Inhibition of spermidine synthase gene expression by transforming growth factor- β_1 in hepatoma cells

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We screened genes responsive to transforming growth factor- β (TGF- β_1) protein in a human hepatoma cell line (Hep3B) using a PCR-mediated differential display technique, in order to investigate the mechanisms involved in TGF- β -induced growth suppression. We found a gene that was down-regulated by TGF- β_1 to be completely identical in an approx. 620 bp segment to the gene for the enzyme spermidine synthase, which mediates the conversion of putrescine into spermidine. Both spermidine synthase mRNA expression and its enzyme activity were decreased after TGF- β_1 treatment of Hep3B cells. The inhibition of spermidine synthase gene expression by TGF- β_1 protein was also observed in other hepatoma cell lines. The expression of genes for other biosynthetic enzymes in polyamine metabolism (ornithine decarboxylase and S-adenosylmethionine decarboxyl-

ase) was also inhibited to the same extent as for spermidine synthase, while the gene expression of spermidine/spermine N^1 -acetyltransferase, a catabolic enzyme, was relatively resistant to TGF- β_1 . Spermine levels in Hep3B cells were decreased by TGF- β_1 treatment, although the levels of spermidine and putrescine were unchanged, probably due to compensation by remaining spermidine/spermine N^1 -acetyltransferase activity. Exogenously added spermidine or spermine, but not putrescine, partially antagonized the growth-inhibitory effects of TGF- β_1 on Hep3B cells. Our data suggest that down-regulation of gene expression of the enzymes involved in polyamine metabolism, including spermidine synthase, may be associated with the mechanism of TGF- β -induced growth suppression.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are thought to be essential for cell growth and differentiation [1–5]. Their concentrations are highly regulated by both biosynthetic and catabolic enzymes [1–6]. Putrescine, the precursor of spermidine and spermine, is formed via decarboxylation of ornithine by ornithine decarboxylase (ODC). Spermidine synthase forms spermidine from putrescine, and spermine synthase forms spermine from spermidine, by transfer of aminopropyl residues from decarboxylated S-adenosylmethionine (AdoMet), which is catalysed by AdoMet decarboxylase (AdoMetDC). The first step of the catabolic pathway is mediated by spermidine/spermine N^1 -acetyltransferase (SAT), which catalyses acetylation in the N^1 position of spermidine and spermine. The second step of oxidation is catalysed by the constitutive FAD-dependent oxidase (polyamine oxidase), resulting in the conversion of spermine into spermidine and of spermidine into putrescine. Of these enzymes, ODC, AdoMetDC and SAT are considered to be essential in the regulation of polyamine levels and have been extensively investigated [2,6]. Spermidine synthase and spermine synthase have been regarded as non-rate-limiting and stable enzymes which are regulated mainly by the availability of the substrate, decarboxylated AdoMet, and by the product, 5'-methylthioadenosine (MTA) [2,4]. However, some previous reports on spermidine synthase have shown it to be inducible during cell proliferation, such as liver regeneration and liver compensatory growth [7], hormone-induced growth of tissues [8,9] and lectin-induced lymphocyte activation [10]. Although the human spermidine synthase gene has been cloned [11,12], there have been few reports concerning its expression.

Transforming growth factor- β (TGF- β) is a potent growth inhibitor for both non-transformed and transformed epithelial cells [13]. The mechanisms of growth suppression by TGF- β are not well understood. In order to understand these mechanisms, it is important to identify genes that are responsive to TGF- β . We have screened TGF- β_1 -responsive genes by a PCR-mediated differential display technique [14] in a human hepatoma cell line (Hep3B) that is highly sensitive to TGF- β . We have identified a gene which is down-regulated by TGF- β_1 as the spermidine synthase gene.

Little is known about the effects of TGF- β on polyamine metabolism. TGF- β has been shown to induce ODC mRNA in H-ras-transformed fibrosarcoma cell lines on which TGF- β acts as a growth stimulator [15]. In contrast, TGF- β suppresses both cell growth and the activities of ODC and AdoMetDC in a human chronic myelogenous lymphoma cell line [16]. However, TGF- β has also been reported not to affect the level of ODC mRNA in T-lymphocytes [17], ODC activity in neuroendocrine cells [18] or the polyamine content of pancreatic β -cells [19]. In the present study we investigated the effects of TGF- β_1 on the gene expression of spermidine synthase and its enzymic activity in Hep3B cells. We also examined the effects of TGF- β_1 on the expression of genes encoding other major enzymes involved in polyamine metabolism (ODC, AdoMetDC and SAT).

EXPERIMENTAL

Cell culture and cell growth assay

Human hepatoma cell lines (Hep3B, PLC/PRF5 and HepG2) were purchased from American Type Culture Collection

Abbreviations used: ODC, ornithine decarboxylase; AdoMet, S-adenosylmethionine; AdoMetDC, AdoMet decarboxylase; SAT, spermidine/spermine N^1 -acetyltransferase; MTA, 5'-methylthioadenosine; TGF- β , transforming growth factor- β .

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(Rockville, MD, U.S.A.). A TGF- β -resistant Hep3B (Hep3B-TR) cell line was established from the original Hep3B (Hep3B-TS) cells by exposure to low TGF- β_1 concentrations (0.1–1.0 ng/ml) as described previously [20]. The Hep40 cell line was established from human hepatoma tissue [21]. All the cell lines were maintained in Eagle's minimum essential medium supplemented with 10% (v/v) fetal bovine serum, penicillin G (50 μ g/ml) and streptomycin (50 units/ml).

The effects of TGF- β and polyamines on cell growth were examined by assaying monolayer cell growth of Hep3B-TS cells. Hep3B-TS cells were plated at 5×10^4 cells per well of 6-well plates. After 24 h, the medium was replaced with control medium or medium containing TGF- β_1 (0.25 ng/ml) with or without various concentrations of polyamines. After 6 days, cells were trypsin-treated and suspended in 1 ml of PBS with 5% (v/v) calf serum. Absorbance at 660 nm was measured spectrophotometrically. Control experiments demonstrated a linear correlation between Hep3B cell density and absorbance at 660 nm [22].

Differential display of mRNA

To find TGF- β -responsive genes in Hep3B-TS cells, we compared mRNA expression patterns of untreated control cells with patterns from cells treated with TGF- β_1 , using the PCR-mediated differential display technique developed by Liang and Pardee [14]. Subconfluently cultured Hep3B cells were treated with 3 ng/ml TGF- β_1 for 48 h. Total RNA from control and TGF- β_1 -treated cells was prepared by using Trizol reagent (Gibco BRL, Division of Life Technologies, Gaithersburg, MD, U.S.A.). After removal of contaminating DNA by digestion with DNase I (MessageClean kit; GenHunter, Brookline, MA, U.S.A.), the samples were reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (RNAmapping; GenHunter). The cDNA was then amplified by PCR using arbitrary 5'-primers (AP4, GGTACTCCAC; AP5, GTTGCGATCC; AP6, CAGGCCCTTC; AP7, TGCCGAGCTG; AP8, AGTCAGCCAC; AP9, AATCGGGCTG; AP10, AGGGGTCTTG; AP11, CGTCAGTGAC; AP12, GCAAGGAGTC) and 3' T₁₂ primers (TTTTTTTTTTTTTGMG, TTTTTTTTTTTTTTMC; where M = A + G + C) in the presence of ³²S-labelled dATP. The PCR reaction was performed for 40 cycles at 94 °C for 30 s, 40 °C for 2 min and 72 °C for 30 s, followed by 72 °C for 5 min. The PCR samples were electrophoresed in 6% DNA sequencing gels, and the gels were dried and exposed to X-ray films (BioMax MR; Eastman Kodak, Rochester, NY, U.S.A.). Selected bands were cut from the gels and reamplified by PCR using the same primer sets as those in the first PCR. The reamplified PCR fragments were purified by sieving agarose gel electrophoresis. The fragment 12C1 (a 640 bp fragment amplified by AP12 and T₁₂MC) was examined in this study.

Direct sequencing of PCR fragments

To generate single-stranded DNA templates for DNA sequencing from the purified cDNA fragments, asymmetrical PCR was performed with an excess of T₁₂MC primer (AP12/T₁₂MC, 1:50) or AP12 primer (AP12/T₁₂MC, 50:1). DNA sequencing was done by the Sequenase (United States Biochemical Corp., Cleveland, OH, U.S.A.) protocols using the AP12 or T₁₂MC primer as a sequencing primer. DNA sequence analysis and homology searches were carried out at the Biomedical Computing Facility of the Pittsburgh Supercomputing Center.

Northern blot analysis

Subconfluent Hep3B-TS, Hep3B-TR, Hep40, PLC/PRF5 and HepG2 cells were treated with various doses of TGF- β_1 . We also treated Hep3B-TS cells cultured at moderate (approx. 40% confluence) and low (approx. 20% confluence) cell density to assess the effect of cell density on TGF- β action. RNA samples (20 μ g) were resolved by electrophoresis using denaturing glyoxal/agarose gel electrophoresis [23]. The separated RNA was transferred to a Hybond N⁺ membrane (Amersham Corp., Arlington Heights, IL, U.S.A.) and was cross-linked with UV light (short wave). The 12C1 fragment (spermidine synthase), ODC [24], AdoMetDC [25] and SAT [26] cDNAs, and 18S ribosomal RNA probes (for loading control), were labelled with [α -³²P]dCTP using the Nick Translation System (Gibco BRL). The membrane was hybridized to the ³²P-labelled probes and washed by standard protocols. Autoradiography was performed at -70 °C using intensifying screens. A GS 300 Transmitter/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) was used for densitometric analysis of autoradiograms.

Assay of spermidine synthase activity

The effect of TGF- β_1 on spermidine synthase activity was examined using subconfluent Hep3B-TS cells. Spermidine synthase was assayed as previously described [27,28] by measuring the conversion of ³⁵S-labelled decarboxylated AdoMet into [³⁵S]MTA in the presence of putrescine, since spermidine synthase catalyses the reaction converting putrescine and decarboxylated AdoMet into spermidine and MTA. The total assay contained about 30000 c.p.m. as decarboxylated [³⁵S]AdoMet. The results were corrected using a blank to which no putrescine was added.

Assay of polyamine levels

Subconfluent Hep3B-TS cells were treated with various concentrations of TGF- β_1 for 48 h, harvested and washed twice with ice-cold PBS. The washed cell pellets were resuspended in 10% trichloroacetic acid and centrifuged. The supernatant was filtered before HPLC analysis. The precipitated protein was dissolved in 0.1 M NaOH for measurement of protein content. Putrescine, spermidine and spermine were separated by reverse-phase HPLC and quantified by using a post-column reaction with o-phthalaldehyde and fluorescence detection [29–31].

Affinity cross-linking analysis of TGF- β receptors

After serum starvation for 24 h, subconfluent Hep3B-TS and Hep3B-TR cells were rinsed with PBS and then treated with 20 pM of ¹²⁵I-labelled TGF- β_1 (Biomedical Technologies, Inc., Stoughton, MA, U.S.A.) in Krebs–Ringer solution with 10% (w/v) BSA (binding buffer) at 4 °C for 3 h. After binding, cells were rinsed with binding buffer and cross-linked using bis-(sulphosuccinimidyl)suberate (Pierce Chemical Co., Rockford, IL, U.S.A.). The cells were lysed, and cross-linked proteins were resolved by SDS/PAGE (linear-gradient of 5–10% polyacrylamide) under reducing conditions. Dried gels were exposed to X-ray films at -70 °C using intensifying screens. We confirmed the binding specificity by adding excess non-labelled TGF- β_1 (100 pM) with labelled TGF- β_1 .

RESULTS

Screening of TGF- β -responsive genes in Hep3B cells by the differential display technique and identification of a fragment of down-regulated cDNA (12C1) as the spermidine synthase gene

To screen for TGF- β -responsive genes, we applied the differential display technique to subconfluent Hep3B-TS cells treated with TGF- β_1 (3 ng/ml) for 48 h. Using a combination of nine different 5' arbitrary primers and two different 3' T₁₂ primers, we identified

Figure 1 Differentially expressed cDNA fragment (12C1) in Hep3B cells after treatment with TGF- β

Hep3B-TS cells were treated with TGF- β_1 (3 ng/ml) for 48 h and total RNA was isolated. Differential display of control cells (–) and TGF- β_1 -treated cells (+) was performed as described in the Experimental section. Shown is a part of the differential display gel using the AP12 and T₁₂MC primers. We designated a cDNA fragment (approx. 640 bp) which was down-regulated by TGF- β_1 as 12C1. Sequence analysis of this fragment revealed it to be completely identical to the human spermidine synthase gene.

Figure 2 Inhibition of spermidine synthase gene expression by TGF- β in Hep3B-TS but not in Hep3B-TR cells

Hep3B-TR (TGF- β -resistant) is a Hep3B-derived mutant cell line that is completely resistant to TGF- β due to the lack of type II TGF- β receptors. TGF- β -sensitive Hep3B (Hep3B-TS) and Hep3B-TR cells were treated with TGF- β_1 (3 ng/ml) for 18 h or 48 h and total RNA was isolated. The expression of the spermidine synthase gene was examined by Northern blot analysis using ³²P-labelled 12C1 as the probe, as described in the Experimental section. For a loading control, an 18S rRNA probe was hybridized to the same membrane.

Figure 3 Effects of TGF- β on gene expression of spermidine synthase, ODC, AdoMetDC and SAT in Hep3B cells

Hep3B-TS cells were treated with TGF- β_1 (3 ng/ml) for various time periods or treated with various concentration of TGF- β_1 for 48 h, and total RNA was isolated. (a) Northern blot analysis of gene expression of spermidine synthase (1.5 kb), ODC (2.2 kb), AdoMetDC (3.4 kb) and SAT (1.5 kb) was performed as described in the Experimental section. For a loading control, an 18S rRNA probe was hybridized to the same membrane. Densitometric analyses of the autoradiograms are shown in (b) (time course) and (c) (dose-response). ■, Spermidine synthase; □, ODC; ●, AdoMetDC; ○, SAT.

28 bands of cDNA fragments that were differentially expressed (10 were up-regulated and 18 were down-regulated by TGF- β_1). One of the down-regulated cDNA fragments (12C1) that was amplified by the AP12 and T₁₂MC primers was analysed further in this study (Figure 1). We sequenced most of cDNA fragment 12C1 by direct sequencing and found complete identity in an approx. 620 bp segment with the gene for human spermidine synthase. There was a sequence CAAGGAGTC at nucleotides 5266–5274 within exon 5 of the spermidine synthase gene [12] to which the 5' primer (AP12) annealed. The 3' primer (T₁₂MC) appeared to anneal to the sequence GCAA (nucleotides 6746–6750) within exon 8.

Inhibition of spermidine synthase (12C1) gene expression and spermidine synthase enzyme activity by TGF- β

We performed Northern blot analyses of spermidine synthase gene expression using the fragment 12C1 as a probe. The amount of transcript (1.5 kb) was decreased within 24 h of TGF- β_1 treatment in TGF- β -sensitive Hep3B cells (Hep3B-TS), while there was no change in a TGF- β -resistant variant of Hep3B cells (Hep3B-TR), which lack surface receptors for TGF- β (Figure 2). The decrease in spermidine synthase mRNA in subconfluent Hep3B-TS cells was evident as early as 6 h after treatment, and there was a clear dose-response relationship (Figure 3). When Hep3B-TS cells cultured at lower cell density were treated with TGF- β_1 , a smaller amount of TGF- β_1 was required for the inhibition of spermidine synthase gene expression (Figure 4). Cycloheximide (10 or 20 μ g/ml) considerably abrogated the decrease in spermidine synthase gene expression caused by TGF- β_1 , indicating that newly synthesized protein(s) were involved in mediating the TGF- β_1 signal (Figure 5). We also assayed the activity of spermidine synthase protein in subconfluent Hep3B-TS cells, and found a decrease in the enzymic activity of spermidine synthase which paralleled the decrease in its mRNA level (Figure 6). A similar decrease in spermidine synthase mRNA in the presence of TGF- β_1 was observed in other human hepatoma-derived cells (Hep40, PLC/PRF5 and HepG2) (Figure 7), indicating that the effect is not restricted to Hep3B-TS cells.

Effects of TGF- β on the gene expression of the other major enzymes in polyamine metabolism

We also examined the effects of TGF- β_1 on the gene expression of two biosynthetic enzymes, ODC and AdoMetDC, and a catabolic enzyme, SAT, to compare with that of spermidine synthase in Hep3B-TS cells. The sizes of the major transcripts were 2.2, 3.4 and 1.5 kb for ODC, AdoMetDC and SAT respectively. The gene expression of ODC and AdoMetDC was down-regulated in a time- and dose-dependent manner by TGF- β_1 treatment, while the expression of SAT was not affected in subconfluent Hep3B-TS cells (Figure 3). As in the case of spermidine synthase gene expression, in Hep3B-TS cells cultured at lower density a smaller amount of TGF- β_1 was necessary for the inhibition of ODC and AdoMetDC gene expression (Figure 4). Whereas SAT gene expression was not affected at moderate cell density, it was inhibited at low cell density (Figure 4).

Effects of TGF- β on polyamine content in Hep3B cells

Subconfluent Hep3B cells were treated with TGF- β_1 and the changes in polyamine content were examined. TGF- β_1 treatment considerably decreased spermine levels, but did not affect the

Figure 4 Effects of cell density on TGF- β -induced inhibition of the gene expression of spermidine synthase, ODC, AdoMetDC and SAT in Hep3B cells

Various concentrations of TGF- β_1 were added to Hep3B-TS cells at moderate cell density (~40% confluence) and low cell density (20% confluence); cells were cultured for 48 h, and total RNA was isolated. (a) Northern blot analysis of gene expression of spermidine synthase, ODC, AdoMetDC and SAT was performed as described in the Experimental section. For a loading control, an 18S rRNA probe was hybridized to the same membrane. Densitometric analyses of the autoradiograms are shown in (b) (moderate cell density) and (c) (low cell density). For the convenience of comparison, the range 0–0.5 ng/ml is shown in the graphs. ■, Spermidine synthase; □, ODC; ●, AdoMetDC; ○, SAT.

Figure 5 Effects of cycloheximide on TGF- β -induced down-regulation of spermidine synthase gene expression in Hep3B cells

To examine whether newly synthesized protein(s) are involved in the down-regulation of spermidine synthase gene expression, subconfluent Hep3B-TS cells were treated with TGF- β_1 (3 ng/ml) for 48 h in the presence (10 or 20 μ g/ml) or absence of cycloheximide (CHX). Northern blot analysis of spermidine synthase expression was performed as described in the Experimental section. For a loading control, an 18S rRNA probe was hybridized to the same membrane.

levels of spermidine or putrescine (Figure 8). The latter finding may be explained by a possible compensatory mechanism via SAT activity, since the gene expression of SAT was not affected by TGF- β_1 in subconfluent cells (see Figure 3).

Effects of exogenous polyamines on TGF- β -induced growth suppression and on TGF- β receptors

We added polyamines to the medium at various concentrations to examine whether exogenous polyamines had antagonistic effects on TGF- β -induced growth suppression in Hep3B-TS cells. In the assay for growth suppression we used Hep3B-TS cells plated at low density (10–20% confluence), which were cultured in the absence or presence of TGF- β_1 for 6 days. The dose of TGF- β_1 for 75% growth suppression was 0.25 ng/ml (Table 1); this dose was sufficient to inhibit the gene expression of major polyamine-metabolizing enzymes in Hep3B cells cultured at low cell density (see Figure 4). Putrescine, spermidine and spermine had no apparent growth-promoting activity. In fact, spermidine and spermine were growth-inhibitory at high concentrations (Table 1). However, spermidine and spermine exerted partial antagonism to the growth-inhibitory actions of TGF- β_1 (Table 1). To check for possible effects of polyamines on the surface receptors of TGF- β , we performed an affinity cross-linking experiment using 125 I-labelled TGF- β_1 in the presence of various concentrations of polyamines in Hep3B-TS cells. We found that polyamines did not affect the affinity of any of the receptors (results not shown).

DISCUSSION

In the present study we screened genes responsive to TGF- β_1 protein in Hep3B cells using the differential display technique. We sequenced a cDNA fragment that was down-regulated by TGF- β_1 , and found complete identity with the spermidine synthase gene [11,12]. However, the 3' end of the cDNA fragment was approx. 400 bp upstream of the putative polyadenylation signal (AATACA) of the spermidine synthase gene [12]. This

Figure 6 Inhibition of the enzymic activity of spermidine synthase by TGF- β in Hep3B cells

The effect of TGF- β on the enzymic activity of spermidine synthase was examined using TGF- β_1 -treated subconfluent Hep3B-TS cells. Hep3B-TS cells were treated with TGF- β_1 (3 ng/ml) for various time periods or with various concentrations of TGF- β_1 for 48 h. The activity is shown as the amount of [35 S]MTA (c.p.m./ μ g of protein) produced from decarboxylated [35 S]AdoMet in the presence of putrescine. (a) Time course; (b) dose-response. Results are shown as means $\pm$ S.E.M. from five separate experiments. Statistical analysis was performed by an unpaired *t* test (two tails) between the control (a, 0 h; b, 0 ng/ml) and treated cells. The presence of significant differences is indicated by: **P* < 0.05; ***P* < 0.01.

Figure 7 Effects of TGF- β on spermidine synthase gene expression in other human hepatoma cell lines (Hep40, PLC/PRF5 and HepG2)

Subconfluent Hep40, PLC/PRF5 and HepG2 cells were treated with various concentrations of TGF- β_1 for 48 h and total RNA was isolated. Northern blot analysis of spermidine synthase gene expression was performed as described in the Experimental section. For a loading control, an 18S rRNA probe was hybridized to the same membrane.

Figure 8 Effects of TGF- β on polyamine levels in Hep3B cells

Subconfluent Hep3B-TS cells were treated with various concentrations of TGF- β_1 for 48 h and polyamine content was assayed as described in the Experimental section. The data from a typical experiment are presented. Results are shown as means $\pm$ S.E.M. of triplicate measurements. We performed three separate experiments and obtained similar results.

Table 1 Effects of exogenous spermidine, spermine and putrescine on the growth of Hep3B-TS cells in the presence or absence of TGF- β

The effects of exogenous polyamines on cell growth in the presence and absence of a growth-inhibitory concentration of TGF- β_1 (0.25 ng/ml) were examined by assaying monolayer cell growth of Hep3B-TS cells as described in the Experimental section. Cell numbers are shown as percentages of control (no treatment). The values are means $\pm$ S.E.M. from five separate experiments. Statistical analysis was performed by a paired *t* test (two tails) between the group treated with TGF- β_1 alone and the groups treated with both TGF- β_1 and polyamines; significant differences are indicated by: **P* < 0.05; ***P* < 0.01.

Polyamine (μ M)	Cell number (% of control)	
	Without TGF- β	With TGF- β (0.25 ng/ml)
None	100	24.9 $\pm$ 3.5
Spermidine		
2	103.6 $\pm$ 2.8	29.1 $\pm$ 2.4*
5	102.0 $\pm$ 3.8	32.0 $\pm$ 2.3*
10	103.5 $\pm$ 2.6	40.1 $\pm$ 6.5**
20	79.2 $\pm$ 3.9	33.4 $\pm$ 6.1
Spermine		
1	105.7 $\pm$ 3.2	29.2 $\pm$ 6.5
2	101.4 $\pm$ 2.3	30.1 $\pm$ 6.1
5	94.5 $\pm$ 4.1	33.4 $\pm$ 4.1*
Putrescine		
10	102.5 $\pm$ 4.2	31.1 $\pm$ 6.0
20	105.0 $\pm$ 5.4	29.5 $\pm$ 5.1

might have been caused by mis-annealing of the T₁₂MC primer to the complementary sequence (GCAAA) of the spermidine synthase gene at the 3' end of the cDNA fragment due to the low annealing temperature (40 °C) during the PCR cycle [14,30]. In previous reports using the differential display technique, genes lacking a polyadenylation signal have been isolated frequently [32–35]. The possibility of cloning of portions of cDNAs other than their 3' ends has been pointed out recently [36].

Although spermidine synthase has been regarded as a non-rate-limiting and stable enzyme [2,4], previous reports have also

shown the inducible nature of its enzymic activity during cell proliferation in several experimental systems [7–10]. To our knowledge, the present study is the first to show that the gene expression of spermidine synthase is also highly regulated. Furthermore, we showed a parallel decrease in the gene expression of other biosynthetic enzymes (ODC and AdoMetDC) due to TGF- β_1 . The gene expression of SAT, a catabolic enzyme, was less affected by TGF- β_1 compared with that of the biosynthetic enzymes, although it was inhibited when cells were treated at low cell density. The effects of TGF- β_1 on the gene expression of spermidine synthase, ODC and AdoMetDC were also dependent on cell density. This is consistent with previous reports demonstrating that the response of cultured cells to TGF- β is influenced by cell density [37–39]. Our data suggest that TGF- β -induced growth inhibition may be associated with the down-regulation of gene expression of the enzymes involved in polyamine metabolism.

Polyamines, especially spermidine and spermine, are highly cationic molecules in cells. They can bind to anionic molecules, such as nucleic acids and phospholipids, and affect fundamental processes in DNA replication, transcription and translation [1,5]. Polyamines are thought to have multiple roles in various cell types, including some specific functions, such as post-translational modification of lysine residues in eukaryotic initiation factor-4D by synthesis of hypusine [*N*^ε-(4-amino-2-hydroxybutyl)lysine] [40], gate-controlling of K⁺ channels [41] and modulation of *N*-methyl-D-aspartate-type glutamate receptors [42,43]. A number of studies with various inhibitors of the enzymes of polyamine metabolism and with cell mutants have established that polyamines have essential roles in cell growth [1–6]. In particular, spermidine has been shown to be the preferred polyamine for maintaining cell growth [44].

TGF- β is a prototype growth inhibitor for many cell types, although it has a variety of actions, including growth stimulation and matrix formation for some mesenchymal cells [13]. A previous report showed that growth suppression by TGF- β in leukaemic cells was associated with a decrease in ODC and AdoMetDC activity [16]. However, others have reported that TGF- β -induced growth suppression did not cause changes in ODC mRNA in T-lymphocytes [17], ODC activity in a functional neuroendocrine cell line [18] or polyamine content in pancreatic β -cells [19]. Although it is difficult to explain the above discrepancy, it is possible that variations in polyamine metabolism might be involved in different cell systems. In particular, the actions of TGF- β might be different in transformed cells, such as the hepatoma cells used in the present study, in which the expression of the biosynthetic and catabolic enzymes of polyamines is maintained continuously at high levels.

The mechanisms involved in the decrease in expression of the gene for spermidine synthase are not clear. Since cycloheximide significantly blocked the TGF- β_1 -mediated decrease in the expression of this gene, newly synthesized protein(s) might be involved in the mechanism of TGF- β action. The partial antagonistic effects of exogenous spermidine and spermine on the TGF- β_1 -induced cell growth suppression suggests that the decreased expression of the spermidine synthase gene and inhibition of its enzymic activity might be participating in the growth-inhibitory mechanism. The antagonistic effects of polyamines are not due to modulation of the binding affinity of TGF- β to its receptors, as shown in the ¹²⁵I-TGF- β affinity labelling experiments in the presence of exogenous polyamines. Much attention has been paid to the actions of TGF- β on cell cycle control [45–47]. TGF- β has been shown to induce a block at some point of the G1 phase of the cell cycle by inhibiting G1 cyclins [48,49] and cyclin-dependent kinases [48,50,51], and by

induction of several cyclin-dependent kinase inhibitors, such as p21 [52] and p15 [53]. However, the pathway from the activation of the serine/threonine protein kinase of types I and II TGF- β receptors to the resulting G1 arrest is largely unresolved. At present, it is not clear whether polyamines have specific actions on cell cycle control mechanisms.

In summary, by examining differentially expressed genes in TGF- β_1 -treated human hepatoma cells, we have cloned a fragment of the human spermidine synthase gene and showed that the gene expression of enzymes of the polyamine metabolic pathway (spermidine synthase, ODC, AdoMetDC and SAT) was inhibited during growth suppression by TGF- β . Our data suggest a link between TGF- β -induced growth suppression and the negative regulation of polyamine synthesis.

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