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COOPERATIVE EFFECT OF ANTISENSE-Rb AND ANTISENSE-p53 OLIGOMERS ON THE EXTENSION OF LIFE SPAN IN HUMAN DIPLOID FIBROBLASTS, TIG-1

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SUMMARY: Normal human diploid fibroblasts, TIG-1, which have a replicative life span of about 62 population doublings (PD), tended to senesce after about 50 PD with a gradual decrease in sensitivity to serum. Treatment of TIG-1 cells with the antisense-Rb oligomer, which completely depleted the retinoblastoma susceptibility gene product (RB), extended life span by about 10 PD. Treatment with the antisense-p53 oligomer alone had no effect; however, cotreatment with the antisense-Rb oligomer further potentiated the extension and the increased sensitivity to serum caused by the antisense-Rb oligomer alone, suggesting that p53 and RB function in separate, yet complementary pathways in signal transduction to senescence. The c-*fos* expression, which is presumed to be regulated negatively by RB, was not stimulated in partially senescent TIG-1 cells by treatment with the antisense-Rb oligomer.

Human diploid fibroblasts (HDF) have limited life span and cease their proliferation after 20 to 60 PD (1) in G1 phase. The senescent cells are no longer responsive to any mitogen (2). The loss of proliferative potential seems to be caused by the presence of one or more inhibitors of entry to S phase, since the senescent phenotype is dominant over the proliferative phenotype in dikaryons formed between young and senescent HDF (3) and microinjection of mRNA from senescent HDF into young fibroblasts inhibits the ability of young cells to synthesize DNA (4). The viral oncoproteins encoded by DNA tumor viruses, SV40 large T antigen, adenovirus E1A and human papillomavirus E7 have ability to immortalize primary

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Abbreviations: HDF, human diploid fibroblast; PD, population doublings; RB, retinoblastoma susceptibility gene product; PDL, population doubling level; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; HPV, human papillomavirus.

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rodent cells. The immortalization has been assumed to occur by an interaction of these oncoproteins with the products of tumor suppressor genes such as RB and p53, thereby inactivating these negative factors for cell proliferation (5). HDF, however, could not be immortalized by these viral oncogenes but were conferred the extended life span by about 20 PD (6,7). Involvement of RB in the process of cellular senescence has recently been reported. The senescent HDF were unable to phosphorylate RB, which is presumed to be required for entry to S phase (8). However, direct involvement of RB and p53 in the control of replicative life span has not yet been demonstrated. Here, we report the direct involvement of these tumor suppressor genes and their cooperative functions in the process of cellular senescence.

MATERIALS AND METHODS

Cell Culture: Normal human diploid fibroblasts, TIG-1 (9) were cultivated at 37°C in DMEM with 10% FCS that was heated at 60°C for 1 h to reduce nuclease activity.

Preparation of oligodeoxynucleotides: Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. To overcome degradation of oligonucleotides by nuclease, the oligonucleotides on the back bone were modified to phosphorothioate (10). Phosphorothioates were synthesized according to the H-phosphonate procedure and purified by ion-exchange chromatography. The sequences of oligomers designed to recognize 15 nucleotides from the translation initiation codon (ATG) were as follows: antisense Rb (5'-GGTTTTGGGCGGCAT-3'), antisense p53 (5'-CTGCGGCTCCTCCAT-3'), control sequence (5'-GGTGCATCAGGATTC-3').

Immunoprecipitation: Cells were labeled with 400 μCi/ml of [^{35}S] methionine for 4 h in methionine-free DMEM and lysed on ice for 15 min with 1 ml of lysis buffer (10 mM Tris-HCl (pH7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 4°C at 16,000 xg for 30 min and supernatants were collected. Each supernatant was incubated with 20 μl of normal rabbit serum at 0°C for 1 h and the complex formed was removed by centrifugation after addition of 50 μl of 10 % formalin-fixed protein A. Aliquot of one fourth of the supernatant was incubated with rabbit anti-RB serum or mouse anti-p53 monoclonal antibody (PAb421) at 0°C for 4 h. The immunoprecipitate was dissolved in loading buffer and subjected to electrophoresis on a SDS-10 % polyacrylamide gel.

RNA dot hybridization: Cells grown in 24-well microtiter plates were lysed in 2 M guanidinium isothiocyanate lysis buffer and cellular RNA was transferred directly to nylon filters. Hybridization was performed with ^{32}P-labeled human c-*fos* cDNA.

RESULTS AND DISCUSSION

To investigate the role of the Rb and p53 genes in senescence of HDF, TIG-1 which have life span of about 62 PD (9), the effects of antisense oligodeoxynucleotides to Rb and p53 transcripts (anti-Rb and anti-p53 oligomers) on the response of TIG-1 cells at different PDL to serum factors were analyzed by labeling the cells with [^{3}H]thymidine. Subconfluent cultures of TIG-1 cells at PDL 46, 53, 56 and 58 were made quiescent by maintaining the cells in low serum medium for 4 days in the presence of the anti-p53 oligomer or the anti-Rb oligomer or both at a final concentration of 200 μg/ml. The quiescent cells were then serum-stimulated, labeled with [^{3}H]thymidine at 6 h intervals and the radioactivity incorporated into acid-insoluble fraction was measured. In TIG-1 cells at PDL 46 not treated with the antisense oligomer (Fig.1A), DNA synthesis began to be observed 10 to 12 h after

addition of serum and reached its maximal rate after 24 h. The same pattern of DNA synthesis was observed with young TIG-1 cells at PDL 30 (data not shown). In TIG-1 cells at PDL 53 and 56, the maximal rate of DNA synthesis was reached after 36 h and was lower than half of that attained in TIG-1 cells at PDL 46. Senescence of TIG-1 cells became apparent around PDL 50. In TIG-1 cells at PDL 58, DNA synthesis was induced very poorly as a result of reaching crisis.

Induction of DNA synthesis by serum in these TIG-1 cells treated with the anti-p53 oligomer is shown in Fig.1B. The patterns of DNA synthesis induced were nearly identical with those induced in the untreated cells indicating that depletion of p53 alone has no effect on the response of TIG-1 cells to serum. Treatment of TIG-1 cells at PDL 46 with the anti-Rb oligomer showed no significant effect on the pattern of DNA synthesis (Fig.1C). However, in TIG-1 cells at PDL 53 and 56 treated with

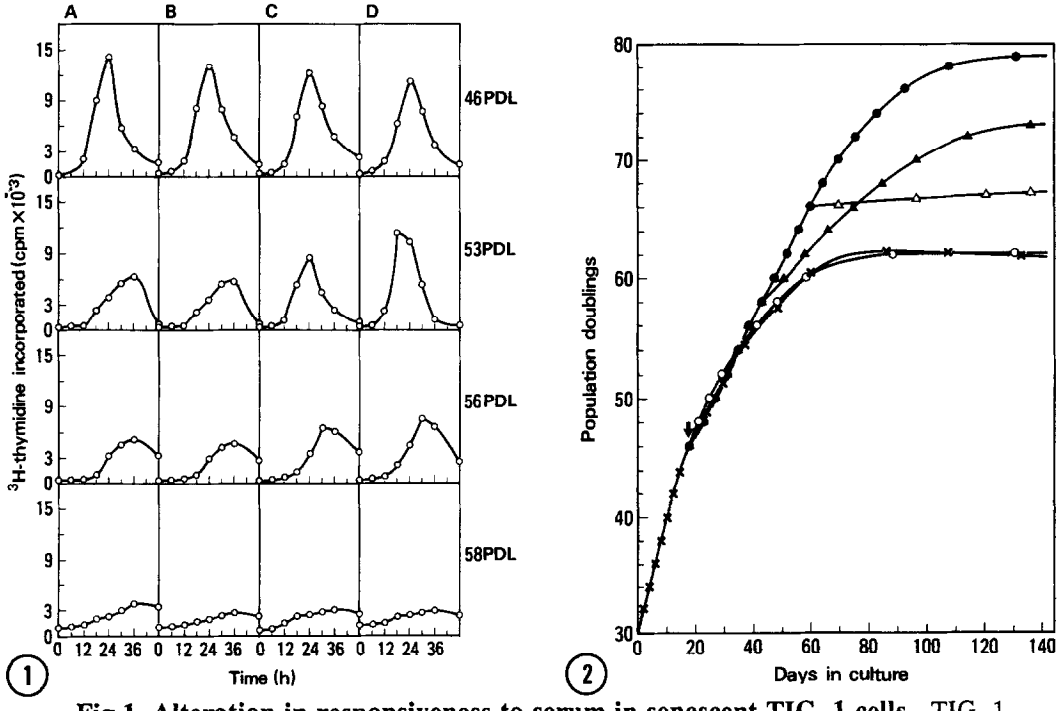

Fig.1. Alteration in responsiveness to serum in senescent TIG-1 cells. TIG-1 cells at PDL 46, 53, 56 and 58 were cultivated in 48-well microtiter plates at 37°C. The medium was replaced with low serum (0.5% FCS) medium when the cells had reached subconfluence and 120 µg/ml of the anti-p53 oligomer (B), the anti-Rb-oligomer (C) or both (D) were added. Additional 20 µg/ml of these oligomers were added daily to make a final concentration to 200 µg/ml (35 µM). (A) The cells were not treated with antisense oligomers. The quiescent cells were serum-stimulated by changing the medium with the fresh one containing 20 % FCS and 200 µg/ml of the same antisense oligomers. At the times indicated, the cells were labeled with 10 µCi/ml of [^{3}H]thymidine for 1 h and TCA insoluble radioactivity was counted.

Fig.2. Extension of TIG cell life span by anti-Rb and anti-p53 oligomers. TIG-1 cells were cultivated in 96-well plates as described in Fig.1 and the medium was changed every 3 days. The cells were replated when they had reached confluence and PD were calculated after each passage. At PDL 46 and each cell passage, 200 µg/ml (35 µM) of the anti-p53 oligomer (○), the anti-Rb oligomer (▲) or both (●) were added to the cultures and additional 33 µg/ml of the same oligomers were added daily. The control culture not treated with the oligomers (×). At PDL 66, the antisense oligomers were removed from aliquots of the culture which had been treated with both oligomers and maintained in fresh medium not containing these oligomers (△). The results are presented as an average of three cultures.

the anti-Rb oligomer, the maximal rate of DNA synthesis was reached 12 h earlier than that reached in the untreated cells and the level was increased by 30 to 40 %. The results suggest that the frequency of reentry to G1 phase from quiescence and/or the frequency of entry to S phase through G1 was increased and a fraction of cells withdrawn from cell cycle was decreased by depletion of RB. No significant increase in the rate of DNA synthesis was observed in TIG-1 cells at PDL 58 by the anti-Rb oligomer treatment indicating that TIG-1 cells at this stage were no longer respondent to external stimuli.

The increase in the rate of DNA synthesis was marked when TIG-1 cells were treated with both anti-p53 and anti-Rb oligomers (Fig.1D). In TIG-1 cells at PDL 53, the maximal rate of DNA synthesis was reached in a much shorter time and was comparable to that reached in younger TIG-1 cells at PDL 46. An increased stimulation of DNA synthesis was also observed in TIG-1 cells at PDL 56, although the response was decreased. The cooperative effect of the anti-p53 oligomer with the anti-Rb oligomer suggests that p53 modulates the function of RB to suppress cell proliferation, since depletion of p53 alone had no effect. The addition of these oligomers to the culture of TIG-1 cells expressing SV40 T antigen had no effect on the serum-induced DNA synthesis (data not shown).

The effect of the anti-p53 and the anti-Rb oligomers on the extension of life span was subsequently analyzed. Young TIG-1 cells at PDL 30 were propagated successively and the culture was divided into four groups at PDL 46. The anti-p53 oligomer and/or the anti-Rb oligomer were added daily to aliquots of these cultures and the number of cells propagated was counted (Fig.2). The addition of the anti-p53 oligomer did not extend the life span and the cells entered crisis at PDL 60 to 62 just as did the untreated TIG-1 cells. In contrast, daily addition of the anti-Rb oligomer extended the life span by more than 10 PD and the cells gradually entered crisis at about PDL 72. The simultaneous addition of the anti-p53 and the anti-Rb oligomers extended the life span further by about 20 PD and the cells entered crisis at PDL 78 to 80. This cooperative effect is consistent with that observed in the induction of DNA synthesis in senescent cells. Removal of these oligomers from an aliquot of the culture continuously fed with both oligomers at PDL 66 resulted in an immediate cessation of cell proliferation, suggesting that p53 and RB were acting as strong growth inhibitors in senescent TIG-1 cells.

The antisense oligomer is relatively stable in culture, and effectively inhibits translation of mRNA by forming the duplex after entering cells (11,12,13). To confirm the effect of the antisense oligomers, p53 and RB in the treated cells were analyzed by immunoprecipitation (Fig.3). TIG-1 cells at PDL 46 were cultured in the presence of increasing concentrations of the anti-Rb oligomer (Fig.3 a) or the anti-p53 oligomer (Fig.3 b) and labeled with [^{35}S]methionine. RB and p53 were immunoprecipitated with specific antisera against these proteins, and proteins eluted from the precipitate were analyzed by electrophoresis followed by autoradiography. Significant amounts of RB and p53 were immunoprecipitated from the untreated cell extract (Fig.3, lane 1). Similar amounts of RB and p53 were immunoprecipitated from senescent TIG-1 cells at PDL 56. Treatment of the cells with either of these antisense oligomers at a final concentration of 5 μM resulted in a reduction of these proteins immunoprecipitated (Fig.3, lane 2). At increasing concentrations of 35 and 50 μM, no RB and p53 were immunoprecipitated (Fig.3, lanes 3 and 4).

The serum-stimulation of senescent HDF induces the expression of a variety of growth related genes including c-*myc* and c-Ha-*ras* just as do quiescent young HDF (14) except for the c-*fos* expression (15). The results suggest that senescent

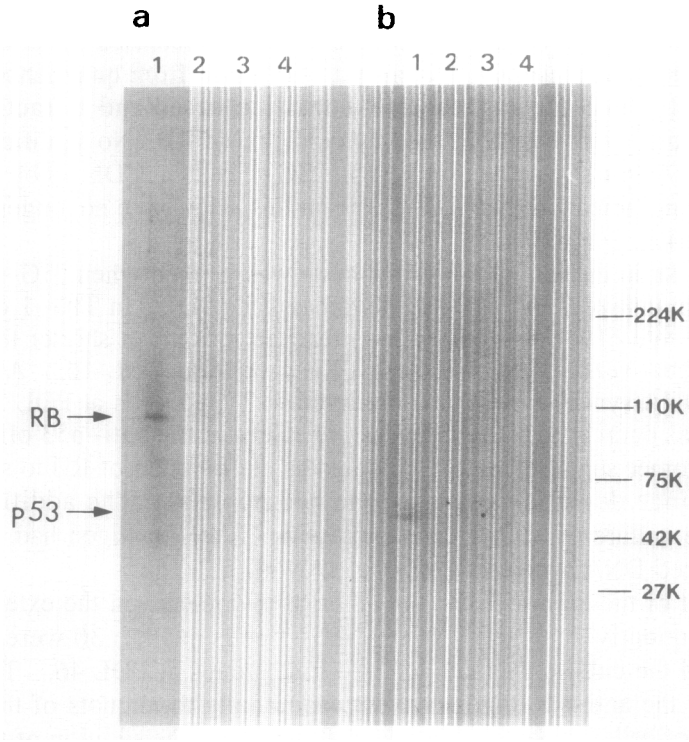

Fig.3. Immunoprecipitation of RB and p53 from TIG-1 cells treated with antisense oligomers. TIG-1 cells at PDL 46 were plated in 30 mm-dishes and increasing concentrations of the anti-Rb oligomer (a)or the anti-p53 oligomer (b) were added. Lane 1, 0 μM; lane 2, 5 μM; lane 3, 35 μM; and lane 4, 50 μM. One sixth amount of the same oligomer was added daily. After 4 days, the medium was changed with the methionine-free medium containing the same concentration of the antisense oligomer and labeled with 400 μCi/ml of [^{35}S] methionine.

fibroblasts are unable to enter S phase owing to, at least in part, the repression of c-*fos* expression. The repression seems to occur by a specific transcriptional block (15). In fact, the repression of c-*fos* expression by RB has recently been reported (16). To see a possible induction of the c-*fos* expression by the anti-Rb oligomer in senescent cells, TIG-1 cells at different PDL were treated with the anti-Rb oligomer and the induction of c-*fos* expression by serum was analyzed by RNA-dot hybridization. As shown in Fig.4 a, the c-*fos* expression was induced 1 h after serum stimulation in untreated quiescent TIG-1 cells at PDL 46 which still retained the ability to respond to serum. Similar level of c-*fos* expression was induced in the anti-Rb oligomer-treated cells (Fig.4 b). In senescent TIG-1 cells at PDL 53 and 58, no c-*fos* expression was induced irrespective of the treatment with the anti-Rb oligomer. Under these conditions, the levels of β-actin gene expression were nearly constant, although the results were somewhat variable. These results suggest that repression of the c-*fos* expression in senescent cells is not merely caused only by RB but caused also by a factor(s) other than RB and that the induction of c-*fos* expression is not directly related to the induction of DNA synthesis in senescent TIG-1 cells, since DNA synthesis was stimulated in TIG-1 cells at PDL 53 and 56 by the treatment with the anti-Rb oligomer (Fig.1C).

The cooperative effect of p53 and RB on the responsiveness of partially senescent TIG-1 cells to serum and on the extension of life span suggests that p53

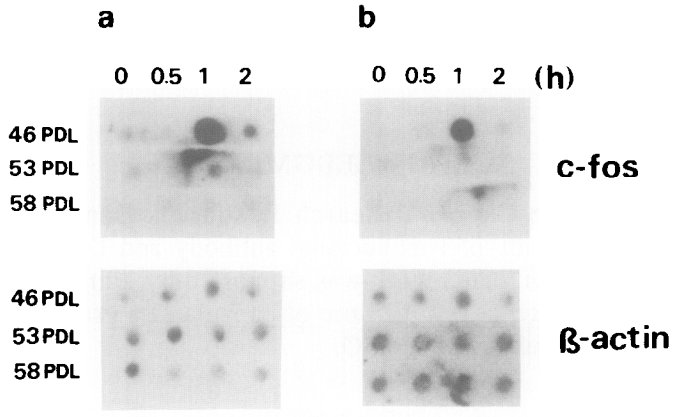

Fig.4. Expression of the c-*fos* gene in TIG-1 cells treated with the anti-Rb oligomer. TIG-1 cells at PDL 46, 53 and 58 were cultivated in 24-well plates and maintained in low-serum medium in the absence (a) or presence (b) of the anti-Rb oligomer at 200 µg/ml. After 4 days, the cells were serum-stimulated as stated in Fig.1. At the times indicated, the cells were lysed in 2 M guanidinium isothiocyanate lysis buffer and cellular RNA was transferred to the nylon filter. Filters were hybridized with ^{32}P-labeled c-*fos* cDNA. β-actin probe was used as a control.

and RB function in separate yet complementary pathways in signal transduction to senescence. This cooperation is reminiscent of recent reports on the modulation of the HPV16 E7 induced immortalization of baby rat kidney cells and human epitherial cells by p53 (17,18). The HPV16 E7, which is thought to inactivate RB by binding to it, can immortalize these primary cells without the cooperation of the viral E6 gene. However, coexpression of E6 significantly stimulates the E7 immortalizing function although E6 alone has no immortalizing activity (18). The HPV16 E6 binds to p53 and has recently been shown to degrade it (19).

RB and p53 may control cellular senescence at the G1/S boundary. Hypophosphorylated form of RB in G0 to G1 phase has been assumed to lose its negative role in cell proliferation upon phosphorylation at the G1/S boundary and enables late G1 cells to enter S phase (5). This is consistent with the finding that RB present in senescent HDF exclusively consisted of hypophosphorylated form (8). Similar cycle of hypophosphorylated and phosphorylated forms of p53 has been observed. Human p53 is phosphorylated by $p34^{cdc2}$ kinase at the G1/S boundary (20) presumably rendering late G1 cells to enter S phase and participate in the initiation of DNA synthesis.

Depletion of p53 and RB by antisense oligomers did not result in unlimited extension of TIG-1 cell life span just as did the SV40-transformed HDF (6). Extension of the life-span of human endothelial cells by an interleukin-1α antisense oligomer was also limited (21). The results suggest that cellular senescence is regulated at multiple steps in cell cycle and the loss of regulation at the G1/S boundary is not the sole step in cellular senescence. One of the properties of senescent cells is their inability to induce c-*fos* expression upon stimulation by mitogens (15). The property is quite contrast to that of quiescent cells. It has been recently reported that the human c-*fos* expression is negatively regulated by RB (16). However, the present result indicated that a complete depletion of RB do not stimulate the c-*fos* expression efficiently in senescent TIG-1 cells. The expression of c-*fos* seems to be negatively regulated by multiple factors including RB in senescent HDF.

Human cells seem to have two different mechanisms which decide their replicative life span, while rodent cells have only one (7). The negative regulator, RB and p53 may be involved in one of these restriction mechanisms common to human and rodent cells.

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