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Transforming Growth Factor β Triggers Two Independent-Senescence Programs in Cancer Cells

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Transforming growth factor-β (TGF-β)TG has been shown to play a multifunctional role in tumorigenesis. Here we demonstrate that TGF-β induces a morphological change and expression of senescence-associated β-galactosidase activity in the human lung adenocarcinoma cell line A549 cells within a week after the addition. These TGF-β induced phenotypic changes are thought to characterize the rapid onset of senescence. When A549 cells were treated with TGF-β, cell growth was not completely arrested, but the activity of telomerase was down regulated via transcriptional repression of telomerase reverse transcriptase, which led to a shortening of the telomere during long-term culture and finally resulted in replicative senescence. These results indicate that TGF-β is able to induce a rapid senescence in A549 cells without significantly inhibiting cell growth and can further direct A549 cells to a replicative senescence state via the suppression of telomerase which culminates in telomere shortening. All these experimental results suggest that TGF-β transmits several separate and independent signals to shift A549 cells back to a normal senescent cell.

Normal somatic cells do not divide infinitely because they do not contain telomerase, which has the activity to add hexameric repeats to the ends of chromosomal DNA (telomere) to compensate for the progressive shortening of telomere that occurs with successive rounds of replication (1). Normal somatic cells lose their proliferative potential depending upon the degree of telomere shortening, which is termed replicative senescence. Recently, several groups have reported another type of senescence, termed premature or rapid senescence, which is provoked by the induced expression of specific genes and is independent of telomere shortening. Serrano *et al.* have succeeded in provoking premature cell senescence in primary human and rodent cells by expressing oncogenic *ras* (2). Other groups have reported that overexpression of tumor suppressor genes such as p53 or pRB in tumor cells resulted in induction of rapid senescence (3, 4). Premature or rapid senescence is commonly characterized by demonstrating a flat enlarged morphology, growth arrest and activity for SA-β-gal within a week from the start of induced expression of such genes. The ability to induce senescence within such a short time period means that the telomere of these prematurely senesced cells could not shorten to the threshold length, therefore induction of this rapid senescence state occurred independently of the telomere shortening. Thus, we can summarize cellular senescence mechanisms as that the replicative senescence is provoked dependently upon the telomere shortening, and the rapid senescence is induced via the augmentation of specific signals without the shortening of telomere.

We have previously demonstrated that as much as 1% of the human lung adenocarcinoma derived A549 cells reverted to show normal cell phenotypes concomitant with the expression of major histocompatibility complex antigen class II upon stimulation with interferon-γ (5). A5DC7 cells, one of the sublines of A549, showed no telomerase activity, shortened telomere length depending upon the number of cell divisions, expressed SA-β-gal activity and entered the replicative senescence state after a limited number of cell divisions (6). These results suggest that signaling cascades required for inducing cellular senescence can still function in A549 cells, and can be reactivated via an exogenous signals thereby initiating progression of the cellular senescence program. We then screened for novel active molecules which could induce cellular senescence in A549 cells.

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Abbreviations: SA-β-gal, senescence-associated bg-galactosidase; TGF-β, Transforming growth factor b 1; CDK, cycline dependent kinase; hTERT, human telomerase reverse transcriptase; TEP1, telomerase-associated protein 1; TRF, terminal restriction fragment; PDL, population doubling level.

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TGF-β has a multifunctional role in tumorigenesis. Several tumor cells show an antiproliferative response to TGF-β, where TGF-β acts as a tumor suppressor. However, when cells acquire an insensitivity to growth inhibition by TGF-β, TGF-β functions as a tumor promoter by stimulating angiogenesis, immunosuppression and synthesis of extracellular matrix (7). Growth inhibition by TGF-β is demonstrated to be elicited by up regulation of CDK inhibitor $p15^{INK4b}$ and/or repression of Cdc25A, a CDK tyrosine phosphatase which activates CDK (8). Even though A549 cells show a weak antiproliferative response to TGF-β, this effect could not be ascribed to the up regulation of $p15^{INK4b}$ and repression of Cdc25A (8). Thus we assumed that other signaling molecules or mechanisms may participate in this TGF-β induced growth inhibition in A549 cells. Considering together with our results that A549 cells cultured with TGF-β rapidly showed a flat enlarged morphology and SA-β-gal activity characteristic to normal senescent cells, we expect that TGF-β triggered another type of tumor suppression program in A549 cells, which we tried to elucidate from the viewpoint of cellular senescence in this study.

MATERIALS AND METHODS

Cell culture. Human lung adenocarcinoma cell line (A549 cells) was cultured in ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 5% fetal bovine serum at 37°C in a 95% air/5% CO_2 atmosphere. Recombinant human TGF-β (AUSTRAL Biologicals, San Ramon, CA) was added every other day to the culture at a final concentration of 10 ng/ml where indicated.

Senescence-associated β-galactosidase activity. SA-β-gal staining was performed according to the method described by Dimri *et al* (9). Briefly, cells were fixed in 3% formaldehyde and then incubated with fresh SA-β-gal staining solution (1 mg of 5-bromo-4-chloro-3-indolyl-bg-*d*-galactoside (X-gal) per ml of sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$). Staining was carried out at 37°C for 12 hr.

Cell cycle analysis. The percentage of the cells in different cell cycle phases was determined by Flow cytometer analysis of the DNA content by using the FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Briefly, 5×10^6 cells were suspended in 0.2% Triton X-100/12 mM sodium phosphate, pH 7.2, 2.7 mM KCl, 0.14 M NaCl solution (PBS) containing 0.5% ribonuclease A. After incubation for 20 min, DNA was stained with 50 μg/ml of propidium iodide, then applied to the Flow cytometer. The percentage of cells for each cell cycle phase was calculated using the ModFit LT software (Verity, Topsham, ME).

Immunoblot analysis. Cells were washed with ice-cold PBS and lysed in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium vanadate and 1 mM EDTA). Protein quantification was performed using the Protein Assay kit (BIO-RAD Labs, Hercules, CA). Seventy five μg of total cell protein was subjected to 10% SDS-PAGE and transferred to an nitrocellulose membrane (BA85; Schleicher & Shuell, Keene, NH). The membrane was then blocked with 5% Skim Milk/0.1% Tween/PBS followed by incubation with anti-p53 antibody (Ab-2; Oncogene Sci., Cambridge, MA) or anti-Cdc25A polyclonal antibody (144; Santa Cruz Biotech., Santa Cruz, CA), and immunodetected using the ECL system (Amersham, Buckinghamshire, UK).

Telomerase assay. We used the PCR-based TRAP (Telomeric Repeat Amplification Protocol) assay for detecting telomerase activity with some modifications (1, 6). Briefly, 1×10^6 cells were pelleted by centrifugation and resuspended in 200 μl of cold lysis buffer, which was composed of 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 0.1 mM AEBSF and 5 mM 2-mercaptoethanol. After a 30-min incubation on ice, the lysate was centrifuged for 20 min at 13,000 $\times$ g, and the supernatant was snap frozen in liquid nitrogen and stored at -80°C. Protein concentration of the lysate was determined by using the Protein Assay kit. The TRAP reaction mixture, which was composed of 50 μM.M dNTP, 1 $\times$ PCR buffer (Boehringer Mannheim, Indianapolis, IN), 0.1 μg TS primer (5g-AATCCGTCGAGCAGAGTT-3-A), 1.0 μg T4 gene 32 protein (Wako, Kyoto, Japan) and 2 units of Taq polymerase (Boehringer Mannheim), and the lysate which was appropriately diluted as described below were mixed. Following a 30 min incubation at 20°C, the samples were heated at 94°C for 3 min. During this heating, 0.1 μg of CS primer (5g-CCCTTACCCTTACCCTTACCCTAA-3-C) was added to each samples and gently mixed. Then, the samples were subjected to 29 PCR cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s. The PCR products were then run on 10% nondenaturing polyacrylamide gels and visualized by SYBR GREEN I (TAKARA, Shiga, Japan) staining. To measure telomerase activity semiquantitatively, cell lysates prepared from 10^6 cells were titrated between 10 and 10^4 cells equivalent per assay, and a linear range of telomerase activity versus cell number was determined to estimate the appropriate cell number used in each assay. In our case with A549 cells, lysate equivalent to 10^3 cells per reaction was used because telomerase activity can be quantitatively determined by the TRAP assay when lysate equivalent to a range from 50 to 1000 cells per reaction was used (data not shown).

RT-PCR. Human $p15^{INK4b}$, hTERT and TEP1 mRNAs were detected by RT-PCR method. Total RNA was prepared from each subconfluent culture by using TRIzol reagent (Gibco BRL, Gaithersburg, MD) and further purified with ice-cold 4 M LiCl. Three μg of total RNA was used as template for cDNA synthesis reaction using SuperScript II Rnase H^- reverse transcriptase (Gibco BRL). Subsequent PCR cycles was performed by using 1/20 vol. of cDNA synthesis reaction mixture. Human $p15^{INK4b}$ mRNA was amplified using oligonucleotide primers (AGAACAAGGGCATGCCCAG and GTTGACTCCGTTGGGATCC) for 29 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 90 s). As reported by Nakamura *et al.* (10), hTERT mRNA was amplified using oligonucleotide primers LT5 (CGGAAGAGTGTCTGGAGCAA) and LT6 (GGATGAAGCGGAGTCTGGA) for 26 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 90 s), and TEP1 mRNA was amplified using oligonucleotide primers TEP1.1 (TCAAGCCAAACCTGAATCTGAG) and TEP1.2 (CCCGAGTGAATCTTTCTACGC) for 26 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 90 s). Reaction products were resolved on an 8% polyacrylamide gel and stained with SYBR GREEN I.

DNA extraction and analysis. Genomic DNA was prepared using the DNA Extractor WB Kit (Wako). Briefly, pelleted cells (3×10^6 cells) were lysed with Lysis solution and treated with protease in the presence of 1% SDS. Genomic DNA was prepared from the resulting solution with NaI. Telomere length distributions were analyzed at a series of time points during serial passaging of TGF-β-treated A549 cells. Length of the TRF was determined by Southern blot analysis with a telomeric sequence probe and used to measure the length of the telomere as described previously (6). Briefly, 5 μg of DNA digested with *Hin*fI and *Eco*RI was loaded onto a 0.8% agarose gel and the separated DNA was transferred onto Hybond N^+ membrane (Amersham). The blot was hybridized with the non-radioisotopic probe $(TTAGGG)_4$, the 3′-end of which was labeled with fluorescein-11-dUTP by using the ECL 3′-oligolabelling system (Amersham). Signal detection was carried out by using the *Gene Images* CDP-Star detection module (Amersham). Blots hybridized with the probe were incubated with the anti-fluorescein-alkaline phosphatase conjugate, and subsequently with the CDP-Star detection reagent, then directly

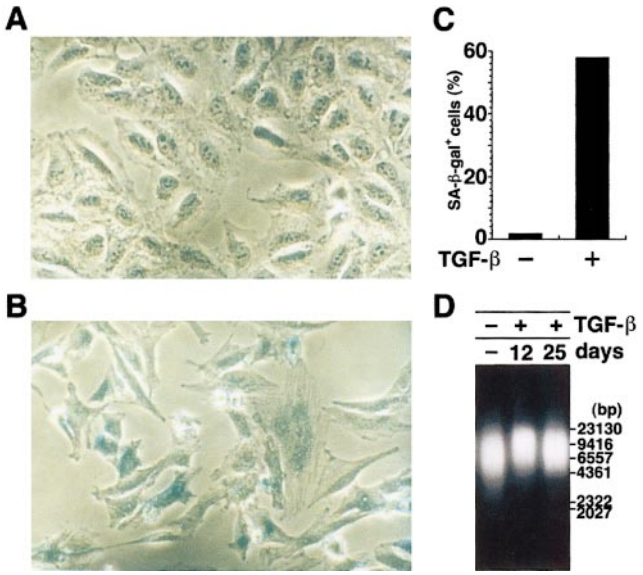

FIG. 1. Telomere length-independent senescence inducing effect of TGF-β on A549 cells. SA-β-gal staining was performed on A549 cells (A) and A549 cells cultured with TGF-β for 7 days (B). The number of SA-β-gal positive cells were microscopically counted in these cells (C). A549 cells cultured with TGF-β were evaluated for the telomere length as described in Materials and Methods (D). Fluorescein-labeled λ*Hin*dIII marker was used for estimating the length of TRF.

exposed onto Polaroid film (Hertfordshire, UK). Fluorescein-labeled λ*Hin*dIII marker (Amersham) was used for estimating the length of TRF. The TRF length was measured with the Kodak EDAS system (Eastman Kodak Co., Rochester, NY), where the center of the peak was taken as the mean TRF length (11).

RESULTS AND DISCUSSION

TGF-δ induced premature senescence in cancer cells. When cultured in the presence of 10 ng/ml TGF-β, A549 cells rapidly showed a morphological change. A549 cells cultured with TGF-β adopted a flat enlarged morphology which is characteristic to normal senescent cells (Fig. 1 A and B) (12). To assess whether A549 cells acquired senescent cell phenotypes when culturing with TGF-β, we investigated for the expression of SA-β-gal activity. SA-β-gal positive A549 cells dramatically increased after culturing with TGF-β for 7 days (Fig. 1 B and C), which was demonstrated by the formation of blue precipitates upon cleavage of X-gal. Considering together with the result that the telomere length of chromosomes from A549 cells did not shortened until day 25 (Fig. 1D), this rapid expression of SA-β-gal activity in A549 cells cultured with TGF-β suggests that TGF-β induces a rapid onset of senescence, which is independent of the telomere shortening. Serrano *et al.* has reported that oncogenic *ras* provokes a premature senescence state in primary human and rodent fibroblasts within a shorter period than is expected from the total PDLs normally required for entering the replicative senescent state, suggesting that there exist signaling cascades for senescence induction that is independent of telomere shortening (2). Two other groups have reported that induced expression of certain tumor suppressor genes can trigger rapid senescence in tumor cells. Xu *et al.* indicated that re-expression of the functional pRB protein in RB/p53-defective tumor cells resulted in the induction of cellular senescence within 4–5 days (4). Sugrue *et al.* demonstrated that overexpression of wild-type p53 in tumor cells that have lost the functional p53 protein is able to trigger rapid senescence within 5 days (3). These rapid senescence inductions via activation of functional tumor suppressor genes are also thought to be independent of the telomere shortening, because the telomere does not shorten to the threshold length within such a short period of time. These reports indicate that oncogenic *ras* or tumor suppressor genes such as pRB and p53 can rapidly induce cellular senescence independent of the telomere shortening. Considering together with these reports, our results suggest that TGF-β emits certain signals to induce the premature senescence in A549 cells.

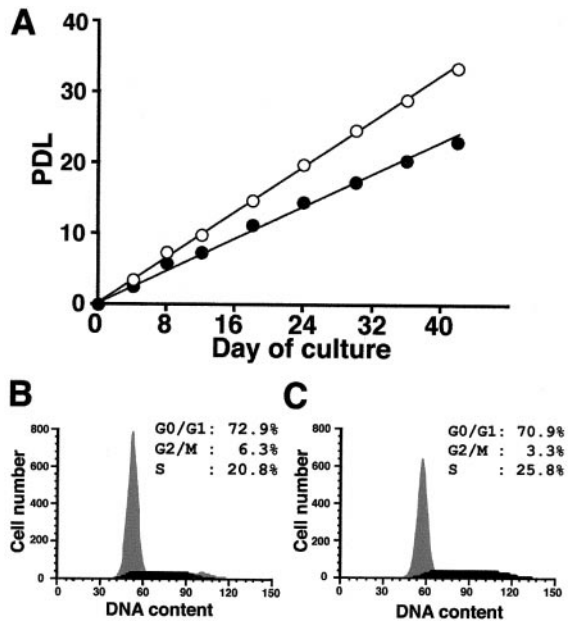

FIG. 2. Growth phenotypes for A549 cells and A549 cells cultured with TGF-β. A549 cells (○) and A549 cells cultured with TGF-β (●) were inoculated at a concentration of 1×10^5 cells/ml. Cell number was counted at the indicated passages to calculate the PDL (A). X axis denotes the culture period (days) and Y axis shows the PDL. Cell cycle analysis was performed on a fluorescence-activated cell sorter (FACS Calibur, Becton Dickinson) for A549 cells (B) and A549 cells cultured with TGF-β (C). Data was analyzed using ModFit LT software (Verity). Cell number is depicted on Y axis and DNA content, as measured by propidium iodide fluorescence, is depicted on X axis.

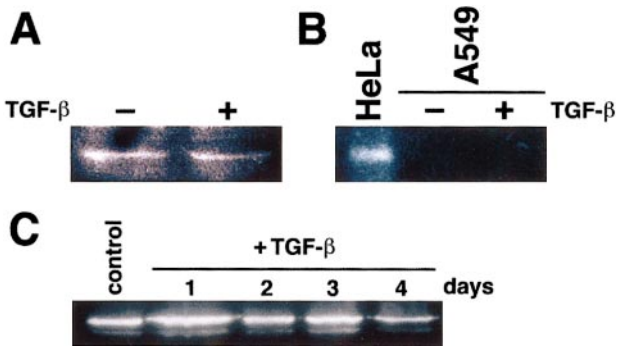

FIG. 3. Effect of TGF-β on cell cycle regulatory proteins in A549 cells. (A) Immunoblot analysis of p53 protein in cell lysates from A549 cells and A549 cells cultured with TGF-β. Cell lysates was prepared from A549 cells and A549 cells cultured with TGF-β for 123 days and applied to immunoblot analysis using the anti-p53 antibody. (B) RT-PCR analysis of $p15^{INK4b}$ transcript in A549 cells and A549 cells cultured with TGF-β. Total RNA was prepared from A549 cells and A549 cells cultured with TGF-β for 167 days, then applied to RT-PCR analysis. Cell lysates from HeLa cells was used as a positive control. (C) Expression of Cdc25A protein in response to TGF-β treatment. At day 0, TGF-β was added to A549 cells, then cell lysates were prepared at the indicated days and immunoblotted using the anti-Cdc25A antibody.

A549 cells displayed only weak growth inhibition against TGF-β. Common features for cells in premature senescence are a flat enlarged morphology, expression of SA-β-gal activity and initiation of an arrested growth rate earlier than expected from a residual replicative life-span (2–4). We therefore investigated growth phenotypes for A549 cells cultured with TGF-β. Our results show that A549 cells displayed only weak growth inhibition against TGF-β (Fig. 2A), which coincides with the result reported by Iavarone *et al.* (8). Furthermore, cell cycle phase distribution of A549 cells was not greatly changed by the addition of TGF-β (Fig. 2B and C). These results suggest that the induction of premature senescence and growth inhibition are independently regulated, and that molecules participating in growth inhibition could be functionally impaired in A549 cells. The tumor suppressor gene products p53 and $p16^{INK4a}$ has been reported to accumulate in the rapidly senesced cells (2). Furthermore, antiproliferative effect of TGF-β has been ascribed to the up regulation of $p15^{INK4b}$, one of the CDK inhibitors, and/or the down regulation of Cdc25A, a tyrosine phosphatase of which the repression causes cell-cycle arrest in a human mammary epithelial cell line via an increased tyrosine phosphorylation level of CDK4 and CDK6 (8). However in our case, TGF-β could not induce an increase in the p53 protein level (Fig. 3A). As expected from a report which stated that $p15^{INK4b}$ and $p16^{INK4a}$ genes are homozygously deleted in A549 cells (8), $p15^{INK4b}$ transcript could not be detected even after the addition of TGF-β (Fig. 3B). Furthermore, TGF-β could not suppress Cdc25A expression in A549 cells (Fig. 3C). These functional impairments of signal molecules which participate in growth inhibition would explain why the growth inhibition observed in rapidly senesced A549 cells upon the treatment with TGF-β was so weak. We can further propose the possibility that the premature senescence induction as well as the weak but detectable growth inhibition is thought to be elicited by other molecules than those described above. We should then address the possibility that even though A549 cells enters the rapid senescence state upon the treatment with TGF-β they still have an infinite replicative life-span because the growth potential still remains.

Telomerase activity was suppressed in TGF-β-treated cells. The re-expression of pRB in pRB/p53 deficient tumor cells suppresses telomerase activity as well as initiates expression of multiple senescence phenotypes (4). We then investigated the telomerase regulation mechanism in the TGF-β induced rapid senescent A549 cells. We measured telomerase activity by the TRAP assay which was established by Kim *et al.* with some modifications (1, 6). To estimate the telomerase activity in A549 cells semi-quantitatively, we titrated

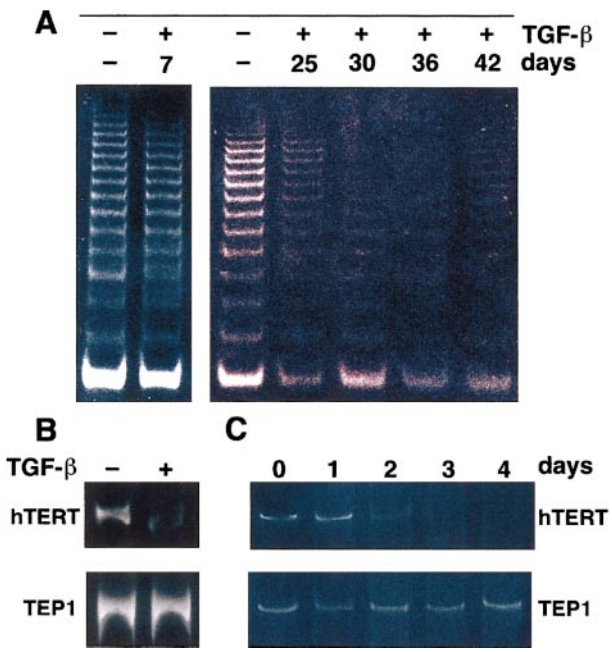

FIG. 4. Effect of TGF-β on telomerase activity and telomerase component expression in A549 cells. (A) Telomerase activity in A549 cells and A549 cells cultured with TGF-β. Cell lysates prepared from A549 cells and A549 cells cultured with TGF-β at several points during serial passagings were applied to the TRAP assay to measure telomerase activity. TRAP assay products were visualized by SYBR GREEN I staining. (B) Expression of telomerase components in A549 cells and A549 cells cultured with TGF-β. Total RNA was prepared from A549 cells and A549 cells cultured with TGF-β for 57 days, then applied to RT-PCR analysis using hTERT and TEP1 specific primers. (C) Repression of hTERT expression in response to TGF-β treatment. At day 0, TGF-β was added to A549 cells, then total RNA was prepared at the indicated days and applied to RT-PCR analysis.

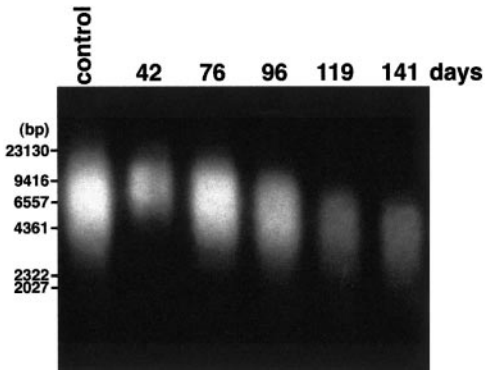

FIG. 5. TRF length of A549 cells cultured with TGF-β. A549 cells cultured for a long-term with TGF-β was evaluated for the telomere length distributions by measuring the length of TRF. Prepared DNA was digested with *Eco*RI and *Hin*fI, and analyzed by Southern blot analysis using a non-radioisotopic probe $(TTAGGG)_4$, the 3′-end of which was labeled with fluorescein-11-dUTP. Signal detection was carried out using *Gene Images* CDP-Star detection module as described in Materials and Methods. Fluorescein-labeled λ*Hin*dIII marker was used for estimating the length of TRF.

the number of cells used for the assay so that the band intensity for the TRAP assay product and the number of cells used in the assay were linearly correlated with each other (data not shown) (6). The result demonstrated that although A549 cells cultured with TGF-β for 7 days had a similar level of telomerase activity to non-treated A549 cells, telomerase activity of TGF-β-treated A549 cells gradually decreased from day 25 and completely vanished by day 30 (Fig. 4A). We further investigated the transcriptional regulation of telomerase components in the TGF-β-treated A549 cells. To date, two component proteins for telomerase were identified in human cells, hTERT (10, 13) and TEP1 (14, 15). Nakamura *et al.* have reported that hTERT expression is completely correlated with telomerase activity in cultured cell lines, but TEP1 was not, suggesting that telomerase activity is regulated at the transcriptional level of the hTERT gene (10). Thus, we investigated the transcriptional regulation of the hTERT and the TEP1 gene in A549 cells cultured with TGF-β using the RT-PCR method as described by Nakamura *et al.* As expected, hTERT but not TEP1 mRNA expression was repressed in A549 cells cultured with TGF-β for 57 days, when the telomerase activity was completely repressed (Fig. 4B). Furthermore, hTERT mRNA transcription was shown to be repressed at day 2 from the start of the culture with TGF-β (Fig. 4C). Repression of hTERT transcription could be observed before down regulation of telomerase activity. Telomerase activity remained unchanged over 1 week after the repression of hTERT transcription was observed, which could be ascribed to the higher stability of telomerase activity as previously reported (166). This advanced repression of hTERT transcription also suggests that TGF-β emits signals to repress hTERT transcription as well as to induce a premature senescence in A549 cells. This delayed but complete repression of telomerase activity and remaining proliferative potential observed in the TGF-β-treated A549 cells suggest that telomerase can be down regulated independently of the growth and/or cell cycle in A549 cells (16–18), and further implicate that TGF-β induced rapid senescent A549 cells could undergo passage-number dependent telomere shortening during long-term culture.

TGF-β-treated cells entered a replicative senescent state. We then measured the telomere length at a series of time points during serial passaging of TGF-β-treated A549 cells. The result demonstrated that the telomere length of A549 cells was kept nearly constant until day 42 after the addition of TGF-β, but gradually shortened starting from day 76 (Fig. 5). Parental A549 cells has a telomere length of 8.1 kb but is shortened to about 3.6 kb at day 141 (Fig. 5). The average rate for telomere shortening starting from day 42 was demonstrated to be 86.5 bp per cell division, which is consistent with the report that normal cells shorten the telomere by 50–200 per cell division (199). This result showed that telomerase repression by TGF-β observed after day 30 evoked a passage-number dependent telomere shortening in A549 cells during long-term culture, indicating that A549 cells entered a replicative senescence state dependent upon the telomere shortening by treatment with TGF-β. Actually, TGF-β-treated A549 cells showed a gradual reduction in proliferative potential from day 300 and greatly lost their proliferative potential around day 400 (Fig. 6), which is interpreted as entry into the replicative senescence state depending upon the telomere shortening. Considering together with the results obtained, we can conclude that two separate and independent cellular senescence induction mechanism was triggered by TGF-β. TGF-β can induce a premature senescence in

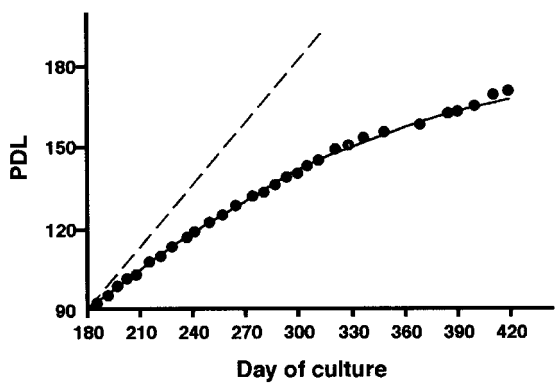

FIG. 6. Growth phenotypes for A549 cells and A549 cells cultured with TGF-β. A549 cells (– – –) and A549 cells cultured with TGF-β (●) were inoculated at a concentration of 1×10^5 cells/ml. Cell number was counted at the indicated passages to calculate the PDL. X axis denotes the culture period (days) and Y axis shows the PDL.

A549 cells independent of the telomere shortening, and can also suppress telomerase activity which leads to a telomere shortening-dependent replicative senescence state that would vest A549 cells with a finite replicative life-span.

Until now, many researchers have observed elevated levels of two CDK inhibitors, $p16^{INK4a}$ and $p21^{CIP1}$, in senescent cells (200–222), and Uhrbom *et al.* reported that induced expression of $p16^{INK4a}$ in glioma cells reverted their immortal phenotype and caused an immediate cellular senescence (23m), suggesting the involvement of these CDK inhibitors in the senescence-associated growth arrest. On the other hand, additional genes encoding growth inhibitors that are up regulated in senescent cells, such as the recently isolated $p33^{ING1}$, were also identified (24), thus signaling pathway and cell cycle machinery which function in the growth arrest correlated with the replicative senescence remain undefined. In our case, $p15^{INK4b}$ and $p16^{INK4a}$ genes are homozygously deleted in A549 cells, suggesting that cell cycle regulatory molecules other than $p16^{INK4a}$ would participate in the growth inhibition observed in TGF-β-treated A549 cells in replicative senescence state. Furthermore, these results indicate that A549 cells can inhibit their growth in response to the telomere shortening, but only partially to TGF-β.

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