p16INK4A Mediates Cyclin Dependent Kinase 4 and 6 Inhibition in Senescent Prostatic Epithelial Cells

Charanjit Sandhu, Donna M. Peehl, and Joyce Slingerland

ABSTRACT

The senescence checkpoint constrains the proliferative potential of normal cells in culture to a finite number of cell doublings. In this study, we investigated the mechanism of cyclin dependent kinase (cdk) inhibition in senescent human prostatic epithelial cells (HPECs). Progression of HPECs from early passage to senescence was accompanied by a gradual loss of cells in S phase and an accumulation of cells containing 2N DNA. Furthermore, G1-S phase-associated kinase activities progressively diminished with increasing cell passage. In senescent HPECs, cdk4 and cyclin E1- and A-associated kinases were catalytically inactive. In contrast to observations in senescent fibroblasts, levels of the kinase inhibitor protein (KIP) inhibitor p21CIP1 diminished over the proliferative life span of HPECs. p27KIP1 levels fell as cells approached senescence, and the association of both p21CIP1 and p27KIP1 with cdk4/6 complexes was decreased. However, the level of cyclin E1-associated KIP molecules was unaltered as cells progressed into senescence. Progression to senescence was accompanied by a progressive increase in both the level of p16INK4A and in its association with cdk4 and cdk6. As HPECs approached senescence, cdk4- and cdk6-bound p16INK4A showed a shift to a slower mobility due to a change in its phosphorylation profile. As p16INK4A increased in cdk4 and cdk6 complexes, there was a loss of cyclin D1 binding. The altered phosphorylation of p16INK4A in senescent prostatic epithelial cells may facilitate its association with cdk4 and cdk6 and play a role in the inactivation of these kinases.

INTRODUCTION

The eventual growth arrest that defines the termination of cellular proliferation of normal cells in culture is referred to as cellular senescence (1). Normal cells can undergo a finite number of population doublings in culture before they stop proliferation at senescence (1, 2). Fibroblasts arrested at senescence have predominantly 2N DNA, reflecting arrest during the G1 phase of the cell cycle (2). Several lines of evidence suggest that the mechanisms regulating cell cycle arrest at senescence are genetically programmed and reflect processes relevant to aging within the organism (3–5). It has been postulated that the senescence checkpoint may function as a critical tumor suppression checkpoint in vivo. Estimations of cellular proliferation have suggested that tumor growth beyond a volume of 1 cm3 requires abrogation of the senescence arrest (6).

Most previous studies investigating senescence have used fibroblasts. The high incidence of prostatic cancer in adult males and our limited understanding of prostatic oncogenesis motivated our study of prostatic epithelial cell senescence. Primary prostatic cancers frequently show telomerase activation (7–10), and it is possible to establish immortal lines from primary prostate cancers (11–13). Thus, prostate cancer development may be associated with loss of the senescence checkpoint. An understanding of the molecular mechanisms whereby the senescence checkpoint is lost in cancers requires an assessment of this checkpoint in normal HPECs.

In this study, we investigated the mechanisms of cdk inhibition in HPEC senescence.

Transition from one phase of the cell cycle to the next requires the orderly activation and inactivation of a family of related cdks. Cdk1–7 are positively regulated by the cyclin-cdk complexes. Recent in vitro experiments demonstrated that a single KIP molecule is sufficient to inhibit cyclin/cdk kinase activity (21). In contrast to the KIP inhibitors, members of the INK4 family (p15INK4B, p16INK4A, p18INK4C, and p19INK4D) bind specifically cdk4 and cdk6, with resulting loss of cyclin D binding and catalytic inactivation.

Cell culture models have identified a role for the cdk inhibitors in senescence. An increased expression of p21 and/or p16 at senescence has been identified in human and murine fibroblasts and melanocytes (22–26). The induction of p21 in senescent fibroblasts led to the initial identification and cloning of this gene by Noda et al. (27). The elevated expression of p16 and/or p21 at senescence is associated with their increased binding and inhibition of G1-S phase cdks. Elimination of p21 expression through homologous recombination extended the life span of human diploid fibroblasts in culture (28). Thus, the loss of p21 expression, although itself not sufficient to abrogate senescence arrest, may represent a key step in the immortalization of cells of fibroblastic lineage. The loss of p21 and p16 expression in human cancers suggests that in vivo, these inhibitors may contribute to the senescence checkpoint and limit tumor development (29, 30).

In human prostatic tumors, although p16 is rarely mutated (31, 32), loss of expression occurs frequently through hypermethylation or deletion (29, 33, 34). Furthermore, loss of p16 expression in human prostatic tumors may have prognostic implications (30). Together, these results suggest that p16 may play an important tumor suppressor role in prostatic epithelial cells. In this study, we show that the progression toward HPEC senescence was associated with an increase in p16 levels. Novel phosphorylated forms of p16 showed increased association with the target cdk complexes, cdk4 and cdk6. In contrast to senescence in fibroblasts, neither the expression of p21 nor its association with target cdk complexes was increased. Phosphorylation
of p16 may represent a novel regulatory pathway for p16 inhibitory activity.

MATERIALS AND METHODS

Cell Culture. Cell cultures were established as follows: Cell strains E-PZ-16 and E-PZ-22 were obtained from men 60 and 61 years of age, respectively, undergoing radical prostatectomy to treat prostate cancer. Neither patient had received previous therapy. Prostatic specimens were transferred to the laboratory within 1 h after surgery. A small wedge of tissue was dissected from the peripheral zone of each specimen, and primary cultures were established as described previously (35). Briefly, tissues were minced and digested overnight with collagenase. The digested tissues were inoculated into dishes coated with collagen type I and containing PFMR-4A medium supplemented with 10 ng/ml choler toxin, 10 ng/ml epidermal growth factor, 40 μg/ml bovine pituitary extract, 4 μg/ml insulin, 1 μg/ml hydrocortisone, 100 μg/ml gentamicin, 0.1 mM phosphoethanolamine, 3 nM selenous acid, 2.3 mM NaCl, 1 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Tween-20, 0.1 mM Na3VO4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.5 mM DTG and 1 mM both leupeptin and aprotinin. CdK4 kinase assays were performed following the method of LaBaer et al. (42), using cdK4 antibody obtained from Santa Cruz for immunoprecipitation and the COOH-terminal fragment of PAP as substrate. Radioactivity was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. Cyclin El- and A-associated kinase assays were performed as described (43). Histone H1 substrate was obtained from Boehringer Mannheim (Laval, Quebec). Background levels of kinase activity were determined for each kinase reaction by immunoprecipitating early passage cellular extract with nonspecific mouse mAbs (Fig. 2, Lane C).

Phosphatase Assays. Dephosphorylation of p16 was carried out by immunoprecipitating cdK6 complexes from 100 μg of cellular extract. Complexes were washed three times with NP40 lysis buffer and twice with potato acid phosphatase (PAP) buffer [40 mM PIPES (pH 6,0), 1 mM DTG, 20 μg/ml aprotinin, and 20 μg/ml leupeptin]. An ammonium sulfate precipitate of PAP (Sigma) was resuspended in 1 ml of PAP buffer and eluted from a desalting column (Pharmacia Nap-5) with 500 μl of PAP buffer. Immunoprecipitated complexes were then incubated with 12 units of PAP for 2 h. The specificity of the phosphatase assay was confirmed by incubating immunoprecipitates with 1 mM Na3VO4 and 100 μM β-glycerophosphate in addition to PAP. The complexes were then resolved by SDS-PAGE and immunoblotted for p16.

Flow Cytometric Analysis. At different passages, cells were pulse labeled with 10 μM BrdUrd for 2 h. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 90°C to expose the labeled DNA. Cells were then stained with anti-BrdUrd-conjugated FITC (Becton Dickinson, Bedford, MA) and propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan using Cell Quest software.

Antibodies. Antibodies to pRb, cdK2, cyclins A and D1, and p21 were obtained from PharMingen (San Diego, CA) or Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin El-specific antibodies (mAbs E12 and E17; Refs. 37, 38) were from E. Lee and E. Harlow (Massachusetts General Hospital, Boston, MA). Monoclonal PSTAIRE antibody (39) was a gift from S. Reed (The Scripps Research Institute, La Jolla, CA), and cyclin D1 antibody, DCS-11, was purchased from Neomarkers (Fremont, CA). Cyclin A mAb E67 was provided by J. Gannon and T. Hunt (ICRF, London, United Kingdom). Monoclonal p27 antibody was purchased from Transduction Labs (Lexington, KY). CdK4 and cdK6 polyclonal sera were provided by J. Gannon and T. Hunt (ICRF, London, United Kingdom). CdK4 and cdK6 polyclonal sera were then transferred to Immobilon, and p16 was detected by immunoblotting.

RESULTS

Cell Cycle Arrest in Senescence. The cell cycle profile of HPECs was determined by BrdUrd pulse labeling and flow cytometric analysis of cells at early passage and senescence (Fig. 1). At early passage, 22% of asynchronously growing cells were in S phase, 66% contained 2N DNA, and the remaining 12% of cells contained 4N DNA. Cellular proliferation ceased after six passages, which corresponded to ~30 population doublings at senescence. Cells were considered to be senescent after they remained subconfluent for >1 month. Flow cytometry of senescent HPECs demonstrated a cell cycle arrest with <1% of cells in S phase and 85% of the cells with 2N DNA. The remaining 14% of cells had 4N DNA at senescence.

p16INK4A MEDIATES cdK4 INHIBITION IN SENESCENT PROSTATE EPITHELIAL CELLS
Cdk Activities Decreased Progressively with Increasing Passage. The kinase activities in cyclin E1, cyclin A, and cdk4 immune complexes from cell populations of increasing passage were assayed using either histone H1 (cyclins A and E) or a pRb fragment (cdk4) as substrates. Results are shown in Fig. 2. The activities of cyclin E1- and cyclin A-associated kinases and of cdk4 decreased steadily as cells progressed from early passage to senescence. Senescent cells showed no kinase activities above that in nonspecific immune controls. Furthermore, analysis of cells of increasing passage revealed a progressive loss of pRb phosphorylation, indicative of cell cycle arrest in G1 (see Fig. 3A). Because pRb is phosphorylated by cyclin E1 and D1-associated kinases, loss of pRb phosphorylation provides further evidence of inhibition of these cdk.

**Increased p16 INK4A Levels in Senescent Prostatic Cells.** The levels of cdk inhibitors, cyclins, and cdk4 associated with the G1-S transition were assayed as HPECs progressed from early passage toward senescence (Fig. 3A). Western analysis revealed no change in cyclin E1 or cyclin D1 levels during the aging of prostatic epithelial cell populations. Cyclin A levels gradually decreased, consistent with the gradual recruitment of cells into G1 arrest at senescence. The decrease in cyclin A levels was likely the consequence of decreased cyclin E1- and cyclin D1-associated kinase activities, resulting in reduced E2F-mediated transcription of cyclin A. The cdk4 and 6 levels remained unchanged. The loss of cdk2 protein with increasing population doublings was largely due to loss of the CAK-activated, Thr-160 phosphoform (faster migrating cdk2 band).

The levels of the KIP and INK inhibitors were also analyzed in asynchronous prostatic epithelial cells at increasing population doublings and at senescence. There was a steady decrease in the protein levels of p21CIP1 and p27KIP1. p18 INK4C expression was not detected (data not shown), and p19INK4B levels remained unchanged, whereas p16INK4A levels steadily increased, reaching maximal levels of expression in senescent cells. In addition, longer exposures of the p16 immunoblots revealed the presence of two additional p16-reactive bands of decreased mobility on SDS-PAGE (Fig. 3B). These slower mobility bands were detected using two different anti-p16 antibodies (the JC-6 monoclonal and the Santa Cruz polyclonal). The abundance of these two novel p16-related bands increased with increasing population doublings.

**KIP Binding to Cyclin E-cdk2 Did Not Increase in Senescent HPECs.** The composition of cyclin E1-cdk2 complexes showed no apparent change during the progression of HPECs toward senescence (Fig. 4A). The amount of cdk2 bound to cyclin E1 was not reduced.
p16INK4A MEDIATES cdk4 INHIBITION IN SENESCENT PROSTATE EPITHELIAL CELLS

Altered Phosphorylation of p16 in Senescent Prostatic Epithelial Cells. To determine whether the novel bands of delayed mobility were different phosphoforms of p16, cdk6 complexes were immunoprecipitated from senescent cellular extract (Fig. 5, Lane 1) and treated with PAP (Fig. 5, Lane 2); immunoblots were reacted with p16 antibodies. PAP treatment of the immunoprecipitates from senescent cells resulted in the loss of the upper two p16 bands visible on long exposures and the formation of two bands of increased mobility (data for senescent cells shown in Fig. 5, middle and bottom panels). Thus, the slower mobility forms of p16, whose association with both cdk4 and cdk6 complexes increased in senescent HPECs, represented novel phosphoforms of p16. These delayed mobility p16 phosphoforms were not detected, even with prolonged exposure in the early passage HPECs. It is notable that the dominant, faster mobility p16 band, detected in both early passage and senescent cells, also shifted to two faster mobility bands when the phosphatase reaction went to completion, indicating that this dominant band also represents phosphorylated p16 (Fig. 5). There was no loss of any of the p16 reactive bands when PAP was preincubated with phosphatase inhibitors (Fig. 5, Lane 3). The formation of two p16-reactive bands of increased mobility after PAP treatment suggested that all of the cellular p16 is phosphorylated, but the pattern of expression of the different phosphoforms differed between early passage and senescent cells.

2D IEF of p16 in cdk6 immunoprecipitates with and without PAP confirmed the existence of multiple p16 phosphoforms (Fig. 6). In early passage cells, the solitary band of p16 seen on one-dimensional Western blots (Fig. 4) resolved on 2D IEF as two forms of p16, which focused to a pH between 5 and 6 (Fig. 6, arrowhead 3). In senescent cells, the phosphorylation pattern of p16 differed between early passage and senescent extracts were immunoprecipitated and treated with PAP in the presence or absence of β-glycerophosphate (inhibitor). Complexes were then resolved and immunoblotted for p16. +, present; −, absent.

Furthermore, the proportion of Thr-160 phosphorylated cdk2 bound to cyclin E1 remained constant. The levels of cyclin E1-associated p21 and p27 remained constant. Thus, the inhibition of cyclin E1-cdk2 activity could not be attributed to increased KIP binding or to a lack of activating phosphorylation at the Thr-160 residue of cdk2.

**p16 Accumulated in cdk4 and cdk6 Complexes as HPECs Approached Senescence.** As total p16INK4A levels increased during the aging of the HPEC population, the association of p16INK4A with cdk4 and cdk6 complexes also increased (Fig. 4, B and C). Although there was no increase in the faster mobility band of p16 in cdk4 and cdk6 complexes (Fig. 4, B and C, short exposure), longer exposures of the cdk-associated p16 immunoblots (Fig. 4, B and C, long exposure) again revealed the existence of two delayed mobility bands that cross-reacted with p16-specific antibodies. The association of these two bands with cdk4 and cdk6 complexes steadily increased with later cell passages. Densitometry showed that association of the slower migrating forms of p16 increased by 2-fold in cdk6 complexes and by 1.6-fold in cdk4 complexes between passage 2 and senescence. The accumulation of these slower mobility p16 bands in cdk4 and cdk6 complexes was correlated with a decrease in the binding of cyclin D1, p27, p21, and p19. None of the anti-p16-reactive bands were cross-reactive with p18 or p19 antibodies.

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cells, in addition to the two forms of p16 observed in early passage cells, two additional p16 isoforms (Fig. 6, arrowheads 1 and 2) were present. These two isoforms showed a delayed mobility on SDS-PAGE (the second dimension) and focused just below pH 6.0. The relative mobilities of isoforms 1 and 2 in the second dimension with respect to the predominant forms of p16 was consistent with these being the two minor p16 bands of delayed mobility that were observed in senescent cells in one-dimensional Western blots in Fig. 4.

With PAP treatment prior to resolution on 2D IEF, all of the p16 isoforms focusing between pH 5.0 and 6.0 were lost. Phosphatase-treated p16 focused at a higher isoelectric point, at approximately pH 6.8, and had increased mobility in the second dimension. These results confirm the results in Fig. 5 and indicate that all three p16 bands detected on Western blotting represent p16 phosphoforms. The pattern differs between early passage and senescence, with isoforms 1 and 2 increasing as cells approach and enter senescence.

**DISCUSSION**

The senescence checkpoint limits the proliferative capacity of cells in a tissue culture environment. Processes analogous to the senescence checkpoint observed in cultured cells are thought to limit cellular proliferation in vivo. A number of observations have suggested that senescence occurs within the organism in vivo. The proliferative capacity of cells with a finite life span in culture is inversely related to the donor’s age (3). The number of population doublings that fibroblasts undergo in culture is related to the longevity of the donor species (4). The limit imposed by senescence on the proliferative capacity of cells has raised the hypothesis that cellular senescence may indeed represent a natural impediment to malignant degeneration. To date, most studies investigating senescence have used human fibroblasts and rodent cells. Because the incidence of malignancies arising from fibroblasts such as sarcomas is relatively rare in humans, we examined the senescence phenomenon in epithelial cells, which undergo malignant transformation more frequently than fibroblasts. Our investigation of senescence arrest in HPECs and in human mammary epithelial cells demonstrates that epithelial cells differ from fibroblasts in the manner whereby cdk inhibition occurs at senescence.

The identification of key inhibitor(s) of cell cycle progression at senescence could potentially indicate a critical regulator whose expression or activity would need to be down-regulated for tumor progression to proceed. Investigations of senescence in fibroblasts suggest important roles for p21 and p16. Here we have demonstrated an increase in p16 and the appearance of novel phosphorylated forms of p16 that bind and inhibit cdk4 and cdk6 activity in senescent HPECs.

The prostatic epithelial cell cultures used in this study moved from a proliferatively active state to a senescent state within six passages or ∼30 population doublings. Senescent HPECs showed predominantly 2N DNA. A small proportion of cells had 4N DNA at senescence. We and others have observed an increase in tetraploidy as cultured cells approach senescence (44). Thus, cells with 4N DNA are most likely tetraploid cells arrested prior to S phase entrance at the senescence checkpoint, as opposed to cells arrested at the G1-M transition. The catalytic activity of the cdk5 associated with the G1-S transition underwent a steady decrease from early passage to senescence. The steady loss of kinase activity implies that the entry of cells into senescence does not occur in a synchronized manner. Rather, there appears to be some heterogeneity with regard to the passage at which epithelial cells enter senescence. Early studies of senescence identified this phenomenon by the progressive reduction of tritiated thymidine incorporation that occurred during the serial passaging of fibroblasts (45). The established relationship between telomere length and the proliferative capacity of cells suggests that variations in telomere length within a population of cells may account for the apparently stochastic manner whereby cells enter senescence (46). The heterogeneity in telomere length may reflect variations in the proliferation of epithelial stem cell populations in vivo.

To investigate the cause of cdk inhibition in senescent HPECs, we examined the steady-state levels of G1-S-associated cell cycle regulators. Of the cyclins examined, only cyclin A diminished with increasing passage, accounting for the corresponding loss of cyclin A-associated kinase activity. The loss of cyclin A in senescent HPECs is likely a consequence of the cells arresting at a point within the cell cycle prior to cyclin A induction. Studies in human fibroblasts have shown that entrance into senescence is similarly associated with a loss of cyclin A (47). However, in senescent fibroblasts there is a significant increase in the expression of cyclins D1 and E1 (48, 49). The levels of cdk4 and cdk6 were unaffected by increasing passage in HPECs, whereas loss of cdk2 was largely due to loss of the Thr-160 phosphorylated form of cdk2. Fibroblasts have a similar loss of the Thr-160 form of cdk2 during senescence (48, 50), and in addition, studies of fibroblast senescence have revealed a reduction in cdk4 levels (49). Thus, it appears that the regulation of both cyclin and cdk expression differs between senescent fibroblasts and prostatic epithelial cells.

In contrast to reports in cells of fibroblastic and melanocytic lineage, the steady-state levels of both p21 and p27 proteins decreased as HPECs moved toward senescence (22, 27, 51). Furthermore, there was no increase in the binding of p21 and p27 to cyclin E1-associated complexes in senescent HPEC extracts. These KIP molecules were also lost from cdk4 and cdk6 complexes during the progression toward senescence. These results contrast with previous findings in senescent human fibroblasts and keratinocytes, where p21 was shown to be induced at senescence and its binding to G1-S-associated cdks was increased (28, 51, 52). Our findings in senescent HPECs and human mammary epithelial cells indicate that p21 does not appear to mediate cdk inhibition in all senescent epithelial cells. The lack of p21 induction in senescent prostatic and mammary epithelial cells may represent a fundamental difference between these epithelial cell types and keratinocytes and fibroblasts at senescence.

Investigations of senescence in fibroblasts, lymphocytes, and uroepithelial cells have implicated p16 in the inhibition of cyclin D1-dependent kinases (22, 26, 53). The high incidence of p16 inactivation in prostatic tumors suggests that p16 may play a role in arresting HPECs at senescence both in vivo and in vitro (29, 33). The present study and the recent report of Jarrard et al. (54) establish that there is an increase in p16 levels in senescent prostatic epithelial cells. Immortal HPEC derivatives, generated by the introduction of human papilloma virus E6 and/or E7, were associated with a loss of p16 or pRB expression (54). We have identified two novel p16-related bands of delayed mobility that accumulate in senescent HPECs. Resolution on both one-dimensional gels and by 2D IEF after treatment with PAP demonstrates that the two novel p16 bands of delayed mobility observed in one-dimensional Western blots were p16 phosphoforms. In both early and late passage cell extracts, PAP treatment also shifted the most abundant (faster mobility) p16 band on the one-dimensional Western blots, suggesting that all detectable p16 is phosphorylated. The failure of p16 to resolve into a single band after prolonged PAP treatment may be a consequence of phosphorylation at sites on p16 that are not recognized efficiently by PAP, or alternatively, a fraction

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of the cellular p16 may undergo other posttranslational modifications, such as glycosylation, that serve to alter its gel mobility.

PAP treatment and 2D IEF confirmed the existence of different p16 phosphorylation patterns in early passage and senescent HPECs. The dominant p16 band seen on immunoblots from both early passage and senescent cdk6 immune complexes was composed of two p16 isoforms. Two additional p16 isoforms of delayed mobility consistently appeared in senescent cells, suggesting that p16 undergoes senescence-specific posttranslational modifications. These senescent-specific isoforms (Fig. 6, arrowheads 1 and 2) of p16 focused at a slightly higher pH, and thus represent p16 isoforms with a lower level of phosphorylation when compared with the two dominant isoforms of p16 (Fig. 6, arrowhead 3). After PAP treatment, all of the p16 isoforms focused at a higher pH. These results are consistent with the interpretation that all of the detected p16 is phosphorylated but that novel phosphorylation patterns appear in senescent cells.

The increased expression of p16 translated into an increased association of p16 with cdk4 and cdk6 complexes in senescent HPECs. In senescent HPECs, as in other forms of G0 arrest, the accumulation of this INK4 molecule in target kinases was associated with loss of cyclin D1 and KIP binding (55, 56). However, only the senescence-specific phosphoforms of p16 showed an increased binding to cdk complexes. The accumulation of these novel p16 phosphoforms in cdk4 and cdk6 complexes suggests a senescence-activated mechanism of posttranslational modification of p16 contributing to kinase inhibition and senescence arrest in HPECs.

Phosphorylation of p16 may represent an important mechanism of p16 regulation. p16 phosphorylation may regulate either the affinity for cdk complexes and/or the localization of p16 within the cell. Phosphorylation of p27 functions to regulate the stability of the protein and its affinity for cdk complexes (57–59). It is tempting to postulate that phosphorylation of specific sites on p16 in senescent HPECs facilitates the binding of p16 to target cdk complexes and contributes thereby to G1 arrest in senescence. The identification of these phosphorylation sites and the pathways that influence phosphorylation of p16 would aid in the understanding of the regulation of the INK family of inhibitors and elucidate further the pathways regulating p16 inhibitory activity.

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