Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts

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ABSTRACT Human diploid fibroblasts (HDFs) can be grown in culture for a finite number of population doublings before they cease proliferation and enter a growth-arrest state termed replicative senescence. The retinoblastoma gene product, Rb, expressed in these cells is hypophosphorylated. To determine a possible mechanism by which senescent human fibroblasts maintain a hypophosphorylated Rb, we examined the expression levels and interaction of the Rb kinases, CDK4 and CDK6, and the cyclin-dependent kinase inhibitors p21 and p16 in senescent HDFs. Cellular p21 protein expression increased dramatically during the final two to three passages when the majority of cells lost their growth potential and neard senescence but p21 levels declined in senescent HDFs. During this period, p16 mRNA and cellular protein levels gradually rose with the protein levels in senescent HDFs reaching nearly 40-fold higher than early passage cells. In senescent HDFs, p16 was shown to be complexed to both CDK4 and CDK6. Immunodepletion analysis of p21 and p16 from the senescent cell extracts revealed that p16 is the major CDK inhibitor for both CDK4 and CDK6 kinases. Immunoprecipitation of CDK4 and CDK6 and their associated proteins from radiolabeled extracts from senescent HDFs showed no other CDK inhibitors. Based upon these results, we propose that senescence is a multistep process requiring the expression of both p21 and p16. P16 up-regulation is a key event in the terminal stages of growth arrest in senescence, which may explain why p16 but not p21 is commonly mutated in immortal cells and human tumors.

Concurrent work has recently shown an increase in p16 protein and mRNA in senescent human fibroblasts (18, 19) but it was not determined if this up-regulation resulted in significant CDK binding. In the present study, high cellular expression of p16 protein was found in multiple strains of senescent HDFs. Further, in a detailed analysis of the senescent process in MRC-5 fibroblasts, elevated p16 expression followed an increase in p21 expression. P16 was found in both CDK4 and CDK6 complexes and was bound to the majority of the CDK4 and nearly all of the CDK6 expressed in senescent MRC-5 fibroblasts. Immunoprecipitation of CDK4 and CDK6 and their associated proteins from radiolabeled extracts from senescent MRC-5 fibroblasts showed no other CDK inhibitors present, suggesting p21 and p16 are the molecules responsible for CDK4/6 inhibition. Finally, these results suggest that the growth arrest in senescence fibroblasts results from a multistep process with an increase in p21 expression as cells enter senescence followed by a sustained elevation in the level of p16 mRNA and protein.

MATERIALS AND METHODS

Cells and Culture Conditions. HDFs used in this study were a strain of HDF from newborn foreskin, NHF1, and three

Abbreviations: HDF, human diploid fibroblast; CDK, cyclin-dependent kinase; LI, labeling index; SV40, simian virus 40; Rb, retinoblastoma gene product; LS, low serum; HD, high density; MAP, mitogen-activated protein.

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strains from embryonic lung tissue, MRC-5, IMR-90, and WI-38, obtained from M. Cordiero-Stone (University of North Carolina, Chapel Hill), American Type Culture Collection, Olivia Pereira-Smith (Baylor College of Medicine, Houston) and American Type Culture Collection, respectively. GM639, an SV-40 immortalized human fibroblast line, was also obtained from O. Pereira-Smith. Cells were serially passaged twice weekly at early passage and at decreasing dilution with increasing age. Medium was changed weekly for senescent cultures. Proliferative potential of the culture was determined as the labeling index (LI) measured by the percentage of cells incorporating BrdUrd following a 48-hr incubation in complete medium containing 10 μM BrdUrd and was detected by immunocytochemistry using anti-BrdUrd antibody (Becton Dickinson catalog no. 347580). Cultures with a LI of <5% were considered senescent. Early passage cells were growth inhibited by three means: (i) growth to high density (HD) with continued cultivation for at least 3 additional days, (ii) incubation in medium containing 0.2% fetal bovine serum for 48 hr (low serum, LS), or (iii) exposure to 4 Gy of gamma-irradiation from a cesium source (Irrad).

Extract Preparation and Protein Analysis. Total cellular protein extracts were prepared by rinsing cultures with cold PBS, then scraping the cells directly into either 1× Laemmli sample buffer or a small volume of cold PBS with 1 mM sodium vanadate, and a protease cocktail consisting of 170 μg/ml phenylmethylsulfanyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 150 μg/ml benzamidine, and 10 μg/ml trypsin inhibitor. For cells scraped in PBS, concentrated SDS was added (final concentration of 1%) and the proteins were rapidly denatured by boiling. Viscosity was reduced by sonication. For extracts in PBS/SDS, protein concentrations were determined using the DC protein assay (Bio-Rad) and diluted with sample buffer. The total number of cells per ml in the final sample was determined by trypsinizing a companion plate and counting on a Coulter counter. Except where noted, all experiments represent analysis based on an equal number of cells.

Western blots were prepared by separation of proteins using the Laemmli discontinuous gel system, and transferred overnight to supported nitrocellulose (Schleicher & Schuell). The membrane was blocked with 5% skimmed milk and then incubated with appropriate primary antibodies for 1 or 2 h at room temperature. After three washes with Tris-buffered saline, blots were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive proteins were detected by chemilumino-graphy using ECL reagents (Amersham). The intensity of the bands was quantitated by densitometry with NIH Image software. In all cases, the exposure of the films to autoradiographic emulsion was standardized.

RESULTS

p16 and p21 Expression in Senescence HDFs. Based upon previous reports of increased expression of the CDK inhibitor p21 in senescent cells (9), the expression of additional inhibitors was examined in early passage and senescent fibroblasts of several strains of HDFs, NHF1, MRC-5, IMR-90, and WI-38. Total cellular protein increased 3- to 5-fold in senescent cells, which increase significantly in size (21). Therefore, we compared young vs. senescent cells on the amount of a given protein per cell (termed cellular level) based on the fact that subcellular localization by immunofluorescence staining showed p21 and p16 proteins primarily within the nucleus (22, 23), which would therefore effectively concentrate their action.

While no increase was seen for p27kg (data not shown), an increase in p21 and p16 protein expression was seen in senescent cultures relative to logarithmically growing early passage fibroblasts (Fig. 1 A and B). The p16 protein was at nearly undetectable levels in log phase cells but increased dramatically upon senescence (Fig. 1 A). Senescent MRC-5 cells express nearly 40-fold higher cellular p16 protein levels per cell than early passage cells, which translates into an 10-fold increase in protein expression based upon a per-microgram protein analysis (see Fig. 4 A). The precise increase is difficult as the basal levels are low. The magnitude of p16 expression was variable from culture to culture of senescent cells, but senescent cells that had been maintained for extended periods of time expressed the highest levels of p16. Further, the relative expression of p21 vs. p16 varied between

![FIG. 1. p16 and p21 protein expression in proliferating and senescent HDFs. Total extracts from the same number of cells from NHF1 (lanes 1 and 2), MRC-5 (lanes 3 and 4), IMR-90 (lanes 5 and 6), and WI-38 (lanes 7 and 8) strains of HDFs were analyzed for expression of p16 (A) and p21 (B) under conditions of log phase growth (lanes 1, 3, 5, and 7) and senescence (lanes 2, 4, 6, and 8).](image-url)
the strains analyzed with less dramatic but significant increases in p16 levels were seen in senescent IMR-90 and WI-38.

A more detailed examination of p21 and p16 mRNA and protein expression under different conditions of growth inhibition and at increasing passage in culture was performed on MRC-5 fibroblasts (Fig. 2). For p21, cellular protein levels increased in HD cultures and following incubation with reduced or LS. Upon serial propagation, cellular p21 levels reached a maximum during the period in which the majority of the cells lost their growth potential (LI ≈ 20%) as shown by the decrease in the LI and decreasing cumulative population doublings (Remaining CPD). The p21 level then decreased as the cells reached a LI of <5%, although its cellular level remained several-fold above the levels found in log phase cells (Figs. 1, 2, and 5B). p16 protein increased modestly upon growth to HD and upon passage to near senescence, but reached maximal levels when p21 levels had declined and the cells showed no remaining growth potential (Fig. 2). p16 protein levels in senescent cells reached similar cellular levels as those expressed in an SV40-immortalized human fibroblast line, GM639 cells (Fig. 4A, lanes 4 and 5). No significant changes in the cellular levels of either CDK4 or CDK6 were observed in MRC-5 fibroblasts of any age or growth-arrest state (Fig. 2). A several-fold decrease in cellular CDK2 was observed in senescent and serum-starved fibroblasts (data not shown).

**Fig. 2.** Growth and age related expression of cell cycle related proteins in MRC-5 fibroblasts. Extracts representing the same number of cells from early passage MRC-5 fibroblasts in log phase (lane 1), arrested by growth in reduced serum (LS) (lane 2) or HD (lane 3) and from MRC-5 fibroblasts at increasing age in culture (lanes 4–8) were analyzed for expression of CDK4 and CDK6, MAP kinase, cyclin D1, the cyclin-dependent inhibitors, p21 and p16. The percentage of the population actively undergoing DNA synthesis during the 48-hr period before the extracts were made is shown as the LI. The degree of senescence is shown as the number of cumulative population doublings remaining before loss of proliferative potential (Remaining CPD) and is denoted as late, near senescent (Near Sen) and senescent (Sen) populations. Lanes 1–8 represent 25, 42, 43, 53, 95, 105, 115, and 100 μg of protein, respectively.

**p16 and p21 in CDK4 and CDK6 Complexes of Senescent MRC-5.** CDK inhibitors regulate CDK activity by forming physical complexes with CDK or CDK-cyclin complexes. To determine which specific CDK inhibitor(s) were involved in senescent cells, we examined the CDK4 and CDK6 complexes. The proteins associated with CDK4 and CDK6 were determined in MRC-5 HDF extracts from growing and growth-arrested early passage cultures and from senescent cultures. When CDK4 was immunoprecipitated from extracts of radio-labeled senescent cells, proteins of 36, 33, 21, and 16 kDa were specifically coprecipitated (Fig. 3A, lane 7) representing cyclin D1, CDK4, and the CDK inhibitors p16 and p21, respectively (Fig. 4A, data not shown for cyclin D1). The specificity of the immunoprecipitations was confirmed by parallel precipitations with sera blocked by competing peptides. While moderate levels of p21 protein were seen in the CDK4 complexes of growth factor deprived and gamma-irradiated cells, p16 was identified as a significantly labeled protein in growth-factor deprived early passage cells, in senescent MRC-5 cells and in GM639 cells (Fig. 3A). In CDK6 complexes, proteins of ≈90, 40, 33, and 16 kDa specifically coprecipitate with CDK6 (Fig. 3B, data not shown). The identity of the p40 and p16 bands are

**Fig. 3.** Radiolabeled proteins in immunoprecipitated native CDK4 and CDK6 complexes from MRC-5 extracts under different growth states. Extracts from radiolabeled early passage MRC-5 fibroblasts logarithmically growing (lanes 1 and 2), arrested by growth in LS (lanes 3 and 4), 20 hr after gamma irradiation (Irrad) (lanes 5 and 6), and senescent MRC-5 cells (Sen) (lanes 7 and 8) or a SV40-transformed immortal human fibroblast line, GM639 (lanes 9 and 10) were immunoprecipitated under non-denaturing conditions with anti-CDK4 (A) or anti-CDK6 (B). Specificity of the precipitations was shown by competing the antibody with either a buffered saline solution (−) or peptides used to generate the antibodies in PBS (+). Extracts from the same number of cells were immunoprecipitated. Lanes 7–10 for both A and B were exposed three times longer to compensate for differences in radiolabel incorporation between extracts.
CDK6 and the CDK inhibitor p16, respectively, while the identities of the other proteins are unknown. Anti-CDK6 immunoprecipitations from radiolabeled lysates revealed no p21 protein present under any conditions analyzed although Western analysis of the immune complex of the unlabeled extracts prepared at the same time showed p21 in CDK6 complexes from irradiated cells (Fig. 4C). Because proliferating-cell nuclear antigen is down-regulated during senescence (ref. 6 and data not shown), its absence in either CDK4 or CDK6 complexes is expected for senescent MRC-5, but we have no explanation for its absence in the early passage extracts. No other antibody specific proteins representing CDK4 or CDK6 inhibitors of either the INK4 or the p21 family were seen in either the CDK4 or CDK6 radiolabeled complexes, suggesting they play little or no role in CDK4 or CDK6 inhibition in senescent human fibroblasts.

Comparison of relative levels of inhibitors in the complexes by Western blot analysis of unlabeled CDK complexes for p16, p21, and the respective CDK revealed no significant differences in p21 levels associated with CDK4 between log, growth factor-deprived, and in cells 20 hr after irradiation when normalized to the amount of CDK4 precipitated, whereas CDK4 complexes from MRC-5 senescent contained 75% less p21 as compared with complexes from log phase cells (Fig. 4). Interestingly, significant levels of p21 accumulated in the CDK6 complex from irradiated cells at a time in which no change in levels of p21 was found in the CDK4 complexes from log phase cells, reduced serum-treated and irradiated MRC-5 fibroblasts.

Senescent CDK4 and CDK6 complexes contain 8- and 13-fold, respectively, higher amounts of p16 protein as compared with logarithmically growing MRC-5 cells (Fig. 4). These levels of complex-bound p16 were similar to the amounts found in GM639 cells. No significant change of p16 levels in complexes from either growth factor deprived or irradiated cells was observed, although a modest increase in cellular p16 and p16/CDK4 complex was found upon growth to HD (data not shown).

Amount of CDK4 and CDK6 Complexed with p16 and p21. The relative contribution of p16 and p21 in inhibiting CDK4 and CDK6 in senescence was determined by quantitation of the amounts of CDK4 and CDK6 remaining following immunodepletion of p21 and p16 from non-denatured extracts. p16 depletion removed the majority of CDK4 and nearly all of the CDK6, while p21 depletion removed some of the CDK4 and little of the CDK6 (Fig. 5A and B, lanes 4 and 5). When normalized to the amount of MAP kinase present in the immunodepleted extracts, ~70% of the CDK4 and 85% of the CDK6, were associated with p16, while complexes containing p21 represented the remaining percentages. Removal of either p16 or p21 did not decrease the amount of the other inhibitor from the final extract (Fig. 5B, lanes 4–6). Further, all of the p21, p16, CDK4, and CDK6 was solubilized by the extraction procedure (Fig. 5B, lanes 3 and 4, data not shown for CDKs).

Expression of p16 and p21 mRNAs. Northern blot analysis of total RNA prepared from MRC-5 cells of increasing age revealed that p16 and p21 mRNA levels generally correlated with the magnitude of their protein expression (Fig. 6). As two promoters generate two p16 mRNAs (24), we used an exon 1α specific p16 probe to detect the p16 protein specific message (p16α mRNA). This probe hybridized primarily to a 1.1-kb mRNA and in young cultures, p16α mRNA levels increased ~4-fold in HD cultures but not under growth factor limiting conditions (Fig. 6A, lanes 2 and 3). Further, expression increased with increasing age, reaching 17- and 12-fold higher levels in two senescent cultures with an average of a 12-fold increase in four independent samples (Fig. 6A, lanes 6 and 7, data not shown). Hybridization with the full-length cDNA for human p21 revealed a 2.1-kb mRNA that showed 2- to 3-fold increases under limiting growth factor and HD conditions as well as with increasing age but upon senescence decreased significantly to an average of only a 1.2-fold increase in the four independent samples, which reflects the changes seen in p21 protein expression (Figs. 2 and 6B, data not shown).

DISCUSSION

Isolation and characterization of the CDK inhibitor p21 as a gene with increased expression in senescent human fibroblasts (9), and its characterization as a protein that interacted with
and inhibited the CDKs (25, 26) initially suggested a mechanism for why Rb was hypophosphorylated in senescent fibroblasts. Further, the finding that p21 transcription was activated by p53 (27), suggested a mechanism through which SV40 Tag inactivation of p53, and therefore suppression of p21 protein levels, could extend the life span of HDFs in culture (2). In this study we confirm that p21 expression is increased in old passage cells (4). One possibility is that with the inactivation of the Rb kinases, CDK2/4, Rb is dephosphorylated and may no longer interact with transcription factors that may require partial Rb (hypo)phosphorylation for binding. Dephosphorylated Rb may allow the activation of p16 transcription. Alternatively, in senescent cells, p16 increases may result from Rb inactivation through a yet undefined pathway involving the activity of additional proteins interacting with Rb, as suggested by the formation of unique senescent-specific Rb/E2F and p107/E2F complexes containing p21, cyclins, and CDK2 (31).

Further, suppression of p16 expression may require a specific Rb-E2F complex such as E2F1 or require Rb in conjunction with another transcriptional corepressor that may be down-regulated upon extended growth suppression. E2F1 expression has been shown to be down-regulated (31) or not expressed in senescent cells (32). An additional possibility is that the p16 promoter may contain additional regions that are Rb-independent and are activated in senescent cells possibly through a p21-initiated mechanism. Finally, increased stability of p16 mRNA in senescence has been reported (18) and may explain the increased p16 mRNA levels, although this mechanism would have to be selective for p16.

p16 may function as a tumor suppressor through its role as a senescence gene. The p16 gene corresponds to the melanoma cancer gene, MTS1, on chromosome 9 (12, 33) and p16 mutations have been found in multiple tumor types (34), unlike the p21 gene where no mutations have been found in screens of a wide range of tumor types (35, 36). Ablation of p16 expression by homozygous deletion in mice produces viable offspring but leads to dramatically increased spontaneous tumor formation and enhanced susceptibility to carcinogens.
The cultured embryonic fibroblasts of these mice lack the crisis event before immortalization and are transformed by the expression of a single oncogene (13). However, deletion of the p16 gene also prevents the expression of an alternatively spliced mRNA containing exons 2 and 3 in common with p16. This mRNA produces an unrelated, 19-kDa protein that also suppresses growth (37). In humans, a similar mRNA may generate a 13 kDa protein (24) whose protein expression and activity have yet to be determined. Deletion of p21 in mice also produces viable normal offspring without any apparent phenotype except cells from these mice have a reduced growth-arrest response following gamma-irradiation (14). These results suggest that both p16 and p21 are not developmental growth regulators but checkpoint proteins, with p16 playing a critical role in cellular immortalization and tumor prevention.

In conclusion, we have shown that replicative cellular senescence of HDFs is a multistep process involving the sequential expression of p21 and p16 resulting in p16 becoming the major CDK4/CDK6 inhibitor in senescent MRC-5 fibroblasts. Current models explain a possible mechanism for the initiation of p21 expression, while the mechanism of p21 down-regulation and increased p16 expression have not been delineated. Current work is focusing upon defining these pathways and toward defining the relative roles p21 and p16 may play in the growth arrest found in senescent fibroblasts.

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