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SELECTIVE REPRESSION OF GROWTH-REGULATING cdk2, cyclin E AND E2F1 GENES IN HUMAN CELL SENESCENCE

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INDEXING WORDS

cell senescence; cyclin E; cdk2; E2F1; pRB

SYNOPSIS

Normal human diploid TIG-1 fibroblasts underwent replicative senescence around 64-68 population doubling levels (PDL) by the irreversible serum-unresponsive G1-growth arrest. Repression of growth-promoting genes was searched in this study. The RT-PCR and Western blot analyses have shown that in senescent TIG-1 cells at PDL64-67, cdk2 and cyclin E were selectively repressed at the mRNA and protein levels even after serum stimulation, and cdc2 and cyclin A were less repressed than cdk2 and cyclin E. Such a specific lack of cdk2 and cyclin E proteins correlated with unphosphorylation of the retinoblastoma gene product (pRB) in senescent cells. Transcription factor E2F1 was also completely repressed at the mRNA and protein levels in senescent TIG-1 cells. Middle-passage cells exhibited active expressions of all the above genes and pRB phosphorylation. Therefore, the present results have indicated the selective repressions of cdk2, cyclin E and E2F1 in senescent cells.

INTRODUCTION

Normal human diploid cells in culture invariably undergo replicative senescence, whereby the cells stop DNA synthesis and proliferation at the G1/S boundary of the cell cycle.[4,5,17] At the cellular level, senescence in culture

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reflects aging in vivo.[4,5,17] Mechanisms of replicative senescence resembling a process of terminal differentiation[1] are not fully clear. Senescent cells cannot be stimulated to enter the S phase by any combination of growth factors or physiological mitogens.[5] Seshadri and Campsi[17] first indicated selective repression of immediately early c-fos gene in senescent cells even after serum stimulation, and later, decreases in expressions of c-jun and jun-B (AP-1 activity) were found.[8,13] In addition, unphosphorylation of the retinoblastoma gene product (pRB) was demonstrated in G1-arrested senescent cells.[18] In this respect, unphosphorylated pRB interacts with the pRB-control element (RCE) of the c-fos promoter and represses the c-fos transcription,[14] and the pRB/E2F (transcription factor) complex represses several genes (dhfr, thymidine kinase, DNA polymerase α, cdc2, cyclin A, etc) required for the S-phase entry and progression.[6,7]. However, little is known about the E2F expression in senescent cells. Regarding cyclins and cyclin-dependent kinases (cdks), the cyclin A, cyclin B, cdc2 and cdk2 genes are significantly repressed in senescent cells.[12,19] However, the products of these genes are not directly related with the Go/G1 to S transition. The cdk4/cyclin D and cdk2/cyclin E kinases are thought to act differentially on the Go to G1 and G1 to S transitions, respectively.[2,11,16] Further, the cdk2/cyclin E activity is responsible for pRB phosphorylation related with the G1 to S progression in cycling cells.[2,11] A recent study has shown a great reduction of cyclin E/cdk kinase activity by the selective repression of cdk2 and cdk4 rather than cyclins in late-passage WI-38 cells.[12] Molecular mechanisms of senescent G1-growth arrest remain yet to be further studied.

We attempted to elucidate the repressions of cyclin E, cdk2 and E2F1 genes in late G1 and their relation to unphosphorylation of pRB in senescent TIG-1 fibroblasts.

MATERIALS AND METHODS

Cells and Culture TIG-1 normal embryonic lung fibroblasts were cultured in Eagle's MEM with non-essential amino acids and 10% fetal bovine serum (FBS). For exact population doublings (PDL), a fixed initial number (= *No*) of 2,000 cells/cm^2 in 35- or 50-mm dish was inoculated, and the number of cells after 6-7 day-growth (= *N*) was calculated for assessing PDL (*xi*) per each passage by an equation, $xi = log(N/No)/log2$, followed by estimation of the maximum cumulative PDL for senescence by Σxi[3]. At various PDLs until senescence, cloning efficiency of cells, based on the ability of forming colonies with 50 or more cells ($xi \geq 6$), was also determined for assessing decrease in replicative potential with PDL. The 1:2 split passage of mass culture was also adopted for rough PDL and molecular studies.

Anti BrdU assay for DNA-synthesized cells Middle-passage and senescent cells made quiescent by FBS starvation for 72 h were growth-stimulated by addition of 10% FBS and 10μM BrdU. After incubation for 28 h, a fraction of DNA-synthesized cells was determined on 1,000 cells by the anti BrdU staining method.

Western blot analysis of cell-cycle gene products Actively growing middle passage cells (PDL23-37) and growth-arrested senescent cells (PDL64-68) were lysed in NET or RIPA buffer.[15] Lysates were centrifuged at 14,000g for 10 min by a microcentrifuge. For Western blot, an equal aliquot (25-75μg) of the lysate protein per lane was electrophoresed in 4-20% gradient SDS-PAGE, and transferred to PVDF membrane. For immunoprecipitation, 50-100μg precleared lysate was incubated with specific antibody (Ab) (see below) for ≥ 4 h at 4℃, and the immune complexes bound to protein A-sepharose (for rabbit Ab) or protein A/G-sepharose (for mouse Ab) were collected for SDS-PAGE. Abundance of cyclins (A, E), cdks (cdc2, cdk2) or E2F1 was measured using the respective specific antibodies [rabbit anti human cyclin-A (Oncogene Science) and -E (UBI); rabbit anti human cdc2 and cdk2 (UBI) and rabbit anti human E2F1 (UBI)]. Anti pRB (mouse monoclonal Ab; Oncogene Science)-immunoprecipitated pRB was also analyzed for 116 kD phosphorylated form (pRB^{116}) and ~110 kD unphosphorylated form (pRB^{110}). The alkaline phosphatase chemiluminescence detection was used for visualization.

RT-PCR Total RNA was extracted from middle-passage and senescent cells using 4 M guanidium isothiocyanate and 5.7 M diethyl pyrocarbonate (DEPC)-treated CsCl. First, oligo$(dT)_{18}$-primed synthesis of the first strand cDNA (reverse transcripts; RT) was performed in a 40μl reaction buffer containing 10 μg RNA and RNase H-defective Superscript™ II reverse transcriptase (GIBCO-BPL) according to the manufacturer's manual. A 2.5-5.0μl aliquot of RT was used for PCR (20-26 cycles) using a Takara PCR kit to quantitate transcripts. PCR primers and annealing temperatures were searched by the Oligo Primer Analysis software (National Biosciences). The 5'- and 3'-PCR primers used in this order were: cyclin A: 5'-AGAATATCAACCCGGAAAAGG, 5'-GGCTGTTT CTTCATGTAAACC (product length, 521 bp); cyclin E: 5'-TATCCCCAGCAAATC TT, 5'-GAAGGATGGGGTGGTCA (length, 440 bp); cdc2: 5'-TTCCATGGATCT GAAGAAAGAA, 5'-GCCTATACTCCAAATGTCAAC (length, 450 bp); cdk2; 5'-G AGAACTTCCAAAAGGTGGAA, 5'-CAGCAGCTTGACAATAATAGG (length, 487 bp); E2F1: 5'-CAGGAGGTCACTTCTGA, 5'-CCCTGTCAGAAATCCAG (length , 480 bp). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (product length, 550 bp) was also amplified as a reference marker of cell cycle-independent transcripts. The primers were synthesized by a Model 391 DNA synthesizer

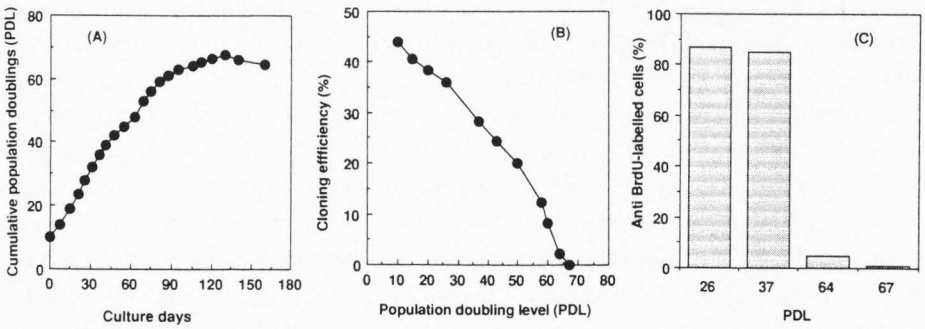

Fig. 1. TIG-1 cell growth and replicative senescence. (A) Cumulative PDL (Σ xi) against days in culture with 10% FBS. Replicative lifespan was PDL68 around 130 days where cells senesced. (B) Cloning efficiency against PDL. A linear decline of replicative potential was -0.6%/PDL with an abrupt loss at senesced PDL60-68. (C) Anti BrdU-stained DNA synthesized cells (see Methods) in actively growing PDL27 and 37 and senescent PDL 64 and 67.

(Applied Byosystems) and purified.

RESULTS AND DISCUSSION

Replicative lifespan and senescence of TIG-1 cells

Fig. 1A plots the cumulative PDL of TIG-1 cells starting with PDL10 against days in culture with 10% FBS. The limited replicative lifespan of TIG-1 cells due to senescent growth arrest was about 68 PDL around 130 days in culture. Thereafter, some fraction of differentiated epithelioid cells survived longer without dividing, but the other died (Fig. 1A). Fig. 1B shows that cloning efficiency declined linearly with progress of PDL at a rate of -0.6%/PDL, indicating the continuous appearance of growth-arrested senescent cells. At the end of lifespan (PDL64-68), almost all cells became senescent by G1-arrest, since DNA-synthesized cells after incubation in 10% FBS and 10 μM BrdU following starvation were 0.4-4 %, compared to ~85% for middle-passage PDL26 and 37 cells when assayed by the anti-BrdU staining method (Fig. 1C). The main cellular senescent phenotype is the FBS-unresponsive, irreversible G1-growth arrest .

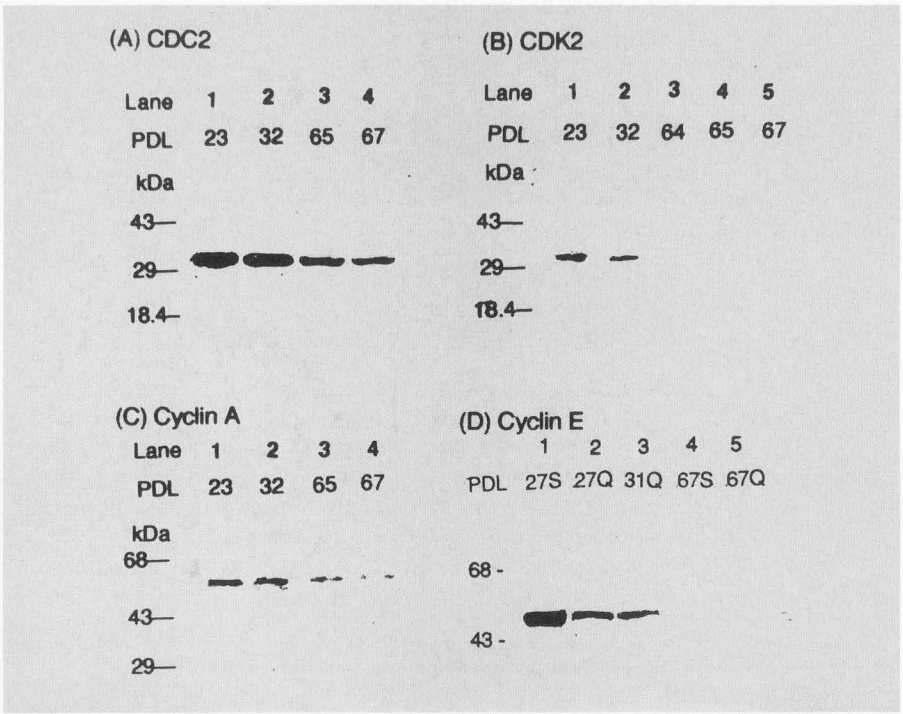

Fig. 2. Western immunoblots for cell-cycle gene products. (A) cdc2 (50 mg lysate); (B) cdk2 (50 mg lysate); (C) cyclin A (30 mg lysate); (D) cyclin E (50 mg lysate). Each lane was specified with the PDL used. Postscripts S and Q of the PDL number in (D) are for FBS-stimulated and quiescent cell-lysates, respectively.

Repression of cdk and cyclin proteins in senescent cells

To elucidate some basic molecular mechanisms for the senescent G1-arrest, we have studied the expression of cell cycle genes especially in the late G1 and G1/S transition phases. For that purpose, middle-passage cells were made quiescent by FBS starvation for 72 h, and then cell growth was stimulated by addition of 10% FBS. Middle-passage cells progressed to late G1 and early S phases around 18 h after stimulation (unpublished data). Senescent cells were treated similarly.

First, Fig. 2 indicates the Western blot analysis for protein levels of pertinent cdks (cdc2 and cdk2) and cyclins (cyclin A and cyclin E) using the respective specific antibodies. Proteins of cdk2 (Fig. 2B) and late-G1 cyclin E (Fig. 2E)

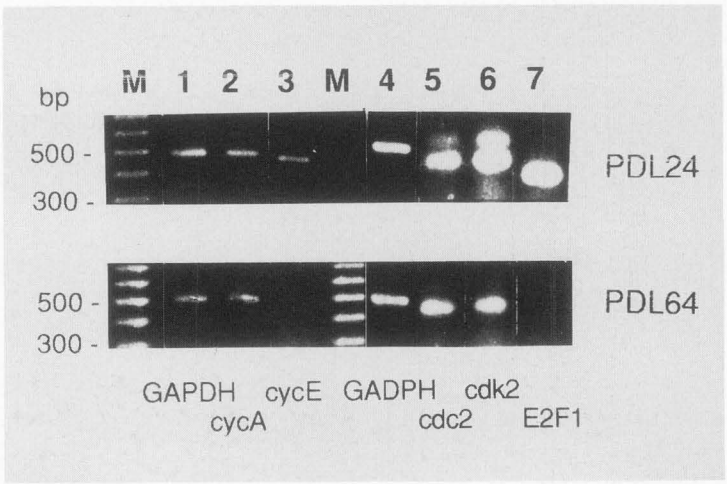

Fig. 3. RT-PCR. *Upper*, RNA from FBS-stimulated PDL24 cells; *lower*, RNA from FBS-stimulated PDL64 cells. Lanes: M, 100 bp ladder marker; 1 and 4, GAPDH; 2, cyclin A; 3, cyclin E; 5, cdc2; 6, cdk2; 7, E2F1. The RT products used were 2.5 ml for lanes 1 to 3 (20 cycles), and 5.0 ml for lanes 4 to 7 (26 cycles).

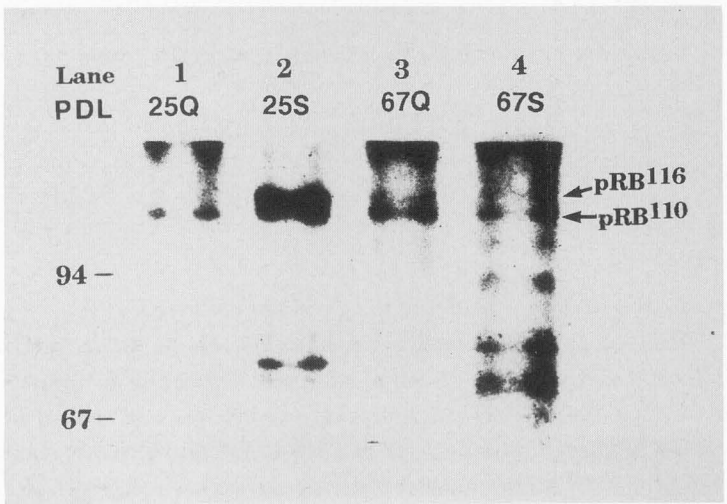

Fig. 4. SDS-PAGE for pRB. The 50-mg precleared lysates of PDL25 and PDL67 cells were immunoprecipitated with mouse monoclonal anti human pRB and protein A/G-sepharose, and electrophoresed in 4-20% SDS-PAGE. Postscripts Q and S indicate "quiescent" and "FBS-stimulated for 24 h", respectively. pRB^{116}, phosphorylated pRB; pRB^{110}, unphosphorylated pRB.

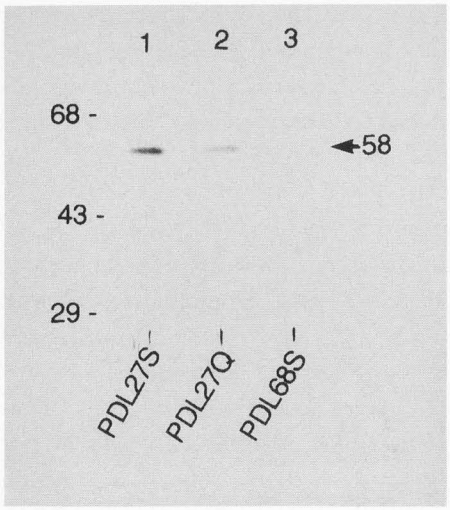

Fig. 5. Western blot for 58 kD E2F1 protein using anti E2F1. Lysates (60 mg) of PDL27Q (quiescent), 27S (FBS-stimulated for 18 h), and senescent PDL68S cells were electrophoresed and blotted with anti E2F1.

were not detected in both FBS-starved and -stimulated senescent PDL64-67 cells under the conditions. High level expressions of both proteins were observed in middle-passage PDL23-32 cells around late-G1/early-S after FBS-stimulation for 18 h (Fig. 2B and 2D). As expected, abundance of cyclin E in FBS-starved quiescent PDL27 and PDL31 cells (Fig. 2D, lanes 2 and 3) was ~1/3 of that in FBS-stimulated middle-passage PDL27 cells at the late-G1/early-S boundary (Fig. 2D, lane 1).

The cdc2/cyclin A kinase activity has been shown to be required for the S-phase progression.[2,11,16] cdc2 (Fig. 2A, lanes 3 and 4) and cyclin A (Fig. 2C, lanes 3 and 4) were still present in FBS-stimulated senescent PDL65 and PDL67 cells at a low level of 1/3~1/4 of those in middle-passage PDL23 and PDL32 cells (Figs 2A and 2C, lanes 1 and 2). Both proteins were less repressed compared to cdk2 and cyclin E in senescent TIG-1 cells. Thus, Fig. 2 indicates the selective repressions of cdk2 and late-G1 cyclin E in senescent cells. In this respect, a great reduction in the cdk2/cyclin E kinase activity has been reported in late-passage WI-38 cells with less than 2% anti-BrdU-positive cells.[12]

Fig. 3 indicates that an amplified band of cyclin E mRNA by the sensitive RT-PCR was not found in FBS-stimulated senescent PDL64 TIG-1 cells, while it was present in PDL24 cells. At PDL24, amounts of PCR products for cyclin A, cyclin E, cdc2, and cdk2 mRNAs were several-fold higher in FBS-stimulated than quiescent cells (data not shown). Compared to PDL24 cells, however, more decreased amplifications of cyclin A, cdc2 and cdk2 mRNA were observed in senescent PDL64 cells, despite the similar degree of amplification of GADPH in

PDL24 and 64 (Fig. 3). These results are consistent with the previous.[12,19] Such RT-PCR data support our findings at the protein level in Fig. 2. Fig. 3 indicates that senescent cells undergo the selective transcriptional repressions of cyclin E and reduced expressions of the cdk2 and cdc2 genes. Such a feature is important for the irreversible G1-arrest in senescent cells.

Unphosphorylation of pRB in senescent cells

pRB phosphorylation by cdk/cyclin kinases allows G1 cells to enter and progress through S phase.[6] At least the cdk2/cyclin E kinase activity in late-G1/early-S phosphorylates pRB.[6] Stein et al.[18] have first indicated unphosphorylation of pRB in senescent human diploid MRC-5 cells. In this respect, almost all of pRB in FBS-starved quiescent PDL25 TIG-1 cells arrested in Go/G1 were un- or hypophosphorylated (pRB^{110}) (Fig. 4, lane 1), while PDL25 cells in late G1 and S phases after a 24 h FBS stimulation had both of phosphorylated pRB^{116} and unphosphorylated pRB^{110} (Fig. 4, lane 2). In senescent PDL67 cells, pRB^{110} predominated in both FBS-starved (Fig. 4, lane 3) and -stimulated states (Fig. 4, lane 4), as reconciled with Stein et al.[18] Therefore, the specific lack of cdk2 and cyclin E and a great suppression of cdc2 and cyclin A (Figs. 2 and 3) may cause hypo- or unphosphorylation of pRB in senescent cells.

Repression of transcription factor E2F1 in senescent cells

E2F1 of the E2F family members is an important cell cycle regulatory transcription factor.[6,7,9,10]. The E2F1 protein recognizes and binds to the E2F1-recognition sequences of TTT(G/C)(G/C)CG(G/C) in the promoters of several cell-cycle genes such as dhfr, E2F, DNA polymerase α, thymidine kinase, thymidylate synthetase, c-myc, c-fos, c-myb, cyclin A, cdc2 and RB[6,7,9,10] for their transactivation. It has been demonstrated that E2F1 forms complexes with unphosphorylated pRB by specific binding to the pRB "pocket" domain and with other cellular proteins (p130, p107) in mid- and late-G1.[7,10] The E2F1/pRB complex in late G1 acts as a transcriptional repressor for the above cell cycle genes to arrest cells in G1.[6,7,9,10] However, SV40 T-antigen, adenovirus E1A and human papilloma virus E7 in virally transformed cells disrupt the E2F1/pRB complex[6,7,9,10] to release free active E2F1 for transactivation of the above cell cycle genes and for cell growth. Therefore, it is of primary importance to study whether or not and to what extent growth-arrested senescent diploid cells express E2F1, because little is known about E2F1 in senescent cells.

Fig. 3 shows that a 480 bp-product for E2F1 mRNA by RT-PCR was not observed in FBS-stimulated senescent PDL64 cells, in contrast to a high PCR

signal for middle-passage PDL24 cells. The Western blot analysis indicates clearly the absence of 58 kD E2F1 even in FBS-stimulated senescent PDL68 cells (Fig. 5, lane 3). The E2F1 protein expression was greater in middle-passage PDL27 cells at the G1/S boundary after FBS stimulation for 18 h (Fig. 5, lane 1) than in FBS-deprived quiescent state (Fig. 5, lane 2). Therefore, transcriptional repression of E2F1 will be related to permanent block of the S-phase entry in senescent cells. In senescent TIG-1 cells, pRB^{110} exists at a nearly normal level (Fig. 4) in the absence of E2F1 (Fig. 5). Cellular molecules other than E2F1 which bind to pRB and result in its unphosphorylation in senescent cells are currently unknown. Nevertheless, the extremely low cdk2/cyclin E activity is most likely to be responsible for unphosphorylation of pRB in senescent cells. In addition to a decrease in the AP-1 activity,[8,13] low levels of E2F1 and cdk2/cyclin E constitute an important characteristic molecular environment for cellular senescence featuring permanent G1-growth arrest. Cell aging may involve both repression of such growth-regulating genes and the reciprocal expression of anti-proliferating genes. Studies on the latter are progressing worldwide.

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