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Transforming growth factor-β1 inhibits the growth of primary adult rat hepatocyte cultures by increasing cAMP levels

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Abstract

We investigated the mechanisms of transforming growth factor-β1 (TGF-β1) inhibition on transforming growth factor-α (TGF-α)-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. TGF-α (1.0 ng/ml) produced a 4.2-fold elevation of DNA synthesis during 3 h of culture and a 1.2-fold increase in nucleus number (proliferation) during 4 h of culture. TGF-β1 dose dependently inhibited the TGF-α-induced hepatocyte DNA synthesis and proliferation: half-maximal inhibition occurred at a TGF-β1 concentration of 0.08 ng/ml. The inhibitory effects of 1.0 ng/ml TGF-β1 were almost completely reversed by adenylate cyclase inhibitors, 2,4-dideoxyadenosine (10^{-6} M), and somatostatin (3×10^{-7} M), or by a specific inhibitor of protein kinase A, H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; 10^{-7} M). In addition, while TGF-α did not affect the basal cellular adenosine 3′,5′-monophosphate (cAMP) levels, TGF-β1 was found to produce dose-dependent increases in cellular cAMP levels. The cAMP-elevating effects of TGF-β1 were also reversed by 2,4-dideoxyadenosine (10^{-6} M), and somatostatin (3×10^{-7} M), but not by H-89 (10^{-7} M). The present results suggest that the specific mechanisms involved in the growth inhibitory effect of TGF-β1 on TGF-α-induced hepatocyte DNA synthesis and proliferation are via stimulation of adenylate cyclase, which increases intracellular cAMP and subsequently activates protein kinase A. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: TGF-α (transforming growth factor-α); TGF-β1 (transforming growth factor-β1); 2,4-Dideoxyadenosine; Somatostatin; H-89; Hepatocyte proliferation

1. Introduction

Most hepatocytes in adult animals exist in a quiescent state, yet are capable of being stimulated into DNA synthesis and subsequent mitosis in vivo and in vitro. The key to understanding the control of hepatocyte proliferation and differentiation may lie in elucidating actions and interactions among various stimulatory and inhibitory growth factors and growth modulators (Fausto et al., 1995; Michalopoulos and DeFrances, 1997).

Transforming growth factor-β (TGF-β) was first identified as a factor that induced phenotypic transformation of some fibroblast cell lines (Roberts et al., 1981). Since then, it has been shown that TGF-β is a mutifunctional cytokine involved in controlling cell cycle progression, cell adhesion, and extracellular matrix deposition in a variety of cell types (Roberts et al., 1985; Carr et al., 1986; Barnard et al., 1990; Sporn and Roberts, 1992; Derynck, 1994; Alexandrow and Moses, 1995). The three distinct molecular forms of TGF-β that have been discovered in mammals have been designated as TGF-β1, TGF-β_2, and TGF-β_3, respectively. Of the three mammalian forms of TGF-β, TGF-β1 has been studied most extensively over the past 10 years (Attisano et al., 1994). TGF-β1 has been shown by several laboratories to have a bifunctional action on cell growth. For instance, TGF-β1 stimulates the proliferation of various mesenchymal cell types, but acts as a growth inhibitor of many other cell types, including epithelial cells (Derynck, 1994).

In the liver, TGF-β1 appears to be an important cytokine under both normal and pathological conditions (Russel et al., 1988). TGF-β1 has been shown to be a strong inhibitor of hepatocyte DNA synthesis in vivo and in vitro (Nakamura et al., 1985; Wollenberg et al., 1987).

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Several studies have been aimed at defining interactions between strong growth stimulators, such as transforming growth factor-α (TGF-α), or epidermal growth factor (EGF), and a strong growth inhibitor, TGF-β. Although several recent reviews have emphasized the role of TGF-β in the control of cell cycles, there have been few studies on the signal transduction mechanisms involved in the antagonistic effects of TGF-β1 on hepatocyte DNA synthesis and proliferation induced by EGF or TGF-α (Houck et al., 1988; Howe et al., 1989; Moses et al., 1990; Geng and Weinberg, 1993). An understanding of the receptor-mediated signaling pathways of TGF-β1 will not only provide an insight into the mechanisms of growth arrest, but also help to explain the activities of these growth-stimulating factors.

Therefore, we have now examined the effects of exogenous TGF-β1 on TGF-α-induced hepatocyte DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. The present results demonstrated that TGF-β1 could rapidly and potently inhibit TGF-α-induced hepatocyte DNA synthesis and proliferation by increasing the levels of intracellular adenosine 3′,5′-monophosphate (cAMP). The effects of TGF-β1 were almost completely reversed by adenylate cyclase inhibitors, 2,4-dideoxyadenosine, and somatostatin, or a protein kinase A inhibitor, H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride). Thus, it was confirmed that TGF-β1 inhibits hepatocyte growth through stimulation of adenylate cyclase, which increases the cellular cAMP level and subsequently activates protein kinase A.

2. Materials and methods

2.1. Animals

Male Wistar rats (weight 200–220 g) were obtained from Saitama Experimental (Saitama, Japan). They were allowed to adapt to a humidity- and temperature-controlled room for at least 3 days before the experiment was started. They were fed a standard diet and given tap water ad libitum. The study reported upon this manuscript was carried out according to the Josai University guidelines for ethical animal care and the Guide for Care and Use of Laboratory Animals as adopted and published by the US National Institutes of Health.

2.2. Hepatocyte isolation and culture

The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Hepatocytes were isolated from normal liver by the two-step in situ collagenase perfusion technique created by Seglen to facilitate disaggregation of the adult rat liver as described previously (Seglen, 1975). In brief, hepatocytes were washed three times by slow centrifugation ($50 \times g$, 1 min) of the cell suspension in order to remove cell debris, damaged cells, and non-parenchymal cells. The viability of hepatocytes was more than 97% as assessed by trypan blue dye exclusion. Unless otherwise indicated, isolated hepatocytes were plated onto collagen-coated plastic culture dishes (Sumitomo Bakelite, Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2 (3.1×10^5 cells/35-mm dish), and allowed to attach for 3 h on collagen-coated dishes in Williams' medium E containing 5% fetal bovine serum, 0.1 nM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.10 μg/ml aprotinin in 5% CO_2 in air at 37°C. The medium was then changed, and the cells were cultured in serum- and dexamethasone-free Williams' medium E supplemented with TGF-α. When appropriate, the following agents were added: TGF-β1 with or without 2,4-dideoxyadenosine, somatostatin, H-89, and cAMP-increasing agents.

2.3. Measurement of DNA synthesis

Hepatocyte DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into acid-precipitable materials (Morley and Kingdon, 1972). Briefly, after an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free Williams' medium E and cultured in a medium containing TGF-α (1.0 ng/ml) and/or TGF-β1 (0.01–1.0 ng/ml) for an additional 4 or 21 h. The cells were pulsed at 1, 2, 3, or 19 h post-TGF-α stimulation for 2 h with [^{3}H]thymidine (1.0 μCi/well) followed by trichloroacetic acid precipitation as described previously. [^{3}H]Thymidine incorporation into DNA was measured in a liquid scintillation counter and normalized for cellular protein. Aphidicolin (10 μg/ml) was added to some wells to establish the level of non-replicative DNA synthesis. Hepatocyte protein content was measured by a modified Lowry procedure with bovine serum albumin as a standard (Lee and Paxman, 1972). The data were expressed as dpm/h/mg cellular protein.

2.4. Counting nuclei

The number of nuclei were counted utilizing a slightly modified version of the procedure previously described by Nakamura et al. (1983). Briefly, the primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4). Then, isolated liver cell nuclei were prepared for quantitation by exposure of hepatocyte cultures to 0.25 ml of 0.1 M citric acid containing 0.1% Triton X-100 for 30 min at 37°C. An equal volume of the nucleus suspension was mixed with 0.3% trypan blue in Dulbecco's phosphate-buffered saline (pH 7.4) and the number of nuclei were counted in a hemocytometer. This procedure was performed because the hepatocytes firmly attached to the collagen-coated plates and were not dispersed sufficiently by 0.02% EDTA–0.05% trypsin treatment.

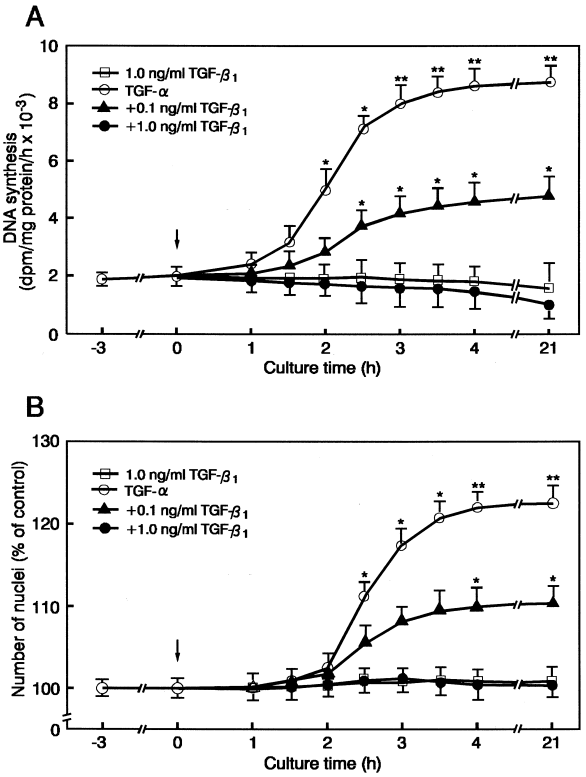

Fig. 1. Time course of TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation induced by TGF-α. Isolated hepatocytes were plated onto collagen-coated plastic culture dishes at a density of 3.3×10^4 cells/cm^2 and allowed to attach to the dishes for 3 h. The medium was then changed, and the cells were cultured for various lengths of time with serum-free Williams' medium E containing 0.1 and 1.0 ng/ml TGF-β1 in the presence of 1.0 ng/ml TGF-α. Arrowhead indicates the time of TGF-α and/or TGF-β1 addition (zero time). Hepatocyte DNA synthesis (A) and proliferation (B) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control (medium alone).

2.5. Assay of cellular cAMP

At each time point, the cultured hepatocytes were washed twice with ice-cold Dulbecco's phosphate-buffered saline (pH 7.4) and the incubation was terminated by adding 0.1 ml of 1.0 N HCl. The cells were scraped off with a rubber policeman and were transferred with the medium into small test tubes. The test tubes were heated in boiling water for 3 min to obtain a deproteinized extract. The suspension was centrifuged at $1500\times g$ for 5 min. The cAMP content of the supernatant was determined with a sensitive radioimmunoassay procedure (Honma et al., 1977). The data were expressed as pmol cAMP/mg cellular protein.

2.6. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): 2,4-dideoxyadenosine, 8-bromo cAMP (8-br-cAMP), aphidicolin, metaproterenol hemisulfate, butoxamine hydrochloride, forskolin, dexamethasone, somatostatin, and aprotinin. Recombinant human TGF-α was obtained from Pepro Tech (London, England). Recombinant human TGF-β1 was obtained from Pharma Biotechnologie Hannover (Hannover, Germany). H-89 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical (Freehold, NJ, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was obtained from DuPont–New England Nuclear (Boston, MA, USA). The assay kit for cAMP was obtained from Yamasa Shoyu (Chiba, Japan). All other reagents were of analytical grade.

2.7. Statistical analysis

The data are expressed as means ± S.E.M. and were analyzed with the unpaired Student's *t*-test. *P* values less than 0.05 were regarded as statistically significant.

3. Results

3.1. Time course of the inhibition of TGF-α-induced hepatocyte DNA synthesis and proliferation by TGF-β1

We studied the time course of the effect of TGF-β1 on hepatocyte DNA synthesis and number of nuclei (proliferation) induced by 1.0 ng/ml TGF-α. A significant increase in DNA synthesis occurred 2.0 h after hepatocytes were cultured with 1.0 ng/ml TGF-α, and reached a peak around 3.5 h that was sustained for about 17 h (Fig. 1A). In addition, hepatocytes proliferated under these conditions with a short lag time: a significant increase in the number of nuclei induced by 1.0 ng/ml TGF-α was observed

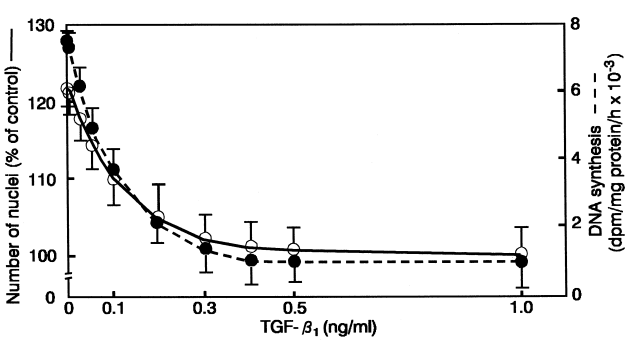

Fig. 2. Dose-dependent effect of TGF-β1 on hepatocyte DNA synthesis and proliferation in primary culture of adult rat hepatocytes. Following cell attachment (zero time), hepatocytes (3.3×10^4 cells/cm^2) were cultured for 4 h more with various concentrations of TGF-β1 in the presence of 1.0 ng/ml TGF-α. Hepatocyte DNA synthesis (a) and proliferation (b) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. TGF-β1 was added at 0 h immediately after 1.0 ng/ml TGF-α addition.

about 2.5 h after TGF-α addition, reached a peak at 4 h and was sustained for an additional 17 h (Fig. 1B). Although TGF-β1 alone had no significant effect on hepatocyte DNA synthesis and proliferation, the TGF-α-stimulated hepatocyte DNA synthesis and proliferation were significantly inhibited with 0.10 and 1.0 ng/ml of TGF-β1 in a time-dependent manner (Fig. 1A,B). There was a good correlation between the dose of TGF-β1 and the ability to inhibit TGF-α-induced hepatocyte DNA synthesis and proliferation.

3.2. Dose-dependent effects of TGF-β1 on TGF-α-induced hepatocyte DNA synthesis and proliferation

We also examined the dose-dependent effects of TGF-β1 on TGF-α-induced hepatocyte DNA synthesis and proliferation in cultured hepatocytes 4 h after the addition of TGF-β1. As shown in Fig. 2, TGF-β1 induced dose-dependent decreases in hepatocyte DNA synthesis and proliferation after 4 h of culture. The inhibitory effects were significant at a concentration of 0.05 ng/ml or higher (Fig. 1B). The DNA synthesis and proliferation induced by 1.0 ng/ml TGF-α were almost completely abolished by 0.5 ng/ml TGF-β1 (Fig. 2). The TGF-β1 concentration required for half-maximal inhibition (IC_{50}) was 0.08 ng/ml TGF-β1 for DNA synthesis and 0.10 ng/ml TGF-β1 for proliferation. These results of TGF-β1 action were compatible with the findings of earlier studies.

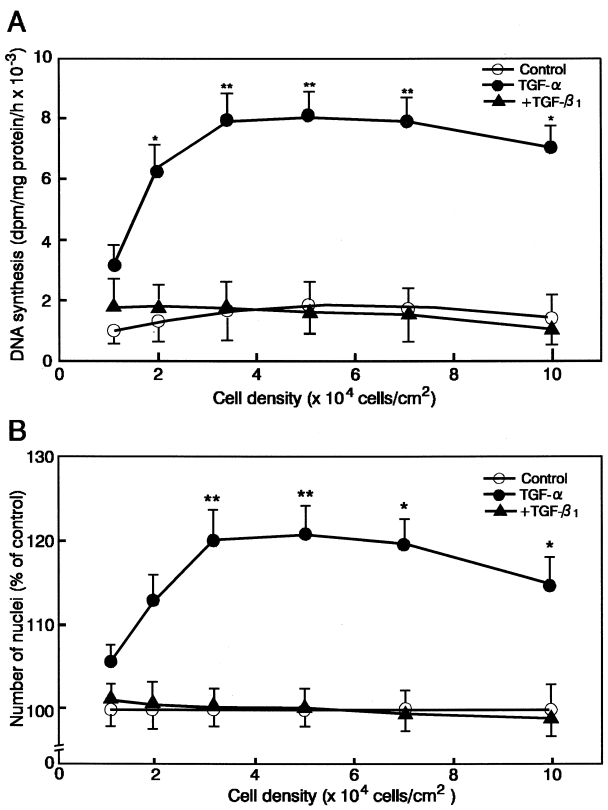

Fig. 3. Influence of cell density on TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation in the presence of TGF-α. Following cell attachment (zero time), hepatocytes were cultured for an additional 4 h with 1.0 ng/ml TGF-β1 in the presence of 1.0 ng/ml TGF-α at various plating densities. Hepatocyte DNA synthesis (A) and proliferation (B) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control (medium alone).

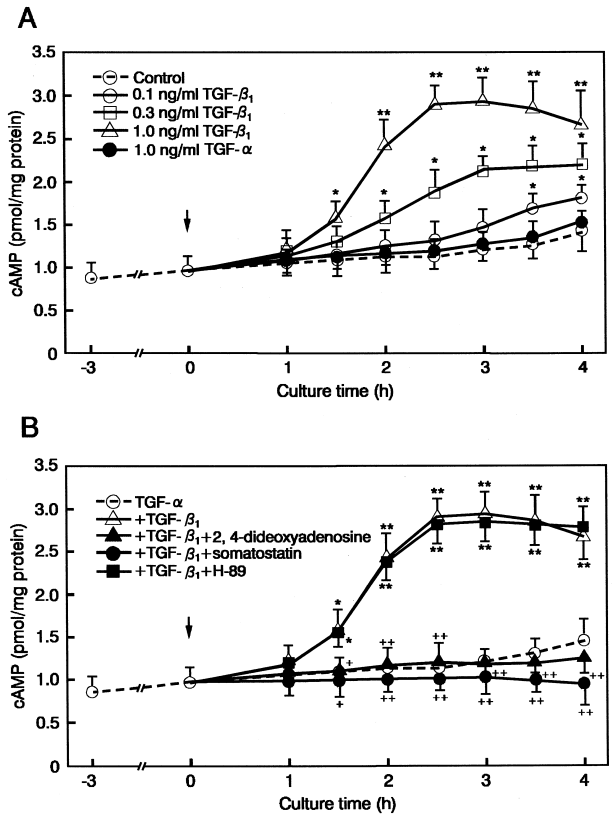

Fig. 4. Effects of TGF-β1 on hepatocyte cAMP levels in the presence or absence of 2,4-dideoxyadenosine, somatostatin, or H-89. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were cultured with various concentrations of TGF-β1 in the presence or absence of 1.0 ng/ml TGF-α for 4 h. Dose-dependent effects of TGF-β1 on hepatocyte cAMP levels (A) and their inhibition by 10^{-6} M 2,4-dideoxyadenosine, 3×10^{-7} M somatostatin, but not by 10^{-7} M H-89 (B) are shown. Hepatocyte cAMP levels were determined as described in Section 2. Arrowhead indicates the time of addition of TGF-α, TGF-β1, 2,4-dideoxyadenosine, somatostatin, and H-89 (zero time). Data are expressed as means ± S.E.M. of three experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control (medium alone).

3.3. Effects of plating density on TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation induced by TGF-α

We next investigated the influence of plating density on TGF-β1 inhibition of the hepatocyte DNA synthesis and proliferation induced by 1.0 ng/ml TGF-α. As shown in Fig. 3A,B, the stimulatory effects of 1.0 ng/ml TGF-α were not affected significantly even at high cell densities. In addition, at high cell densities, the growth inhibitory effects of 1.0 ng/ml TGF-β1 were still observed in primary cultures of hepatocytes, suggesting that the TGF-β1-

induced reduction of hepatocyte DNA synthesis and proliferation is independent of cell density.

3.4. Effects of TGF-β1 on hepatocyte cAMP levels: effects of 2,4-dideoxyadenosine, somatostatin, and H-89

In preliminary experiments, we found that the potent proliferative effects of 1.0 ng/ml TGF-α on cultured hepatocytes were strongly inhibited by β-adrenoceptor agonists and cAMP-elevating agents. Therefore, we were interested in determining whether the mito-inhibitory effects of TGF-β1 effects are mediated by changes in hepatocyte cAMP levels or not. In order to more directly analyze the cAMP involvement in TGF-β1 signal transduction, cAMP levels in hepatocytes were determined during 4 h of culture. As shown in Fig. 4A,B, 1.0 ng/ml TGF-α alone had no significant effect on the basal cAMP level. On the other hand, we found that TGF-β1 led to mild but significant increases in intracellular concentration of cAMP, in a dose-dependent manner, after 4 h of culture. The role of adenylate cyclase and protein kinase A in TGF-β1-induced increases in cAMP levels was determined in the presence of 1.0 ng/ml TGF-α by the addition of 2,4-dideoxyadenosine, somatostatin, or H-89. As indicated in Fig. 4B, treatment of hepatocytes with either 10^{-6} M 2,4-dideoxyadenosine or 3×10^{-7} M somatostatin resulted in almost complete inhibition of the cAMP levels induced by 1.0 ng/ml TGF-β1. In contrast, 10^{-7} M H-89 did not affect the TGF-β1-stimulated cAMP levels. 2,4-Dideoxyadenosine (10^{-6} M), somatostatin (3×10^{-7} M) or H-89 (10^{-7} M) alone, did not significantly affect the basal cAMP levels (data not shown).

3.5. Effects of specific inhibitors and stimulators of β_2-adrenoceptor-mediated responses on the TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation induced by TGF-α

The effects of specific inhibitors of signal transduction on hepatocyte DNA synthesis and proliferation were studied to clarify how TGF-β1 signaling might decrease the hepatocyte mitogenesis induced by TGF-α (Table 1). Consistent with results of a previous study, a β_2-adrenoceptor-agonist, metaproterenol, and 8-bromo-cAMP and forskolin, agents that elevate intracellular cAMP, also produced marked inhibition of the hepatocyte DNA synthesis and proliferation induced by 1.0 ng/ml TGF-α. In addition, the effects of metaproterenol were blocked by a specific β_2-adrenoceptor-antagonist, butoxamine (10^{-7} M). Then, at 4 and 21 h culture points, we examined whether or not 2,4-dideoxyadenosine, somatostatin, or H-89, reversed the TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation stimulated by 1.0 ng/ml TGF-α. As expected, it was found that the 1.0 ng/ml TGF-β1-induced suppression of hepatocyte DNA synthesis and proliferation initiated by 1.0 ng/ml TGF-α was almost completely reversed by simultaneous addition of 10^{-6} M 2,4-dideoxyadenosine, 3×10^{-7} M somatostatin or 10^{-7}

Table 1
Effects of specific inhibitors and stimulators of β_2-adrenoceptor-mediated responses on the TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation induced by TGF-α
Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2. After an attachment period of 3 h, the medium was changed and the cells were cultured for an additional 4 or 21 h with 1 ng/ml TGF-α alone or with avrious agents: TGF-β1, 1 ng/ml; 2,4-dideoxyadenosine, 10^{-6} M; somatostatin, 3×10^{-7} M; H-89, 10^{-7} M; butoxamine, 10^{-7} M; metaproterenol, 10^{-7} M; 8-bromo cAMP, 10^{-7} M; and forskolin, 10^{-7} M. Each value is expressed as mean ± S.E.M. from three independent preparations.

Treatment	DNA synthesis (dpm/mg protein/h × 10^{-3})		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control	1.965 ± 0.210	1.390 ± 0.338	100.3 ± 1.4	99.9 ± 2.7
TGF − β1	1.897 ± 0.135	0.821 ± 0.207	100.1 ± 1.2	100.2 ± 1.0
TGF-α	8.206 ± 0.613[a,b]	8.417 ± 0.603[a,b]	121.1 ± 1.6[a,b]	124.7 ± 1.4[a,b]
+ 2,4-dideoxyadenosine	8.015 ± 1.023[a,b]	8.300 ± 1.032[b,c]	120.9 ± 1.4[a,b]	123.3 ± 2.3[b,c]
+ somatostatin	8.103 ± 1.005[a,b]	8.347 ± 0.908[b,c]	121.3 ± 1.6[a,b]	123.1 ± 2.6[b,c]
+ H-89	8.255 ± 1.006[a,b]	8.402 ± 1.020[b,c]	120.9 ± 1.4[a,b]	123.6 ± 2.9[b,c]
+ butoxamine	8.206 ± 1.203[a,b]	8.330 ± 0.968[a,b]	121.2 ± 1.8[a,b]	122.7 ± 1.0[a,b]
+ metaproterenol	2.207 ± 0.521	2.045 ± 0.608	101.8 ± 3.0	101.4 ± 2.1
+ metaproterenol + butoxamine	8.132 ± 0.586[a,b]	8.312 ± 0.521[a,b]	121.5 ± 1.8[a,b]	124.6 ± 1.5[a,b]
+ 8-bromo cAMP	2.106 ± 0.612	3.307 ± 1.044	102.2 ± 2.7	103.4 ± 1.5
+ forskolin	2.111 ± 0.360	2.217 ± 0.303	100.1 ± 2.6	100.6 ± 2.0
+ TGF-β1	1.803 ± 0.198	1.096 ± 0.284	100.1 ± 1.2	98.3 ± 2.0
+ TGF-β1 + 2,4-dideoxyadenosine	8.321 ± 1.031[a,b]	8.402 ± 1.122[b,c]	122.2 ± 1.6[a,b]	123.5 ± 2.1[b,c]
+ TGF-β1 + somatostatin	8.332 ± 1.037[a,b]	8.321 ± 1.062[b,c]	121.8 ± 1.5[a,b]	123.3 ± 2.0[b,c]
+ TGF-β1 + H-89	8.263 ± 1.027[a,b]	8.321 ± 1.220[b,c]	123.3 ± 1.8[a,b]	123.7 ± 2.6[b,c]

[a] Values significantly different from control indicated by $P < 0.01$.
[b] Values significantly different from TGF-α + TGF-β1 indicated by $P < 0.01$.
[c] Values significantly different from control indicated by $P < 0.05$.

M H-89 to the cultures. In the absence of TGF-β1, 2,4-dideoxyadenosine (10^{-6} M), somatostatin (3×10^{-7} M), and H-89 (10^{-7} M) did not significantly affect TGF-α-induced hepatocyte DNA synthesis and proliferation (data not shown).

4. Discussion

The present study demonstrated that, in a dose-dependent manner, TGF-β1 rapidly inhibited the early proliferative response induced by TGF-α (Figs. 1 and 2). The data are very similar to those obtained by another group that demonstrated TGF-β1 inhibition of primary cultured hepatocyte growth induced by 10 ng/ml EGF (Houck et al., 1988). Together, these findings raise the possibility that this cytokine may regulate proliferation in the liver. In addition, the present results suggest that TGF-β1 inhibition of hepatocyte mitogenesis may be a consequence of the blockade of the late G1 phase (2.5–3.0 h before onset of DNA synthesis) of the cell cycle (Fig. 1A,B). This result was somewhat surprising, because the inhibitory effects of TGF-β1 on DNA synthesis induced by growth factors are recognized to be closely associated with cell cycle arrest at the G0/G1 phase. On the other hand, it has been reported that interference with the cell cycle by TGF-β1 may commence in the late S or G2 phase in another cell line (Kramer et al., 1994). Therefore, the precise stage of the cell cycle at which TGF-β1 exerts its growth inhibitory effects remains to be elucidated.

TGF-β was shown to induce apoptosis in hepatocytes as well as in hepatoma cells by investigators who cultured hepatocytes for a long time (more than 48 h) and treated them with high concentrations of TGF-β1 (5–40 ng/ml) (Diez-Fernandez et al., 1998; Huang et al., 1998). We therefore investigated the question of whether or not cell death, under our conditions, would occur through apoptosis. When we extracted DNA from hepatocytes cultured with or without 1 ng/ml TGF-β1 or 1 ng/ml TGF-β1 plus 1 ng/ml TGF-α and analyzed the 12-μg DNA/lane on 1.2% or 2.0% agarose gel elecctrophoresis (Cohen and Duke, 1984), no significant difference in the DNA fragmentation pattern was seen as compared with that of the corresponding control after 4 or 21 h of culture (data not shown). In addition, viable cells were counted under the microscope and the number of dead cells as judged by trypan blue staining was compared with that from the control cultures (medium alone) at 4 h of culture. The relative adherent cell number (compared with that of the control value) after 4 h of culture in the presence of 1ng/ml TGF-β1 was around 99%. There was no significant difference in the number of dead cells as compared with the control culture (data not shown). According to these observations, we conclude that, under our conditions, 1 ng/ml TGF-β1 did not stimulate DNA degradation and cell death through apoptosis.

Several second messenger systems have been proposed as the mediators of growth factor action: these include tyrosine kinase phosphorylation, ion fluxes, cAMP, and phosphatidylinositol turnover (Diehl and Rai, 1996). Very few studies, however, have reported direct coupling of TGF-β1 binding to subsequent biological responses. For instance, there is a report that the G protein, which modulates the activity of adenylate cyclase, is responsible for mediating some of the biological actions of TGF-β1 (Howe et al., 1989). We found that TGF-β1 produced sustained suppression of hepatocyte DNA synthesis and proliferation under conditions that resulted in a mild but significant increase in intracellular cAMP levels (Fig. 4 and Table 1). Therefore, we hypothesized that cAMP may mediate the mito-inhibitory action of TGF-β1. By using specific inhibitors of signal transducers, such as 2,4-dideoxyadenosine, a direct inhibitor of adenylate cyclase (Holgate et al., 1980), somatostatin, an indirect inhibitor of adenylate cyclase (Grimaldi et al., 1997), or H-89, a specific protein kinase A inhibitor (Zuscik et al., 1994), we demonstrated that TGF-β1 loses its ability to inhibit TGF-α-induced hepatocyte DNA synthesis and proliferation (Table 1). In addition, TGF-α-induced hepatocyte DNA synthesis and proliferation were almost completely inhibited by cAMP-elevating agents, which act via different mechanisms (Christofferesen, 1992). These results support the notion that activation of both adenylate cyclase and protein kinase A is obligatory for the TGF-β1-mediated inhibition of the early proliferative response induced by 1.0 ng/ml TGF-α. Furthermore, a number of studies have shown recently that the second messenger cascades connected to protein kinase A are involved in apoptosis in hepatocytes. For instance, activation of protein kinase A by increasing cAMP levels (forskolin, 1-methyl-3-isobutylxanthine) correlated with decreased proliferation and subsequent apoptosis (Chen et al., 1998). In contrast, agents that increase intracellular cAMP protect against hepatocyte apoptosis induced by hydrophobic bile acid (Webster and Anwer, 1998). Therefore, the precise role of the TGF-β1-induced cAMP/protein kinase A pathway in the regulation of hepatocyte apoptosis remains to be elucidated.

Other specific inhibitors of the intracellular signaling cascade, such as genistein, wortmannin, and rapamycin, seem to be useful probes with which to characterize target proteins involved in the TGF-β1 inhibition of hepatocyte DNA synthesis and proliferation (Kimura and Ogihara, 1997, 1998). However, as previously described, these agents themselves strongly inhibit the hepatocyte DNA synthesis and proliferation induced by 1.0 ng/ml TGF-α (Kimura and Ogihara, 1999). Therefore, the signal transduction pathway of the TGF-β1 action on the TGF-α-induced hepatocyte proliferation cannot be analyzed in the presence of these specific inhibitors.

In conclusion, our results show that TGF-β1 potently inhibits the TGF-α-induced hepatocyte DNA synthesis and proliferation in a dose-dependent manner during short-term

culture (i.e., approximately 3–4 h after attachment period of 3 h). Results of studies with specific inhibitors of signal transducing elements (i.e., 2,4-dideoxyadenosine, somatostatin, and H-89) suggest that, among the elements that link the cell surface receptor to the nucleus, adenylate cyclase and protein kinase A mediate the action of TGF-β1. However, the complete sequence of events by which TGF-β1 decreases hepatocyte proliferation remains to be determined. It is anticipated that an understanding of the molecular mechanisms by which TGF-β1 arrests hepatocyte proliferation will provide important information related to the termination of liver regeneration.

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