng/ml); 6, TGF-β_1 (10 ng/ml) and EGF (10 ng/ml)

Dexamethasone, found to be necessary for the full acute phase protein response in cultured hepatocytes or hepatoma cells [3, 13], had an enhancing effect on the TGF-stimulated production of FBG, CC3, and to a smaller extent of CTN, while being almost without effect on API and ALB (Fig. 3).

The synthesis of FBG and CC3 was increased by treatment with IL-6 in murine hepatocytes but was not significantly affected by the addition of TGF-β_1 to IL-6 (Table 1). CTN and API unaffected by IL-6, respond to TGF-β when treated with a combination of the two factors. EGF showed a decreasing effect on the IL-6-stimulated proteins (especially CC3) but did not change the ALB, CTN, API and FBG production (not shown). In the presence of IL-1, TGF-β only slightly enhanced ALB and the production of remaining proteins was either increased, like in the case of CC3 and FBG, or decreased, like for CTN (Table 1).

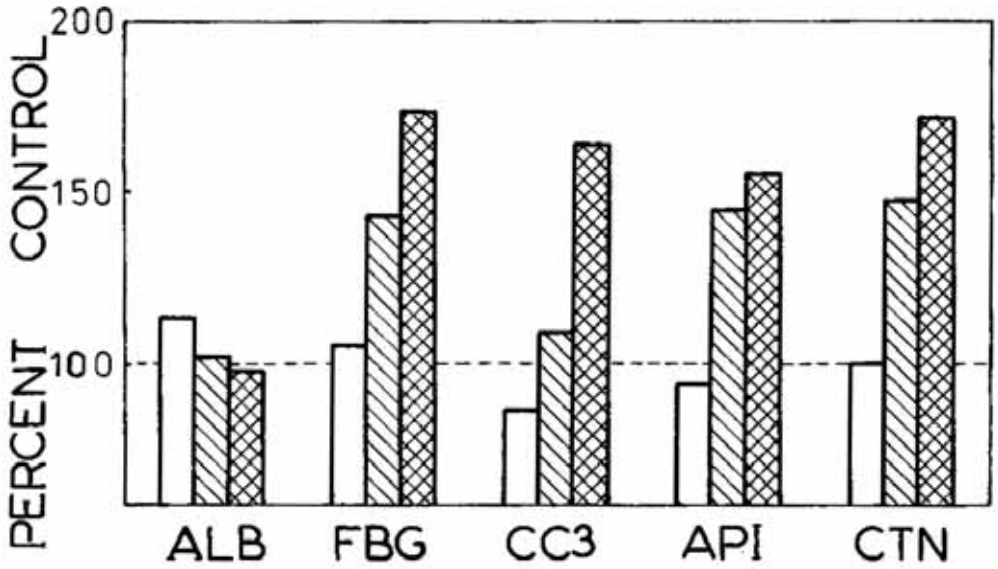

Fig. 3. Influence of dexamethasone on TGF-β_1-stimulated acute phase proteins. Primary cultures of mouse hepatocytes (10^6 cells per well in 1.25 ml medium) were treated twice for 24 h periods with 1 ng of TGF-β_1 with (crossed bars) or without 10^{-6} M dexamethasone (hatched bars). Control cultures without dexamethasone were assumed as 100% (horizontal dashed line) while controls treated with dexamethasone are presented as open bars. The results (from the second day) are expressed as means from three separate experiments

DISCUSSION

Owing to the widespread use of primary cultures of rat and murine hepatocytes and cultures of human hepatoma cell lines, several inflammatory cytokines have been identified [10, 14-16]. Among them IL-6/IFN β_2 is regarded as the most potent stimulator of major human, rat and mouse acute phase proteins *in vitro* [4, 9, 17, 18]. Despite the generally accepted opinion that the regulation of a given acute phase protein expression can be satisfactorily achieved with just three basic factors: IL-1, IL-6, and dexamethasone, current results confirm the participation of TGF-β_1 in the modulation of the acute phase response [19, 20].

TGF-β_1 (0.1-10 ng/ml) inhibited both basal and interleukin-6-induced amino acid uptake by rat hepatocytes with only slight alteration of acute phase protein synthesis [19].

In the experiments reported here, TGF-β_1 was found to be the inducer of a subset of murine plasma proteins, in part different from those reported to be affected by IL-1, TNF, or IL-6. Namely, TGF-β_1 can increase the secretion of not only the murine FBG, and CC3 but also CTN and API that are not regarded as positive acute phase proteins in murine hepatocytes. However, the latter two proteins are increased by IL-6, as well as by TGF-β_1 in two human hepatoma cell lines, HepG2 and Hep3B [21, 22]. Thus TGF-β has been found

to stimulate two serine protease inhibitors as well as other types of protease inhibitors such as the inhibitors of plasminogen activators and metalloproteases [23]. From those data it appears that a possible principal role of this growth factor is to limit proteolysis by stimulating the synthesis of proteinase inhibitors. This also suggests that TGF-β_1 is a major participant in the inflammatory and immunological response to injury and infection and in the subsequent process of tissue repair [24]. The observation by Mackiewicz *et al.* [21] that TGF-β down regulates the fibrinogen synthesis in human hepatoma cells is different from the results obtained for mouse hepatocytes, in which we find an increase in the FBG synthesis and β-fibrinogen mRNA upon TGF-β treatment. Similar elevation of the FBG synthesis has been also reported by Darlington *et al.* [22] for Hep 3B cells.

TGF-β_1 is similar to IL-6 in its effect on the FBG and CC3 synthesis but differs in its enhancing effect on CTN and API. The lack of the effect of TGF on ALB and the increase in CC3 synthesis are shared with the IL-1 activities but the two factors differ in their effect on FBG, API and CTN. In the presence of IL-6, TGF-β retains its activity toward the API and CTN enhancement but does not change the IL-6 effect on ALB and two positive acute phase proteins: FBG and CC3. IL-1α and TGF-β seem to have an additive effect on the complement component C3 synthesis by murine hepatocytes (see Table 1). Northern blot analysis carried out for the mRNA for β-fibrinogen in rat liver during acute inflammation revealed that the acute-phase response dominates during the first 18 h [25]. In our current experiments TGF-β and EGF effects, the most spectacular on proteins at 48 h, were clearly visible on β-fibrinogen mRNA level already at 24 h (Fig. 2).

Although glucocorticoids (including dexamethasone) alone could stimulate, both *in vivo* and *in vitro*, the expression of some acute phase proteins, they were unable to reproduce the entire acute phase response [13, 26]. Dexamethasone prominently enhances the expression of IL-6 responsive genes, e.g., for rat cysteine proteinase inhibitor and alpha-2-macroglobulin [27, 28] as well as of IL-1 responsive genes like that for alpha-1-acid glycoprotein (AGP) [29]. The dexamethasone specificity in mouse liver cells was identified in the regulation of the synthesis of AGP, haptoglobin, CC3, serum amyloid A and P, and FBG [9, 30, 31]. In our recent experiments dexamethasone appeared to be necessary for the FBG, CC3 and CTN enhancement by TGF-β_1 (see Fig. 3).

It is hardly justified to extrapolate the results from tissue culture to the situation *in vivo*. The cellular sources of TGF-β in the liver are only partially known. It appears now that hepatocytes and bile duct epithelial cells do not express TGF$_1$-β_1 mRNA while in endothelial cells and fat storing cells the level of increased TGF-β_1 mRNA was reported [32]. Therefore TGF-β-induced acute phase protein synthesis in cultured hepatocytes might not reflect the physiological effects of TGF-β on the hepatocytes *in vivo*, where the actual concentrations of particular cytokines may be insufficient to induce

the production of acute phase proteins. Moreover, the mechanism of activation of the latent TGF-β_1 form *in vivo* still remains to be elucidated.

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