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Regulation of Cell Cycle-Related Genes in Rat Hepatocytes by Transforming Growth Factor β1

Akihiko Sugiyama, Masahito Nagaki, Yoshihiro Shidoji, Hisataka Moriwaki,[1] and Yasutoshi Muto
First Department of Internal Medicine, Gifu University School of Medicine, Gifu 500, Japan

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Transforming growth factor β (TGF-β) is a potent inhibitor of the proliferation of many cell types. We investigated the effects of TGF-β1 on cyclin D1, cyclin A, p21, p27, and p53 mRNA expressions in primary cultured rat hepatocytes by the reverse-transcription polymerase chain reaction (RT-PCR) method. TGF-β1 decreased the level of cyclin A mRNA in a dose-dependent manner, while it had little effect on the level of cyclin D1 mRNA. p21 mRNA expression was greatly induced by TGF-β1 in a p53-independent mechanism, while p27 mRNA expression was not affected by TGF-β1. These results suggest that TGF-β1 may inhibit liver cell proliferation by regulating p21 and cyclin A mRNAs. © 1997 Academic Press

It has recently been known that various cell cycle-related genes play a role in the regulation of cell proliferation (1). Progression through the cell cycle is governed by a family of serine/threonine kinases that are composed of cyclins and cyclin-dependent kinases (CDKs) (2-4). Each of these CDKs can form binary complexes with several cyclins (3,4), and the enzymatic activity of a CDK is regulated at three different levels: cyclin activation, subunit phosphorylation, and association with members of a group of small regulatory proteins (2). Various cyclin/CDK complexes regulate a distinct phase of the cell cycle (5). It has been shown that cyclin D1 is active in G1 phase, and that cyclin A begins to increase from late G1 and is still present during S phase (5,6). Furthermore, recent studies have revealed that cyclin/CDK activity can be also down-regulated by several CDK inhibitors (7). $p21^{WAF1/CIP1}$ and $p27^{kip1}$ are these kinds of CDK inhibitors, and their binding to cyclin/CDK complex has been shown to block activating phosphorylation of CDK (8-10).

Transforming growth factor β (TGF-β) has been shown to be a potent inhibitor of the proliferation of normal cells as well as tumor cells. TGF-β treatment causes accumulation of retinoblastoma protein (Rb) in the underphosphorylated state (11). Therefore, exposure to the cytokine causes cells to cease proliferation and undergo cell cycle arrest in G1 phase. Nakamura *et al.* reported that TGF-β1 was a strong growth inhibitor of adult rat hepatocytes, blocked their shift from the G1 phase to the S phase and inhibited DNA synthesis (12).

Recent studies have shown that certain G1 cyclins and cyclin-dependent kinase may be targets of the negative signaling pathways induced by TGF-β (13-16). TGF-β1 caused inhibition of cyclin A and cyclin E mRNA expressions in human keratinocyte line (HaCaT) and mink lung epithelial cells (Mv1Lu cells) (15,16). However, it has been reported that TGF-β treatment failed to suppress cyclin E mRNA expressions, although cyclin A mRNA is suppressed in mouse keratinocytes (17). As for cyclin D1, TGF-β1 suppressed cyclin D1 mRNA expressions in rat intestinal epithelial cells (18). However, myoblasts increased cyclin D1 mRNA expressions after TGF-β1 treatment (19). Thus, there may be important differences among cell types in the mechanisms by which TGF-β inhibits cell proliferation. Little is known about the effect of TGF-β1 on the cell cycle-related genes in hepatocytes, because most of the studies were performed using established cell lines. In the present study, we examined the effects of TGF-β1 on the expression of cell cycle-related genes in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Cell culture. Adult rat hepatocytes were isolated from 6-week-old male Wistar rats by *in situ* 0.05% collagenase perfusion method as previously reported (20). Aliquots of the cell suspension were placed into 100-mm plastic dishes (Becton Dickinson Labware, Bedford, MA) at a concentration of 2.5×10^5 cells/ml in Williams medium E (Dainippon Pharmaceutical Co., Osaka, Japan) supplemented with 20 ng/ml epidermal growth factor (EGF; R&D systems, Minneapolis, MN), 10^{-7} M insulin (Wako, Osaka, Japan), 10^{-6} M dexamethasone

[1] To whom correspondence should be addressed. Fax: 81-58-262-8484.

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(Wako), 30 μg/ml kanamycin (Dainippon) and 5% fetal bovine serum (GIBCO, Grand Island, NY) in the presence or absence of TGF-β1 (R&D systems).

DNA synthesis. ^{3}H-thymidine incorporation was used to measure DNA synthesis in hepatocytes cultured in the presence or absence of TGF-β1. At selected times after plating, cells were labeled with ^{3}H-thymidine (2.5 μCi/ml, 0.3 μCi/mmol) (Amersham International plc, Buckinghamshire, UK) and DNA synthesis was determined by counting incorporation of ^{3}H-thymidine into hepatocytes as previously described (21).

RT-PCR. Total RNA was extracted at selected times from hepatocytes using RNA-zol (Biotec Laboratories, Inc., Houston, Texas) as previously described (22). One μg RNA from each treated cells was preincubated with 1 μl random primer (Boeringer Manheim, Manheim, Germany), 1 μl 20 mM deoxynucleoside triphosphate (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP), 4 μl reverse transcriptase buffer containing of 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, and 12 μl H_2O for 2 min at 70 °C. Reverse transcription was performed at 37 °C for 40 min with 200 units (1 μl) Molony murine leukemia virus (M-MLV) reverse transcriptase (GIBCO). The polymerase chain reaction (PCR) was carried out in a final volume of 50 μl containing 1 μl 20 mM deoxynucleoside triphosphate, 5 μl 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.4), 15mM $MgCl_2$), 1 μl 25 pM oligonucleotide primers and 2.5 units of Taq DNA polymerase (Boeringer Manheim). Sequences for cyclin D1 (23), cyclin A (24), p21 (25), p53 (26), p27 (9) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (27) were obtained from Gene Bank. Each PCR cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 63°C (cyclin D1), at 61°C (p21), at 66°C (cyclin A), at 59°C (p53), at 56 °C (p27) or at 55°C (GAPDH) for 30 sec and a denaturation step at 72°C for 2 min. Primers were as follows: cyclin D1; sense GTGCAGAGGGAGATTGTGCC and antisense GCGGCCCAGGTTCCATTTGAG; cyclin A sense CCTGCATTTGGCTGTGAACTAC and antisense CCTGCATTTGGCTGTGAACTAC; p21 sense AGTATGCCGTCGTCTGTTCG and antisense GAGTGCAAGACAGCGACAAG; p53 sense GTGGCCTCTGTCATCTTCCG and antisense CCGTCACCATCAGAGCAACG; p27 sense CAGCTTGCCCGAGTTCTA and antisense TGGGGAACCGTCTGAAAC; GAPDH sense ACCACAGTCCATGCCATCAC and antisense TCCACCACCCTGTTGCTGTA.

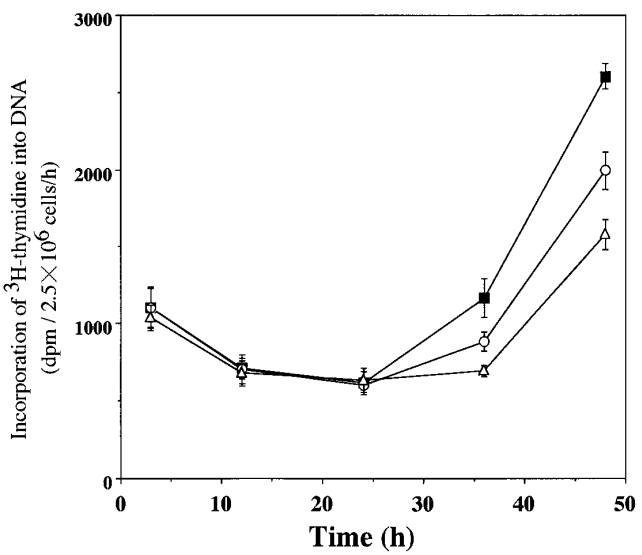

FIG. 1. Effect of TGF-β1 on DNA synthesis. Hepatocytes were cultured at 2.5×10^5 cells/ml on plastic dishes and treated with 0.5 ng/ml TGF-β1 (open circles), with 1.0 ng/ml TGF-β1 (open triangles), or without TGF-β1 (closed squares). ^{3}H-thymidine was added 2h before assay of DNA synthesis. The radioactivity of ^{3}H-thymidine incorporated is expressed as dpm/2.5×10^6 cells/h. Data are expressed as mean ± SD for 3 dishes.

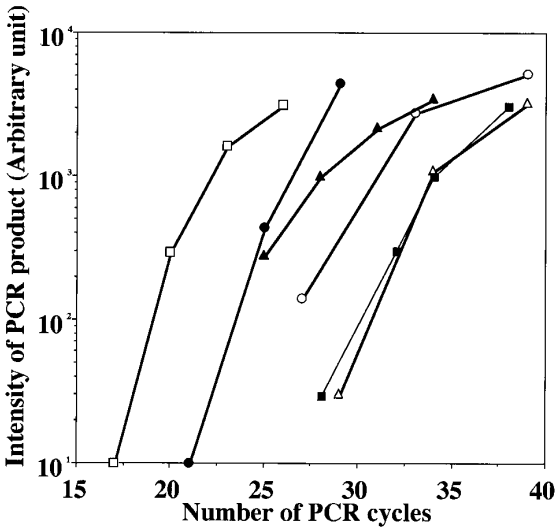

FIG. 2. The intensities of PCR products at various cycles. PCR amplifications of cyclin A (open triangle), cyclin D1 (open circle), p21 (closed circle), p53 (closed triangle), p27 (closed square), and GAPDH (open square) were conducted at various cycles starting with equal RT reaction solutions and the intensity of each PCR band was expressed in arbitrary units.

Detection. A portion (15 μl) of the PCR product was electrophoresed through 2% agarose gel (Takara, Kyoto, Japan) containing 0.2μg/ml ethidium bromide on TBE buffer. Gels were illuminated with UV light, photographed, and analyzed by computer-assisted densitometric scanning of these images using the NIH Image program.

RESULTS

Effect of TGF-β1 on DNA synthesis. TGF-β1 did not affect ^{3}H-thymidine incorporation at 3h, 12h and 24h. DNA synthesis increased from 36h, and TGF-β1 strongly inhibited DNA synthesis in a dose-dependent manner at 36h and 48h after plating (Fig. 1).

Analysis of the intensities of PCR products at various cycles. We conducted PCR amplification at various cycles to choose the adequate number of cycles. The intensity of each band increased in parallel with the increasing number of PCR cycles as shown in Fig. 2. We decided the adequate PCR amplification cycles for cyclin A as 32 cycles, for cyclin D1 as 30 cycles, for p21 as 24 cycles, for p53 as 28 cycles, for p27 as 32 cycles and for GAPDH as 20 cycles.

Effects of TGF-β1 on cyclin D1 and cyclin A expressions. Cyclin A mRNA expression was slightly detectable in freshly isolated hepatocytes. In the absence of

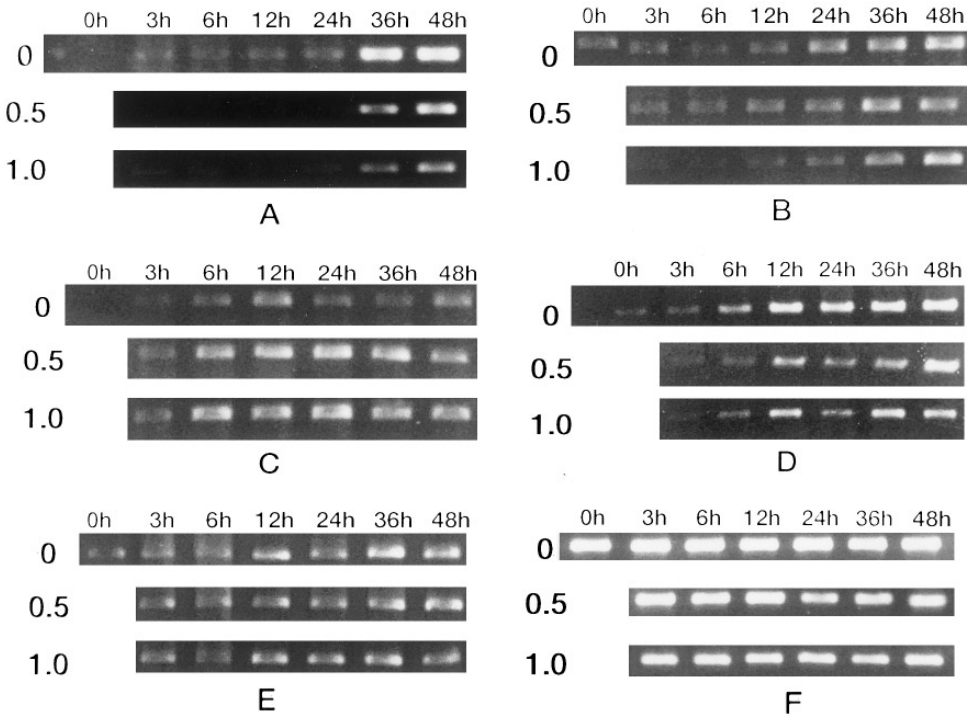

FIG. 3. Effects of TGF-β1 on the time course of cell cycle-related gene expressions in cultured rat hepatocytes. Primary cultured rat hepatocytes were treated with 0.5 ng/ml TGF-β1 (middle lane), with 1.0 ng/ml TGF-β1 (lower lane), or without TGF-β1 (upper lane). Total RNA was extracted at the indicated times from cultured hepatocytes and analyzed by RT-PCR as described in Materials and Methods for cyclin A (A), cyclin D1 (B), p21 (C), p53 (D), p27 (E), and GAPDH (F).

TGF-β1, cyclin A mRNA levels remained the basal level until 24h, but markedly increased thereafter (Fig. 3A and 4A). TGF-β1 strongly inhibited cyclin A mRNA expressions in a dose-dependent manner at 36h and 48h after plating.

Immediately after isolation from the quiescent rat liver, hepatocytes expressed cyclin D1 mRNA minimally. Cyclin D1 mRNA levels gradually increased from 12 h and brought up to 3-fold the basal level at 48 h (Fig. 3B and 4B). Time course of these expressions was similar to that of cyclin D1 mRNA levels in hepatocytes treated by TGF-β1.

Effects of TGF-β1 on p21, p27, and p53 expressions. p21 mRNA expression was not observed in freshly isolated hepatocytes. p21 mRNA quickly increased at 3h after TGF-β1 treatment, and these levels remained high over 48h culture period (Fig. 3C and 4C). During this culture period, p21 mRNA levels were definitely lower in the absence of TGF-β1 than in the presence of TGF-β1. p27 mRNA expression gradually increased but no significant effect of TGF-β1 on p27 mRNA expressions was observed (Fig. 3E and 4E). p53 mRNA gradually increased after plating and there was no significant effect of TGF-β1 on p53 mRNA levels (Fig. 3D and 4D).

GAPDH was used for an internal standard. All of intensities obtained by RT-PCR for GAPDH were almost consistent (Fig. 3F).

DISCUSSION

TGF-β1 is an important factor to inhibit liver regeneration. TGF-β1 inhibits not only regenerative DNA synthesis in rat liver after partial hepatectomy but also epidermal growth factor-induced DNA synthesis in cultured hepatocytes (12,28). Oberhammer *et al.* reported that TGF-β1 decreased the basal DNA synthesis of hepatocytes when it was added at 4h after seeding but not when added 22h after seeding, and showed that TGF-β1 needed to be present at an early stage of the cell cycle to exert its effect on hepatocyte growth (29).

In the present study, TGF-β1 down-regulated cyclin A mRNA expressions in a dose-dependent manner at 36 and 48 h after plating when DNA synthesis in hepatocytes was induced, suggesting that TGF-β1 inhibited the cell cycle progression from G1 phase to S phase. It has been reported that TGF-β1 caused the reduction of cyclin A mRNA expression in many cell lines (16,17,30). Xin *et al.* demonstrated that inhibition of cyclin A transcription by TGF-β required both type I and type II TGF-β receptors (31). Barlat *et al.* reported that cyclin A expression was also down-regulated by TGF-β1 in

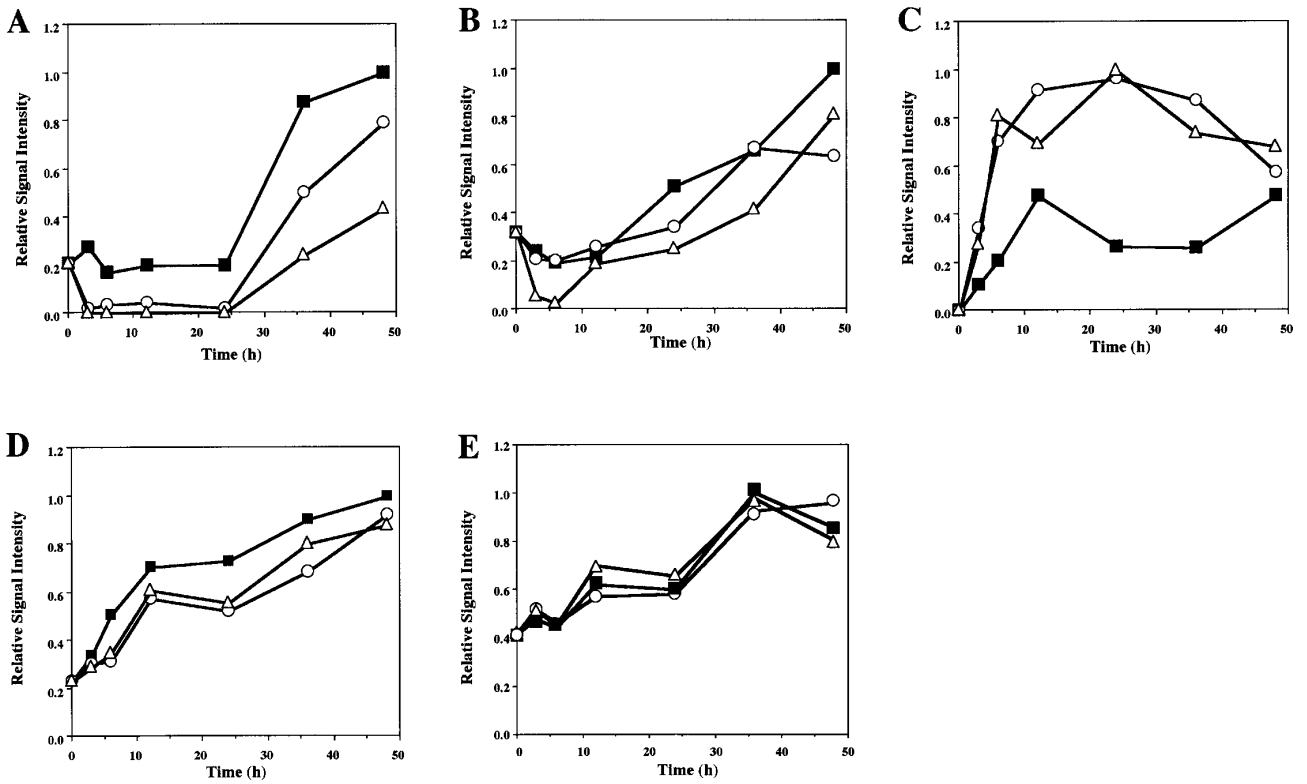

FIG. 4. Quantitative analysis of cell cycle-related gene expressions in cultured rat hepatocytes after TGF-β1 treatment. Relative intensities of the bands shown in Fig. 3 were quantitated by NIH image analysis, and cyclin A (A), cyclin D1 (B), p21 (C), p53 (D), and p27 mRNA levels (E) were standardized to GAPDH mRNA. Primary cultured rat hepatocytes were treated with 0.5 ng/ml TGF-β1 (open circles), with 1.0 ng/ml TGF-β1 (open triangles), or without TGF-β1 (closed squares).

Chinese hamster lung fibroblasts and that this effect was mostly mediated at the transcriptional level through a cAMP-responsive element (CRE) in the cyclin A promoter (32).

Cyclin D1 is one of G1 cyclins and is thought to be an important factor to progress the cell cycle from G1 phase to S phase (5). Over expression of cyclin D1 leads to a shortening of G1 interval and premature entry into S phase in fibroblasts (33). Tien *et al.* showed that TGF-β1 inhibited cyclin D1 mRNA expressions in rat intestinal epithelial cells (18). However, it was reported that cyclin D1 mRNA expressions were not greatly affected by TGF-β1 in mouse keratinocytes and Mv1Lu cells (16,17). Moreover, Sunkara *et al.* showed that cyclin D1 mRNA expression was induced in myoblasts by TGF-β1 (19). Our data showed that TGF-β1 did not affect cyclin D1 expressions, and suggested that cell cycle arrest by TGF-β1 in hepatocytes was not mediated by the down-regulation of cyclin D1 but probably by another mechanism.

p21 appears to be able to inhibit all cyclin-dependent kinases, and has been shown to be a downstream transcriptional target of p53 (34). Usually, transcription of the p21 gene is directly promoted by wild-type p53 in response to DNA-damaging agents that trigger G1 arrest or apoptosis (34, 35). However, in this study, TGF-β1 strongly induced p21 mRNA expression, but not p53 in primary cultured rat hepatocytes. In ovarian cancer and colon cancer cell lines, it has been reported that p21 mRNA expressions were up-regulated by TGF-β1, but these inductions were not mediated through p53 (36, 37). The findings described in these reports suggest that p21 may participate in TGF-β-induced cell cycle arrest in certain cell types, presumably not through p53. Michael *et al.* recently showed that the mechanisms of p21 mRNA induction by TGF-β1 was due to transcriptional activation of the p21 promoter by TGF-β1 (38). In the present study, p27 mRNA expressions in rat hepatocytes were not affected by TGF-β1. It has been reported that the level of p27 is essentially constant in the cell cycle (39-41). p27 is associated with cyclin D1/CDK4 in G1, and on entry into S, p27 is released from cyclin D1/CDK4 complex and bound to cyclin A/CDK2 (39). In this way, p27 is redistributed between CDK4- and CDK2- cyclin complexes in normal cell cycle progression and upon cell cycle arrest. In fact, it was investigated that addition of TGF-β1 induced the release of p27 from cyclin D1/CDK4 (39). Therfore,

TGF-β1 might induce the inhibition of liver cell proliferation through p27.

In conclusion, p21 and cyclin A are crucial targets of TGF-β1 in primary cultured rat hepatocytes. TGF-β1 upregulated p21 through a p53-independent mechanism and downregulated cyclin A but not cyclin D1 in rat primary hepatocytes. Alterations of these gene expressions may be responsible for the impaired liver regeneration due to TGF-β1.

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