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Overexpression of the *p21 sdi1* gene induces senescence-like state in human cancer cells: implication for senescence-directed molecular therapy for cancer

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Abstract

Normal cells in a culture enter a nondividing state after a finite number of population doubling, which is termed replicative senescence, whereas cancer cells have unlimited proliferative potential and are thought to exhibit an immmortal phenotype by escaping from senescence. The *p21* gene (also known as *sdi1*), which encodes the cyclin-dependent kinase inhibitor, is expressed at high levels in senescent cells and contributes to the growth arrest. To examine if the *p21sdi1* gene transfer could induce senescence in human cancer cells, we utilized an adenoviral vector-based expression system and four human cancer cell lines differing in their *p53* status. Transient overexpression of *p21sdi1* on cancer cells induced quiescence by arresting the cell cycle at the G_1 phase and exhibited morphological changes, such as enlarged nuclei as well as a flattened cellular shape, specific to the senescence phenotype. We also showed that *p21sdi1*-transduced cancer cells expressed β-galactosidase activity at pH 6.0, which is known to be a marker of senescence. Moreover, the polymerase chain reaction-based assay demonstrated that levels of telomerase activity were significantly lower in *p21sdi1*-expressing cells compared to parental cancer cells. These observations provide the evidence that *p21sdi1* overexpression could induce a senescence-like state and reduce telomerase activity in human cancer cells, suggesting that these novel *p21sdi1* functions may have important implications for anticancer therapy.

Keywords: p21; senescence; adenovirus vector; human cancer

Abbreviations: Cdk, cyclin-dependent kinase; MOI, multiplicity of infections; SA-β-gal, senescence-associated β-galactosidase; PAI-1, plasminogen activator inhibitor

Introduction

Normal diploid mammalian cells undergo a finite number of population doubling in culture before they accrue replicative senescence.[1] In contrast, tumors often contain immortalized cells that exhibit an unlimited *in vitro* and *in vivo* proliferative potential. These facts suggest that the limited lifespan of normal human cells is a tumor suppressor mechanism and that escape from senescence to yield immortal variants could be an important step in tumorigenesis.[2,3] Fusion of normal and immortalized cells usually results in hybrids with limited proliferative potential, indicating that immortalization is probably due to the loss of normal gene function.[4] Although studies of the transforming proteins of DNA tumor viruses have indicated the involvement of the *p53* gene and the retinoblastoma (*Rb*) susceptibility gene in senescence,[5–7] molecular analyses have identified more changes in gene expression and in the activity of gene products during cellular aging. Differential screening comparing young and senescent human cells has also revealed differences in the expression of several genes.[2]

Noda *et al*[8] has reported that expression of the *sdi1* gene, which encodes a 21 kDa protein product, increases in senescent cells compared with young cells and that the increase parallels the onset of the senescent phenotype, suggesting that *p21sdi1* may be a causal candidate gene for cellular senescence. *p21sdi1* was subsequently cloned as a gene encoding a cyclin-dependent kinase (Cdk) inhibitor (*Cip1*),[9] and as a gene transcriptionally activated by p53 (*Waf1*).[10] *p21sdi1* was also cloned as a melanocyte differentiation-associated factor activated independent of p53 (*mda-6*).[11] These findings concerning *p21sdi1* have elucidated the potent interactions of cellular senescence, cell cycle regulation, cell differentiation, and tumor suppression, and prompted us to study the effect of ectopic *p21sdi1* gene expression in human cancer cells.

The immortalization of normal human cancer cells is often associated with the reactivation of the enzyme telomerase, which adds TTAGGG hexanucleotide repeats on to the telomeres of chromosomes. Telomerase is thought to be present in germ cells, immortal cell lines and cancer cells, but not in normal somatic cells. Telomeres in normal somatic cells are shorter by approximately 50 base pairs with every cell division. It has been proposed that this progressive decrease in

telomere length acts as a mechanism for counting cell divisions and that sufficient telomeric shortening triggers senescence.[12] However, telomerase is reactivated in immortal human cancer cells and, moreover, the enzyme activity is detectable in over 80% of human tumor samples. These observations lead to the hypothesis that terminally nondividing cancer cells in the senescence-like state might have decreased levels of telomerase activity.

In the present study, to investigate the biological significance of $p21^{sdi1}$ on human cancer cells, $p21^{sdi1}$ was exogenously introduced into several cancer cell lines differing in their *p53* status by a recombinant adenovirus vector. Our results demonstrate that ectopic $p21^{sdi1}$ overexpression induces the cellular senescence accompanied with the specific cell cycle arrest, leading to the down-regulation of telomerase activity. These studies may have important implications for developing a novel senescence-directed anti-cancer therapy.

Results

Adenovirally transduced $p21^{sdi1}$ gene expression in human cancer cells

Four human cancer cell lines of different tissue origin were examined for the effects of $p21^{sdi1}$ expression: H1299 (lung) and Saos-2 (osteosarcoma) with deleted *p53*, DLD-1 (colon) with mutated *p53*, and LoVo (colon) with wild-type *p53*. We employed a replication-deficient adenovirus vector system for the $p21^{sdi1}$ gene transfer. Western blot analysis demonstrated that readily detectable $p21^{sdi1}$ protein expression was achieved in all cell lines as early as 24 h after AdCMVp21 infection at a multiplicity of infection (MOI) of 50, whereas parental and AdRSVLuc-infected cells exhibited no detectable $p21^{sdi1}$ protein (Figure 1). A time-course experiment showed that $p21^{sdi1}$ expression was detectable over a period of 15 days after AdCMVp21 infection.

Effect of $p21^{sdi1}$ expression on growth and cell-cycle progression of human cancer cell lines

We looked next for the effect of $p21^{sdi1}$ overexpression on the growth of human lung cancer cells. H1299 cells which remained as an attached monolayer were assessed for viability by measuring trypan blue uptake for 6 days following infection with AdCMVp21 and AdRSVLuc at 50 MOI for 24 h. As shown in Figure 2A, AdCMVp21 markedly suppressed the growth rate of H1299 cells and induced the growth arrest as long as we observed, while AdRSVLuc did not affect the cell growth. This inhibitory effect of transduced $p21^{sdi1}$ on human cancer cells is consistent with previous reports.[13–15]

To investigate the mechanism of $p21^{sdi1}$-mediated growth suppression, we performed the cell cycle analysis on H1299, DLD-1, and LoVo cells. Cells were infected with either AdCMVp21 or AdRSVLuc, harvested at 48 h post-infection, and then assayed for DNA contents by flow cytometry. AdCMVp21-infected cells showed a decreased fraction of S-phase cells resulting from an accumulation of most cells in the G_0/G_1 phase, suggesting arrest predominantly at the G_1 checkpoint (Figure 2B). We also examined the cell cycle distribution of Saos-2 cells, which did not show a typical diploidy pattern. The fraction equivalent for the S- and G_2/M phase decreased and, in contrast, that for the G_0/G_1 phase increased, indicating that progression into S-phase was blocked in Saos-2 cells (data not shown).

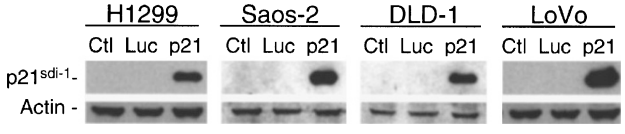


Figure 1 Detection of $p21^{sdil}$ protein in adenovirally transduced cells by Western blot analysis. Human cancer cell lines, H1299, Saos-2, DLD-1, and LoVo, were infected with either AdRSVLuc or AdCMVp21, and collected 48 h after infection. $p21^{sdil}$ protein was expressed in all cell lines transduced with AdCMVp21, but not in untreated and AdRSVLuc-transduced cells

Morphologic effects and SA-β gal expression in human cancer cells transduced with $p21^{sdi1}$ gene

Alterations in cell morphology were also studied after AdCMVp21 infection. $p21^{sdi1}$-transduced H1299 cells showed morphological changes 3 days after infection, such as an enlarged, flattened shape, an increased cytoplasmic-to nuclear ratio, and a decreased cell density, consistent with a senescence phenotype. Identical results were obtained with other cell lines: DLD-1, LoVo, and Saos-2.

To further examine whether the morphological changes indicated cellular senescence, we assessed SA-β-gal activities in four cell lines. SA-β-gal activity at pH 6.0, which is a specific biomarker for senescent cells, are not expressed in presenescent and quiescent cells.[16] Most AdCMVp21-transduced cells processing the senescent morphology expressed intense SA-β-gal activity as early as 2 days after infection. In contrast, little or no SA-β-gal activities were detected in untreated or AdRSVLuc-infected cells. The micrographs of each cell line exhibiting the characteristic morphology and SA-β-gal staining were illustrated in Figure 3A. These results revealed that AdCMVp21 infection induced a senescence-like state in human cancer cells.

We next examined whether withdrawal from the cell cycle by serum deprivation could cause alterations in cell morphology. Although the growth of H1299 and DLD-1 cells cultured in the absence of serum was significantly reduced as evidenced by a decreased cell density, cells exhibited neither a flattened morphology nor enlarged nuclei (Figure 3B). Similar results were obtained with Saos-2 and LoVo cells (data not shown). Thus, the morphologically altered phenotype, indicative of the senescence-like state, appeared not to be due to the withdrawal from the cell cycle.

McConnell *et al*[17] have reported that plasminogen activator inhibitor (PAI-1) mRNA expression is up-

regulated in senescent human diploid fibroblasts by ectopically expressed Cdk inhibitors; Western blot analysis using anti-PAI-1 antibody, however, showed that levels of PAI-1 protein could not be significantly altered in human cancer cells by $p21^{sdi1}$ gene transfer (Figure 4).

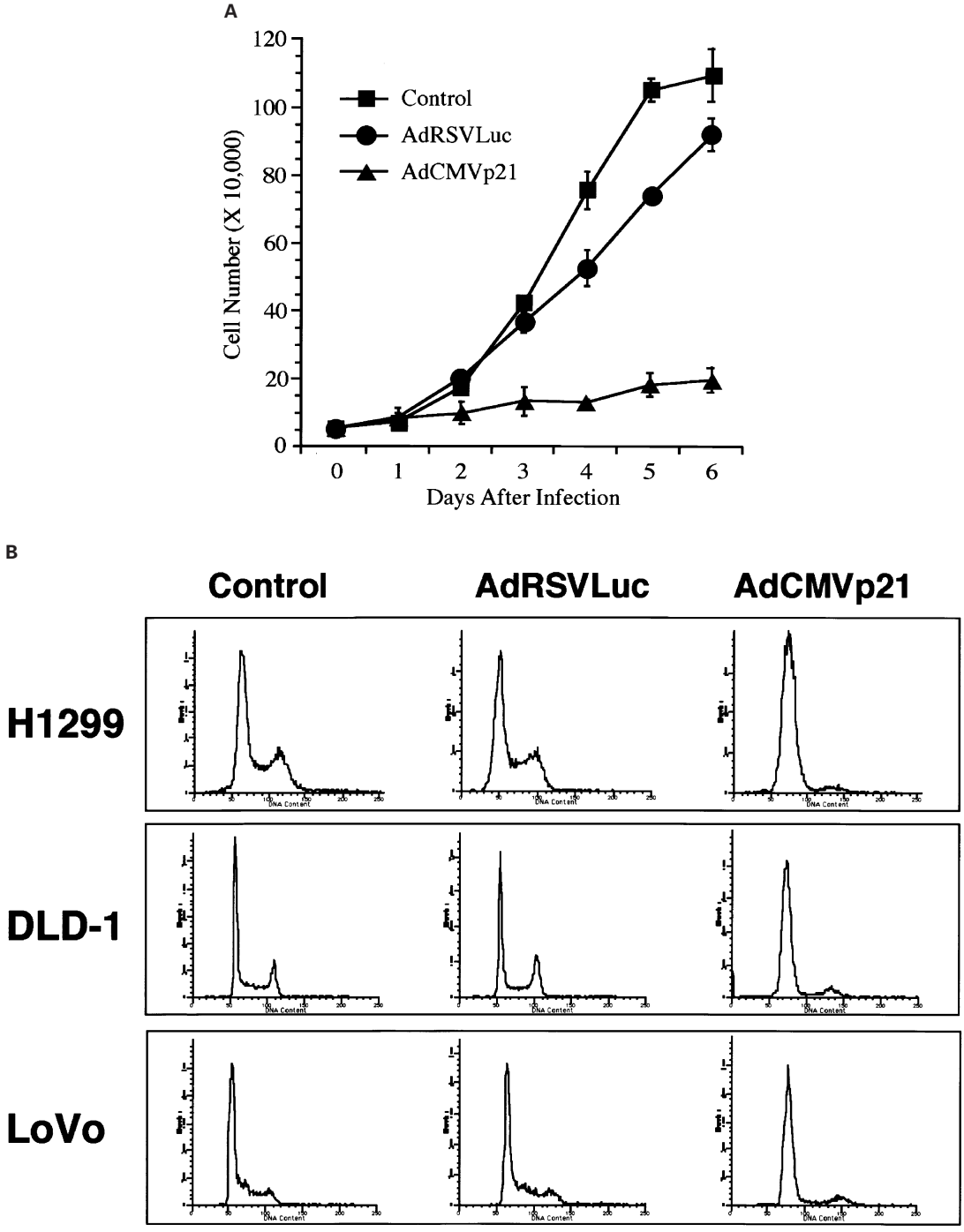


Figure 2 (**A**) Growth curves of H1299 human lung cancer cells transduced with AdCMVp21. Subconfluent H1299 cells (5×10^4) cultured as a monolayer were infected with either AdRSVLuc or AdCMVp21 at a MOI of 50 for 24 h. Cell viability was determined by Trypan blue staining on a daily basis. Each point represents the mean $\pm$ standard deviation of triplicate experiments. Control cells as well as AdRSVLuc-infected cells similarly showed an exponential growth, whereas the growth was markedly suppressed by AdCMVp21 transduction. (**B**) Cell cycle distribution of H1299, DLD-1, and LoVo cells transduced with AdCMVp21 or AdRSVLuc vector. Cells (5×10^5) were infected with either AdCMVp21 or AdRSVLuc at 50 MOI for 48 h, trypsinized, and then analyzed for DNA content by propidium iodide staining using flow cytometry. A normal cell cycle curve was maintained in AdRSVLuc-infected cells, while a marked S-phase reduction was observed in cells transduced with AdCMVp21

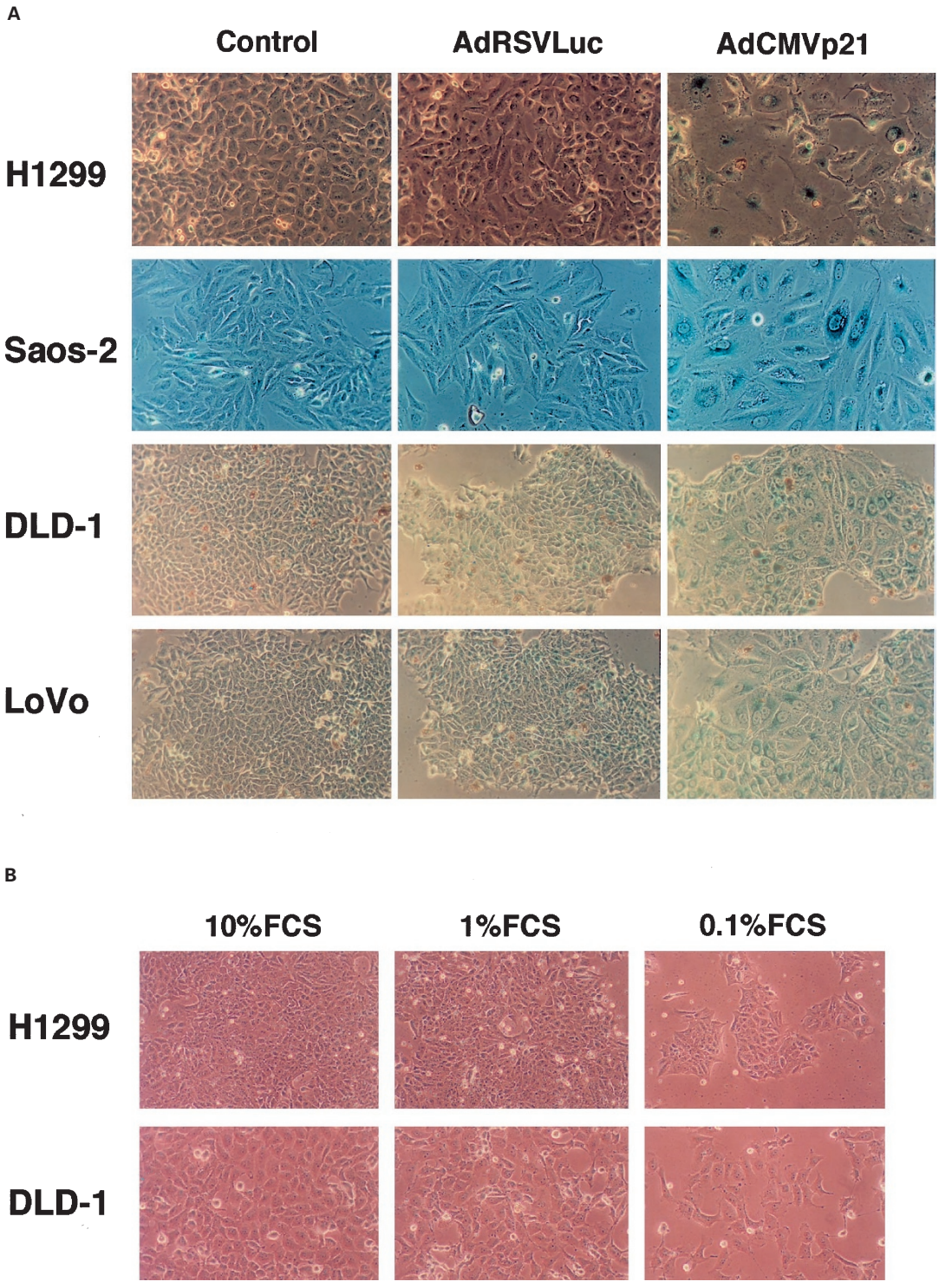


Figure 3 (**A**) Morphology and SA-β gal activity at pH 6.0 of AdCMVp21-infected human cancer cells. H1299, DLD-1, LoVo, and Saos-2 cells were infected with either AdRSVLuc or AdCMVp21 adenoviral vector at 50 MOI, and subjected to SA-β gal staining 72 h after infection. Phase-contrast microscopy showed that most $p21^{sdil}$-transduced cells exhibited flattened, enlarged cell morphology, whereas no remarkable morphologic changes were observed in AdRSVLuc-infected cells. AdCMVp21-infected cells were SA-β gal positive as evidenced by cytoplasmic blue color staining. A few cells grown in control medium expressed SA-β gal activity; the blue color, however, was much weaker than that in cells transduced with AdCMVp21, suggesting that this spontaneous activity may be independent of senescence. (**B**) Photomicrograph of H1299 and DLD-1 cells cultured in serum-depleted medium

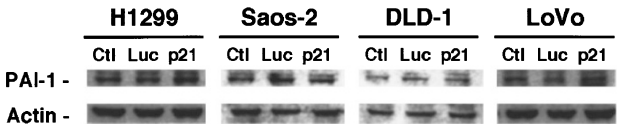
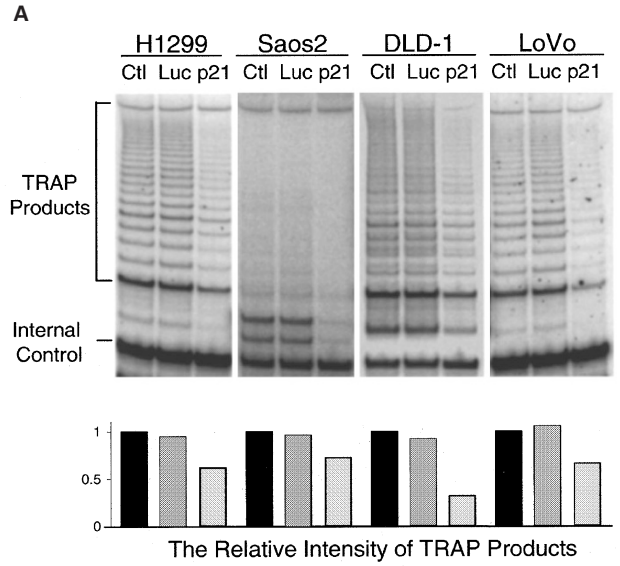


Figure 4 Expression of PAI-1 protein in human cancer cells expressing *p21^{sdil}* gene. Parental human cancer cell lines, H1299, Saos-2, DLD-1, and LoVo cells and cells infected with AdRSVLuc or AdCMVp21 at a MOI of 30 were harvested and analyzed on Western blots using antibodies against PAI-1



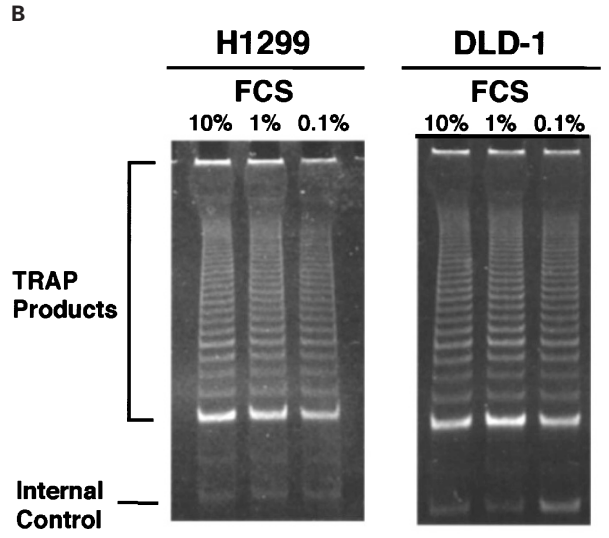


Figure 5 (**A**) Telomerase activity in human cancer cells following ectopic *p21^{sdil}* gene transfer. Cells were assayed for telomerase activity by TRAP assay 72 h after infection with AdCMVp21 or AdRSVLuc (50 MOI). The observation that DNA ladder formation is less clear in AdCMVp21-infected cells compared to untreated or AdRSVLuc-infected cells indicates the reduced telomerase activity. The lower bands demonstrate internal control. The relative intensity of TRAP product bands was quantitated and expressed in bar graphs at the lower panel. (**B**) Telomerase activity of H1299 and DLD-1 cells cultured in the absence of serum. Withdrawal from the cell cycle did not alter the relative telomerase levels in human cancer cells

Effect of *p21^{sdi1}* overexpression on telomerase activity

Progressive loss of telomeres in normal cells has been thought to activate sensing mechanisms responsible for cellular senescence. Therefore, we hypothesized that induction of senescence by *p21^{sdi1}* expression might be associated with a pronounced downregulation of telomerase activity. We assessed telomerase activities of *p21^{sdi1}*-transduced human cancer cells by using a recently developed PCR-based TRAP assay.[18,19] AdCMVp21 infection reduced the relative telomerase levels in H1299, DLD-1, and LoVo cells at 72 h of post-transduction; the level of telomerase activity did not change upon AdRSVLuc control vector infection (Figure 5A). Although, in the case of Saos-2 cells, the telomerase level was quite low, the reduction in telomerase activity was observed.

In accordance with the morphological analysis, H1299 and DLD-1 cells cultured in the absence of serum exhibited similar telomerase levels, despite significant growth inhibition when serum was withdrawn (Figure 5B). These results suggest that quiescent tumor cells could retain telomerase activity and that downregulation of telomerase activity might be a direct effect of *p21^{sdi1}* gene transfer.

Discussion

The present study demonstrates that overexpressed *p21^{sdi1}* arrests human cancer cell growth by blocking cell-cycle progression and induces the senescence-like state, as evidenced by cell morphology, which is similar to that commonly observed with senescent fibroblasts, as well as positive SA-β gal staining, which is histochemically detectable at pH 6.0 upon senescence. We utilized an adenoviral vector system, which is applicable for various cell types with different tissue origin, to introduce the ectopic *p21^{sdi1}* gene into human cancer cells. Our results demonstrated that the expression of the adenovirally transduced *p21^{sdi1}* gene was sufficient to exhibit its biological functions. In addition, our preliminary experiments confirmed that levels of adenovirus-mediated recombinant *p21^{sdi1}* protein expression varied among cell types; those levels, however, did not exceed physiological levels. Thus, our *p21^{sdi1}* expression system may be relevant for studying the cellular responses related to *p21^{sdi1}*.

Many studies comparing young and senescent human cells have attempted to identify molecular mechanisms leading to the induction of the senescence phenotype. Genes that have been implicated in regulating senescence include tumor suppressor genes such as *p53* and *Rb*,[4,20,21] Cdk inhibitors such as *p21^{sdi18}* and *p16*,[22–24] and several currently unidentified genes. Recent studies have shown that ectopic *p16* expression induces senescence on human fibroblasts as well as human cancer cells; induction of senescence by *p21^{sdi1}*, however, has been reported only in fibroblasts.[17,25–27] Overexpression of the wild-type *p53* gene has demonstrated to trigger a rapid senescence program in human bladder carcinoma cells containing a mutant form of *p53*.[20] Growth arrest as well as morphological changes accompanied with activation of SA-β gal activity were induced by an exogenously expressed *p53*

gene in the teracyclin-inducible expression system. These *p53*-mediated cellular responses are very similar to those that we observed by $p21^{sdi1}$ introduction. Together with the fact that *p53* is a direct upstream regulator of $p21^{sdi1}$, $p21^{sdi1}$ rather than *p53* may be thought to play an important role in the cell senescence phenomenon of human cancer cells. We previously reported that adenovirus-mediated transfer of the wild-type *p53* gene into *p53*-deficient H1299 human lunc cancer cells induced apoptotic cell death and resulted in the rapid decrease of cell viability despite endogenous $p21^{sdi1}$ expression;[28] our results described here, however, demonstrated that $p21^{sdi1}$ overexpression triggered a senescence program without apoptosis induction. Thus, *p53* can induce senescence through $p21^{sdi1}$ in some types of cancer cells and may initiate the apoptotic control machinery by direct signaling that can overcome the $p21^{sdi1}$ functions in other types of cells.

Recently, it has been reported that expression of oncogenic ras triggered senescence in primary human or rodent fibroblasts, which was associated with increases in *p53*, $p21^{sdi1}$, and *p16* levels.[29] Moreover, histon deacetylase inhibitors, sodium butyrate and trichostatin A, induced a senescence-like state in human fibroblast accompanied by increased expression of $p21^{sdi1}$.[30,31] The finding that butyrate induced $p21^{sdi1}$ expression by the activation of its promoter[32] provided additional evidence that $p21^{sdi1}$ might participate in the induction of senescence. The observation that expression of a number of genes has been altered during cellular aging brings up the questions of which changes in gene expression cause cellular senescence and which result from the senescence phenotype. Our gene transfer experiments demonstrated that at least human cancer cells transduced with the ectopic $p21^{sdi1}$ gene underwent senescence, suggesting that $p21^{sdi1}$ expression may not be the result of other mechanisms causing senescence. In addition, whether the changes to senescence features, such as morphological changes and the reduction of telomerase activity, is due to senescence induction or is merely accompanied with the cell-cycle arrest was elucidated. We confirmed that cell cycle arrest induced by serum depletion could not induce any changes indicative of senescence.

$p21^{sdi1}$ is known to inhibit the activities of cyclin-Cdk complexes, thereby preventing phosphorylation of the Rb protein, and was initially characterized as an inhibitor of the cell cycle. When phosphorylated, the Rb protein can no longer sequester the transcriptional factors of the E2F family, which in turn regulates many genes required to initiate the S-phase of the cell cycle. Although these cell cycle regulatory mechanisms have been identified recently, the resultant events mediated by $p21^{sdi1}$ for senescence induction is presently unclear. Our study has indicated that $p21^{sdi1}$ activated a commitment to the progression of a senescence program even in Saos-2 human osteosarcoma cells with homozygously deleted *p53* and *Rb* genes. It has been reported that $p21^{sdi1}$ could inhibit E2F activity by direct means in cells lacking a functional Rb protein,[33] suggesting that the Rb protein is not essential for E2F-dependent transcription and that $p21^{sdi1}$ may directly act on E2F activity in Saos-2 cells.

The role of telomeric attrition in $p21^{sdi1}$-mediated senescence was studied in our model, because telomere removal might result in replicate senescence. Our results indicate that cells showing the senescence phenotype following the $p21^{sdi1}$ gene transfer expressed quite low levels of telomerase activity. We and others[34,35] have demonstrated that although telomerase activity is regulated in the cell cycle-dependent manner, withdrawal from the cell cycle alone does not inhibit its activity, suggesting that $p21^{sdi1}$-mediated G1 arrest is not the cause of anti-telomerase effect. Several reports have shown that telomerase activity is downregulated during differentiation in some cell types.[36–40] The fact that $p21^{sdi1}$ is related to differentiation[11] may indicate a direct link between $p21^{sdi1}$ expression and inhibition of telomerase activity, although this remains to be proved by further studies.

The present study shows that the introduction of the $p21^{sdi1}$ gene into human cancer cells can revert their immortal phenotype and cause a cellular senescence. Recently, Brown *et al*[41] reported that inactivation of $p21^{sdi1}$ by targeted homologous recombination caused the bypass of senescence in normal human fibroblasts and that $p21^{sdi1}$ is a key regulator of senescence. Taken together, these data strongly suggest a novel function of the $p21^{sdi1}$ gene as a causal gene of cellular senescence. Senescence is an important tumor suppressive mechanism and the results reported here may provide a clue to a successful senescence-directed cancer therapy.

Materials and Methods

Cells and culture conditions

The following cell lines were used: a human non-small cell lung cancer cell line H1299, which has homozygously deleted *p53*; a human colon carcinoma cell line DLD-1, which exhibits a homozygous *p53* gene mutation; and a human colon carcinoma cell line LoVo, which contains wild-type *p53*.[42] These cells were routinely propagated in monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. The human osteosarcoma cell line Saos-2, which has homozygously deleted *p53* and *Rb*, was grown in monolayer culture in Dulbecco's-modified Eagle's Medium (D-MEM) (Gibco, Grand Island, NY, USA) with low glucose supplemented with 10% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. The transformed embryonic kidney cell line 293 was grown in D-MEM with high glucose (4.5 g/l), supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The 293 cells were used for the production of adenovirus vectors.

Recombinant adenoviruses

The recombinant adenoviral vector expressing human $p21^{sdi1}$ gene was previously constructed and characterized.[28,43] Briefly, the plasmid containing the cytomegalovirus promoter, the wild-type human $p21^{sdi1}$ cDNA, and the SV40 polyadenylation signal was cotransfected with pJM17 into 293 cells by liposome/DNA coprecipitation to generate an adenoviral $p21^{sdi1}$ expression vector. The resultant virus was named AdCMVp21. An adenoviral vector containing luciferase cDNA (AdRSVLuc) was used as a control vector. Culture supernatant of the viral stocks were quantified by a plaque-forming assay using 293 cells. The viruses were stored at -80°C.

Western blot analysis

Cells were collected by trypsinization and washed twice in cold phosphate-buffered saline (PBS). Cells were then lysed in SDS solubilization buffer (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 5% β-mercaptoethanol, and 2% SDS; Sigma, St. Louis, MO, USA). Equal amounts of proteins were boiled for 5 min and electrophoresed under reducing conditions on a 12.5% (w/v) polyacrylamide gel. Proteins were electrophoretically transferred to a Hybond-polyvinylidence difluoride (PVDF) transfer membrane (Amersham, Arlington Heights, IL, USA), and incubated with primary antibody against p21/WAF1 (Ab-1) (Oncogene Science, Manhasset, NY, USA), PAI-1 (Ab-1) (Calbiochem, Cambridge, MA, USA) and actin (Amersham) followed by a peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent Western system (Amersham) was used to detect secondary probes.

Flow cytometric analysis

Trypsinized cells were collected, washed twice with cold PBS, and resuspended in PBS containing 0.1% Triton X-100 and 1 g/l RNase for 5 min at room temperature. Samples were then stained with propidium iodide at 50 μg/ml and analyzed in a cell sorter (FACScan, Becton Dickinson, Mountain View, CA, USA) for DNA content. Cell debris and fixation artifacts were gated out, and G_1, S, and G_2/M populations were quantified using the ModFit LT program for Mac Ver. 1.01 (Verity Software House, Inc.).

Senescence-associated β-galactosidase (SA-β-gal) staining

Senescence-associated β-galactosidase (SA-β-gal) was detected as previously described with slight modifications.[16] Cells were washed once with PBS, fixed for 15 min at 4°C in 2% formaldehyde/0.2% glutaraldehyde, washed four times and incubated with SA-β-gal staining solution: 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal) per ml/40 mM sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$ overnight at 37°C without CO_2.

Telomerase assays

Telomerase assays were assayed by the PCR-based telomeric repeat amplification protocol[18,19] using a TRAP-eze telomerase detection kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cellular extracts from H1299, DLD-1, and LoVo were diluted to 100 ng/μl, and those from Saos-2 were diluted to 750 ng/μl. Two μl of each cellular extracts were subjected to TRAP assay using [γ-^{32}P] dATP (4500 Ci/mmol; ICN, Costa mesa, CA, USA). After the TRAP reaction, samples were loaded on 10% polyacrylamid gel, and those products were visualized by PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) or SYBR Green I staining (Molecular Probes, Eugene, OR, USA). Radioactive signal was quantitated using ImageQuant software (Molecular Dynamics) and the relative intensity was calculated as a ratio of TRAP product bands to an internal control band. These values were expressed in terms relative ratio in untreated control cells, which was arbitrarily set at 1 (Figure 5A, lower panel).

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