Elevated p16 at Senescence and Loss of p16 at Immortalization in Human Papillomavirus 16 E6, but not E7, Transformed Human Uroepithelial Cells

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Abstract

CDKN2/p16 inhibits the cyclin D/cdk-dependent kinase complexes that phosphorylate pRb, thus blocking cell cycle progression. We previously reported that p16 levels are low to undetectable in normal human uroepithelial cells (HUCs) and in immortalized uroepithelial cells with functional pRb, whereas p16 levels are markedly elevated in immortal HUCs with altered pRb (T. Yeager et al., Cancer Res., 55: 493-497, 1995). We now report that elevation of p16 levels occurs at senescence in HUCs, including HUCs transformed by human papillomavirus 16 E7 or E6, whose oncoprotein products lead to functional loss of pRb and p53, respectively. We also report that six of six independently immortalized E7 HUCs show high levels of p16 similar to those observed at HUC senescence, whereas p16 is undetectable in five of five immortal E6 HUCs. Four of the five independent E6 HUCs that lost p16 at immortalization showed hemizygous deletion of the 9p21 region. However, no homozgyous CDKN2 deletions were detected, and only one CDKN2 mutation was identified. For the first time, these data associate elevated p16 with senescence in human epithelial cells. These data also suggest that a component of immortalization may be abrogation, either by pRb inactivation (as in the E7-immortalized HUCs) or by p16 inactivation (as in the E6-transformed HUCs), of a p16-mediated senescence cell cycle block.

Introduction

The most frequent genetic alteration observed in human bladder cancer is a hemizygous chromosome 9 loss (1, 2). Most bladder cancers exhibit LOH for all chromosome 9 markers, consistent with monosomy 9; however, submicroscopic 9p or 9q deletions alone have also been observed (2, 3). This latter observation has led to the hypothesis that there are bladder cancer TSGs on both the 9p and 9q chromosome arms. Although there are no strong candidates for the putative 9q bladder cancer suppressor gene, current data as discussed below indicate that CDKN2/p16 may be the 9p21 TSG (4-7). CDKN2/p16 binds to two cdk5, namely, cdk4 and cdk6, and inhibits their association with cyclin D, thereby preventing cdk-mediated phosphorylation (8-10). One of the target proteins for phosphorylation by cyclin D/cdk5 is the retinoblastoma protein (pRb). Phosphorylation of pRb leads to the release of the E2F-1 transcription factor which leads to cell cycle progression (11, 12).

CDKN2 maps to the minimal 9p21 region in which both hemizygous and homozygous deletions have been detected in bladder cancers and which is homozygously deleted in up to 60% of bladder cancer cell lines (4-7, 13-15). Inactivating mutations in CDKN2 have been described in bladder cancers with 9p21 LOH (4, 14, 16). These data are consistent with a TSG role for CDKN2 in bladder cancers. CDKN2 mutations have also been identified in other malignancies, including head and neck cancer, lung cancer, pancreatic cancer, mesothelioma, and glioma (5, 13). In addition, germline CDKN2 mutations have been identified in families with hereditary melanoma and pancreatic cancer syndromes, providing further evidence that CDKN2 is a TSG (17-19).

Nonetheless, important questions remain regarding the role of CDKN2/p16 in both normal uroepithelial cellular growth and in tumorigenesis. First, the most common mechanism of CDKN2 inactivation in bladder cancer appears to be homozygous deletion, yet the deletions observed are often significantly larger than the CDKN2 locus (4-6). This observation is consistent with the presence of another TSG in the 9p21 region, possibly the closely linked CDKN2B/p15 gene. Second, homozygous germline CDKN2 deletions in humans result in increased cancer risk, but have not been associated with developmental problems, raising the question of the role of p16 in normal cells (19). Third, the mechanism(s) underlying the marked elevation of p16 levels in some tumors and tumor cell lines, notably those with altered pRb, has not been defined (11, 20, 21). In this regard, it has been hypothesized that p16 is up-regulated by a feedback loop in which hyperphosphorylated or mutant pRb plays a role (20). Current data, however, suggest that this mechanism may be insufficient to account for the dramatic difference in p16 levels observed between cells with normal versus mutant pRb (11, 22).

To address these and other questions concerning the role of CDKN2 in HUC growth and tumorigenesis, we used a multistep in vitro HUC transformation system (23). Using this system, normal HUCs can be cultured in vitro routinely, and predictably undergo 50-100 population doublings before spontaneously undergoing cellular senescence (24). Spontaneous immortalization of normal HUCs has not been reported; however, normal HUCs, like other normal diploid human cells, form immortal cell lines at low frequency after transformation with SV40 (25) or with HPV16 E6 and/or E7 (23, 26). We previously reported that two HUC lines immortalized after transformation with HPV16 E7, which binds and inactivates pRb, exhibited elevated p16 levels, whereas two HPV16 E6-transformed HUC lines exhibited both low or undetectable p16 levels (20). Since normal HUCs exhibit low to undetectable p16 levels, the reasons for the elevated p16 levels in the E7-immortalized HUCs and the necessity for p16 loss only in the E6-immortalized HUCs were unclear.
We examined the status of CDKN2/p16 in presenescent and senescent normal HUCs and in presenescent, senescent, and immortalized HUCs transformed by HPV16 E6 and/or E7. We report here for the first time that p16 levels are elevated at the time of senescence in HUCs, including normal HUCs and E6- and E7-transformed and/or E7-transformed HUCs, suggesting that p16 elevation may play an important role in cellular senescence. To further test this hypothesis, we examined a series of independent E6- or E7-immortalized HUCs. Results showed that all of five E6-immortalized, but none of the six E7-immortalized HUCs lost p16 expression at the time of immortalization. This suggests that HUC immortalization requires abrogation of the presumptive p16-mediated cell cycle block, and that this can occur by either pRB inactivation (as in the E7-transformed HUCs) or p16 inactivation (as in the E6-transformed HUCs).

Materials and Methods

Cell Culture. Cultures of normal HUCs were established from explants of normal ureteral uroepithelium left over from kidney transplantation surgery and grown on collagen-coated 100-mm Petri dishes (Corning, Corning, NY) in 1% fetal bovine serum-Ham’s F12 +, a growth factor supplemented Ham’s F12 medium (Life Technologies, Inc., Grand Island, NY) (24). They were dispersed for serial passage using a 1:3 split after incubation for 15 min at 37°C in 0.1% EDTA. Under these conditions, HUCs typically undergo spontaneous senescence after passage 3. HUC irradiation was performed using 18 Gy γ-irradiation at a rate of 6 Gy/min with a 137Cs source. Determination of the cytotoxic effect of the radiation was done 96 h postirradiation by comparing viable cell counts in the exposed cells with those of untreated controls using triplicate samples stained with 0.1% trypan blue (Sigma), as described previously (27).

HPV16 E7 and/or E6 transformation of HUCs was performed as described previously (23, 26). Briefly, HUCs at PI were infected with retroviral vectors carrying HPV16 E7/NEO and/or E6/NEO and after 48 h selected with G418. Immortalized HPV16 E6- and/or E7-transformed HUC lines (αE6-1, αE6-2, βE6-1, βE6-2, βE6-1, αE7-1, αE7-2, βE7-1, γE7-1, and αE6E7-1) were generated from five different tissue samples in five independent experiments (designated by different Greek letters; Refs. 23 and 26). In all cases, normal peripheral lymphocytes were collected and cryopreserved for later DNA extraction and used in the microsatellite assays (see below). Southern blot analysis for HPV16 insertion(s) showed that these 11 lines were independent and clonal in origin. Reverse transcription-PCR showed that each line expresses the appropriate HPV 16 E6 and/or E7 transcript. The HPV16 E6 and E7 immortal HUCs were cultured using the methods described above for HUCs.

β-Galactosidase Staining. Senescent HUCs were identified by their ability to stain positively for β-galactosidase (28). Briefly, cells were grown on chambered slides (Nunc, Naperville, IL) for 3 days, rinsed and fixed, and then incubated in SA-β-galactoside solution as recommended previously (28).

Western Blot Analysis. Western blots were done as described previously (20). Briefly, cells were lysed in EBC buffer containing protease inhibitors, and 100 µg protein/lane for p16 WAP or CDKN2/p16 were resolved on a 15% SDS-polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA), and immunoreactive proteins were visualized using enhanced chemiluminescence. The antibodies were p16 WAP polyclonal antibody (PharMingen, San Diego, CA) and mouse monoclonal antibodies against p21 WAP (Oncogene Science, Uniondale, NY).

Cyto genetic Analysis. Chromosomal analyses, specifically to assess the status of chromosome 9, were done as described previously (23). Briefly, for each cell line, 20 complete karyotypes of Giemsa-banded metaphase chromosome spreads were assessed. Results are presented as the percentage of these 20 cells that showed a deletion that included the 9p21 band.

Microsatellite Repeat Analysis. Detection of LOH was done using highly polymorphic microsatellite probes (20). Normal peripheral lymphocyte DNA was used as a control for each cell line, except for the α series for which no normal control was available. In this latter case, comparison was made to the α-HUC-E7 lines in which we previously showed that there were no 9p21 deletions (20). The markers used were: D9S256, which maps to 9p23; D9S162, which maps to 9p22; IFNA, which maps to 9p21 approximately 2 Mb telomeric to CDKN2; D9S942, which maps 5 kb centromeric of the first exon of CDKN2; D9S171 and D9S161, which map to 9p21 centromeric to CDKN2. The primers for D9S162, D9S171, IFNA, and D9S161 were purchased from Research Genetics (Huntsville, AL), and the remainder were synthesized on an ABI Model 380 DNA synthesizer. Sequences for all primers are available in the Genome Data Base (Johns Hopkins University, Baltimore, MD). To detect LOH, one primer of each pair was end labeled using [32P]ATP and T4 polynucleotide kinase (New England Biolabs). Following PCR, the products were resolved on a 7% formamide/urea polyacrylamide gel, and autoradiography was done for 2 to 24 h.

SSCP Analysis. SSCP was done as described previously (20). Briefly, CDKN2 exons 1 and 2 were amplified with primers 2F/1108R and 4F/551R, respectively. These products were then digested with ScaI (exon 1) or ApaI (exon 2), resolved on a 6% nondenaturing polyacrylamide gel, and autoradiography was performed for 2 to 24 h. Three gels (one at 4°C, one at room temperature, and one at room temperature with 10% glycerol) were run for each sample. Samples with altered mobility were reamplified from total genomic DNA using the same primers, the product was purified over a QIAEX column (Qiagen, Inc., Studio City, CA), and sequenced directly using the same primers used for amplification as well as specific internal primers. All sequencing reactions were performed from at least two independently amplified products and were confirmed in both the forward and reverse direction at least twice.

Results

p16 Is Elevated in Senescent HUCs, E7 HUCs, and E6 HUCs. p16 is low to undetectable in control untreated HUCs at early passage (P1–3), but is elevated in HUCs immortalized by SV40 or HPV16 E7 as well as in bladder cancer cell lines with altered pRB (20). In the present study, we examined the status of p16 in untreated control HUCs at senescence (P3) and compared this to the low to undetectable levels of p16 previously observed in presenescent HUCs (P1–3). We also examined the status of p16 in E7- and E6-transformed HUCs prior to senescence (P1–3) and as cells were entering senescence (P3). Our results show that p16 is reproducibly elevated in independent HUCs from different tissue samples at senescence (Fig. 1). Furthermore, results showed that p16 levels are elevated in E6- and E7-transformed HUCs at senescence (P4; Fig. 1). Presenescent sam-
p16 IN SENESCENCE AND IMMORTALIZATION OF HUCs

I were subjected to an assay that identifies senescent cells by their positive β-galactosidase activity (28). Results showed insignificant β-galactosidase activity in all presenescent samples of HUCs, including all control HUCs and all E6- and/or E7-transformed HUCs (Fig. 2A); strong activity in all samples at senescence, including E6- and/or E7-transformed HUCs (Fig. 2B); and virtually no activity in the HPV16 E6 and/or E7 immortal cell lines (Fig. 2C).

Radiation-induced Cell Cycle Arrest and/or Cytotoxicity Does Not Lead to Elevated p16 Levels. To investigate whether the high levels of p16 observed during cellular senescence could be reproduced in presenescent cells by inducing growth arrest, normal HUCs were exposed to 18 Gy γ-radiation. This highly cytotoxic dose of radiation caused ~58% reduction in HUC survival compared to unirradiated controls when triplicate samples of viable cells were counted 5 days after irradiation. In addition, 18 Gy γ-radiation resulted in cell cycle arrest as determined by a 60% reduction in the number of cells entering the S-phase compared to unirradiated controls (data not shown). Cell cycle arrest was accompanied by p21WAF1 up-regulation peaking at 8 h after 18 Gy γ-radiation exposure, as determined by Western blot analysis (Fig. 3). In contrast, p16 levels remained low to undetectable in the 24 h period after irradiation. Thus, elevated p21WAF1, but not elevated p16, was associated with the normal HUC response to radiation damage.

Loss of p16 at Immortalization in E6-, but not E7-, transformed HUCs. We reported previously that two of two E6-transformed HUCs exhibited p16 loss in association with the immortalization event, whereas two of two E7-immortalized HUCs exhibited p16 elevation (20). To further test the association between loss of p16 expression and HPV16 E6 immortalization, the status of p16 was examined in an expanded series of five independently immortalized E6 HUCs and six independently immortalized E7 HUCs. Results showed that all five E6-immortalized HUCs (αE6-1, αE6-2, βE6-1, βE6-2, and δE6-1) generated from three different tissue samples (designated α, β, and δ) had undetectable p16 (Fig. 4). In contrast, all six E7-immortalized HUCs (αE7-1, αE7-2, δE7-1, γE7-1, εE7-1, and κE7-1) generated from five different tissue sources (designated α, δ, ε, γ, and κ) exhibited high levels of p16 levels similar to those seen in HUCs at senescence (Fig. 4).

Chromosome 9p21 Deletions and CDKN2 Mutations in E6 HUCs. To identify the underlying mechanisms for loss of p16 expression in the five immortalized E6-transformed HUCs (Fig. 4), the status of the CDKN2 gene was examined. First, cytogenetic analysis was done on cells at early passages postcrisis. Results showed that four of five immortal E6 HUCs had cytogenetically detectable 9p deletions in 90–100% cells (Table 1). Even though the region of deletion was different in each of these cell lines, all of the deletions included 9p21-pter. No 9p deletions were seen in any of the E7 HUCs used in this study.
To confirm the cytogenetically visible 9p losses, tests for LOH were done on E6 HUCs using microsatellite markers. Results showed that four of five E6-immortalized lines exhibited 9p21 LOH at the D9S942 locus, which maps within 5 kb of the CDKN2 gene (Table 1). This included the one cell line that did not show a cytogenetically detectable deletion. LOH was not detected in any of the cell lines in which a cytogenetic 9p deletion was observed, suggesting a complex translocation event. No homozygous deletions of the CDKN2 locus were detected, despite maintaining the cells in culture for more than 100 passages and more than 1 year. Finally, SSCP was done to screen for mutations in the first two exons of CDKN2 (data not shown). Only one of the five lines, ßE6-1, exhibited an abnormality using SSCP. Sequencing of ßE6-1 revealed a G to A change in codon 15 of the first exon, thus introducing a new stop codon. The mechanism underlying p16 loss in the other four lines is currently under further investigation.

**Discussion**

We previously reported that p16 levels are elevated in HPV16 E7-immortalized, but not E6-immortalized HUCs (20). We also reported that bladder cancer cell lines with altered pRb, but not with wild-type pRb, exhibited elevated p16 levels. The association between pRb loss and p16 elevation led us to hypothesize that loss of pRb may lead directly or indirectly to p16 up-regulation. It has recently been reported that p16 is elevated in human fibroblasts at senescence (22). We now report for the first time that p16 levels are elevated at senescence in a representative human epithelial cell type, namely, normal HUCs. We also report that this elevation of p16 at senescence becomes irrelevant to cell cycle progression and can be retained, as seen in all of the E7 HUCs and bladder cancer lines with mutant pRb. In the second scenario, reentry into the cell cycle in the presence of wild-type pRb is accomplished by loss of elevated levels of p16 that were observed at senescence, as in all of the E6-transformed HUCs (20). It is important to emphasize here that loss of p16 inhibition of cyclin D/cdk phosphorylation of pRb is insufficient for immortalization. E7-transformed HUCs, as well as E6-transformed HUCs, underwent senescence and remained in a crisis period of low proliferation for many weeks prior to immortalization (26). Thus, overcoming the putative p16-mediated cell cycle/senescent block, which should not affect E7 HUC proliferation, cannot be the only component of HUC immortalization. Indeed, we reported that additional genetic changes, including gain of 20q in E7-transformed HUCs and loss of 3p in E6-transformed HUCs, are nonrandomly associated with immortalization of both E6- and E7-transformed HUCs (23). Whether these additional changes reflect requirements for reactivation of telomerase activity, or some other event, is currently under investigation in many laboratories (30, 31). In summary, our data suggest that HUCs escaping from cellular senescence require at least two and possibly more genetic alterations.

LOH of the 9p21 region in which CDKN2/p16 is mapped correlated with p16 loss in E6 HUCs. Knudson's hypothesis would predict that the remaining CDKN2 allele in the E6 HUCs with 9p21 LOH and p16 loss would frequently show inactivating mutations. However, we detected such a mutation in only one of five immortalized E6 HUCs. Inactivation of the CDKN2 by methylation of upstream regulatory sequences has been reported (32, 33). We are currently examining E6

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**Table 1 9p deletions in E6-immortalized HUCs**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cytogenetic 9p21&gt;pter deletion</th>
<th>Microsatellite analysis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>α-E6-2</td>
<td>0</td>
<td>D9S256: RET D9S157: RET D9S162: RET IFNA: RET D9S942: RET D9S171: RET D9S161: RET</td>
</tr>
</tbody>
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<sup>a</sup> For each cytogenetic analysis, at least 20 complete karyotypes of cells after establishment in culture were analyzed. Data in parentheses, margin deletions. Cells were scored as having or not having a minimal deletion that included 9p21-pter.

<sup>b</sup> Microsatellite markers are arranged from telomere (D9S256) to centromere (D9S161) as described in "Materials and Methods."

<sup>c</sup> NI, noninformative; RET, retention of heterozygosity.
HUCs for such alterations. Homozygous deletions of the CDKN2 locus are a common mechanism for p16 loss in bladder cancer cell lines (4–7). Therefore, it has been suggested that homozygous CDKN2 deletions may be common artifacts of in vitro cell culture. In contradiction to this hypothesis, none of five independent E6 HUCs showed CDKN2 homozygous deletion. Therefore, these data do not support the hypothesis that homozygous deletion of CDKN2 is a common mechanism of p16 loss in cultured uroepithelial cells. Thus, the homozygous CDKN2 deletions observed in bladder cancer cell lines may have originated in vivo, and the population of cells with these deletions may form cell lines in vitro because they had a selective advantage. Finally, these data indicate that the frequency of CDKN2 mutations or homozygous deletions in a cancer may underestimate the frequency of p16 loss.

Senesence of normal HUCs, a predictable event in their in vitro life span, is accompanied by increased p16 levels. Immortalization of cells with functional pRb, such as E6-transformed HUCs, is highly correlated with p16 loss. The high correlation of p16 loss with 9p21 deletion and LOH suggest that the remaining allele is inactivated, but we have not yet defined the mechanism by which this occurs in most of our uroepithelial cell lines. E7-induced immortalization of HUCs, although requiring additional genetic events, aside from pRb inactivation as reported elsewhere (23), does not require p16 loss. This is reasonable since p16 depends on pRb for its inhibitory function. The same rationale would predict that human tumors in which a pRb inactivation occurs early in development might fail to show p16 loss, whereas tumors with wild-type pRb should frequently show p16 loss.

References

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