Association of p16\textsuperscript{INK4a} and pRb inactivation with immortalization of human cells

Takeki Tsutsui\textsuperscript{1}, Shin-ichi Kumakura\textsuperscript{1}, Akito Yamamoto\textsuperscript{1}, Hideaki Kanai\textsuperscript{1}, Yukiko Tamura\textsuperscript{1}, Takashi Kato\textsuperscript{1}, Masanori Anpo\textsuperscript{1}, Hitodoshi Tahara\textsuperscript{2} and J.Carl Barrett\textsuperscript{3,4}

\textsuperscript{1}Department of Pharmacology, The Nippon Dental University, School of Dentistry at Tokyo, Tokyo 102-8159, Japan. \textsuperscript{2}Department of Cellular and Molecular Biology, Hiroshima University, Graduated School of Biomedical Sciences, Hiroshima 734-8551, Japan. \textsuperscript{3}National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709, and National Cancer Institute, Bethesda, MD 20892, USA

\textsuperscript{4}To whom correspondence should be addressed
Email: barrett@mail.nih.gov

To examine the association of cell cycle regulatory gene inactivation with human cell immortalization, we determined the expression status of INK4a, Rb, and WAFI/CIP1, in eleven in vitro immortalized human cell lines, including fibroblasts and keratinocytes. Two human papillomavirus type 16 E6 expressing cell lines with telomerase activity, including a fibroblast cell line and a keratinocyte cell line, expressed no detectable p16\textsuperscript{INK4a}. These cell lines had a hyperphosphorylated pRb and reduced expression of p21\textsuperscript{WAF1/CIP1}. All of seven fibroblast cell lines immortalized either spontaneously or by \textsuperscript{60}Co, X-rays, 4-nitroquinoline 1-oxide or aflatoxin B\textsubscript{1}, maintaining their telomeres by the ALT (alternative lengthening of telomeres) pathway, displayed loss of expression of p16\textsuperscript{INK4a} and hyperphosphorylation of pRb. Levels of p21\textsuperscript{WAF1/CIP1} expression varied among the cell lines. Two fibroblast cell lines that became immortalized following infection with a retrovirus vector encoding human telomerase catalytic subunit (hTERT) cDNA were also accompanied by inactivation of p16\textsuperscript{INK4a} and pRb pathways. Acquisition of telomerase activity alone was not sufficient for immortalization of these cell lines. Taken together, all the cell lines including fibroblasts and keratinocytes, with either telomerase activity or the ALT pathway for telomere maintenance showed loss of expression of p16\textsuperscript{INK4a} and hyperphosphorylation of pRb. These demonstrate the association of inactivation of both p16\textsuperscript{INK4a} and pRb with immortalization of human cells including fibroblasts and epithelial cells and telomerase-positive cells and ALT-positive cells.

\textbf{Introduction}

Normal human cells have a limited proliferative potential \textit{in vitro}. After a finite, cell-type specific number of cell divisions, cells cease to divide and arrest in a viable G\textsubscript{0}/G\textsubscript{1} state termed replicative senescence (1,2). On the other hand, most human tumor-derived cells grow indefinitely and are designated immortal (3), suggesting that senescence is a mechanism of tumor suppression (4), and that immortalization of normal cells is a significant step in carcinogenesis.

Inactivation by SV40 large T antigen or human papillomavirus type 16 (HPV-16) E6 and E7 oncoproteins of cell cycle regulatory gene products, such as p53 and the retinoblastoma gene product (pRb), can extend the replicative lifespan of human fibroblasts, but rarely induce immortalization of the cells (5), suggesting that additional genetic event(s) are required for human cell immortalization. Inactivation of the other cell cycle regulatory gene product p16\textsuperscript{INK4a}, which is an inhibitor of an enzyme complex containing a cyclin D and cyclin-dependent kinase (CDK) 4/6 that phosphorylates pRb (6), is also implicated in mediating human cell immortalization (7,8). p16\textsuperscript{INK4a} is inactivated in many tumors through mutation, deletion or hypermethylation of the gene, leading to phosphorylation and inactivation of pRb (8). Loss of p16\textsuperscript{INK4a} protein expression is not only common in tumor-derived cell lines (6,9), but observed in several \textit{in vitro} immortalized human cell lines (9,10). Several lines of evidence using human normal cells (11) and tumor-derived cells (12–14) indicate an inverse relationship of p16\textsuperscript{INK4a} and pRb function in a common pathway. Kiyono \textit{et al.} (15) found that introduction of either HPV-16 E6 or human telomerase catalytic subunit (hTERT) cDNA alone into human foreskin keratinocytes or mammary epithelial cells fails to immortalize the cells, but that inactivation of the p16\textsuperscript{INK4a}/pRb pathway by HPV-16 E7 or down-regulation of p16\textsuperscript{INK4a} expression in combination with activation of telomerase is able to immortalize the cells. Requirement of inactivation of p16\textsuperscript{INK4a} and pRb functions accompanied by activation of telomere maintenance mechanisms, including telomerase or an alternative mechanism known as ALT (alternative lengthening of telomeres) (16), is also demonstrated in human fibroblast cell lines immortalized by introduction of HPV-16 E6, E7 or both E6 and E7, as described previously (17,18).

Loss of p16\textsuperscript{INK4a} expression is also demonstrated in non-virally immortalized human cells, i.e. spontaneously immortalized human fibroblasts derived from patients with the Li–Fraumeni syndrome (18) and the human fibroblast cell line SUSM-1 immortalized by a chemical carcinogen 4-nitroquinoline 1-oxide (4-NQO) (9). These immortal cells maintain or lengthen their telomeres by either telomerase or the ALT mechanism (9,10), but the status of pRb phosphorylation in these cells is unclear. All these findings suggest the association of p16\textsuperscript{INK4a} and pRb inactivation accompanied by activation of telomere maintenance mechanisms with immortalization of human cells including epithelial cells and fibroblasts and prompted our experiments to determine the expression status of p16\textsuperscript{INK4a} and pRb \textit{in vitro} established human cell lines. In addition, we compared the expression status of p16\textsuperscript{INK4a} and pRb between telomerase-positive and ALT-positive immortal cell lines. Furthermore, we studied the

---

© Oxford University Press 2002

\textbf{Abbreviations}: ALT, alternative lengthening of telomeres; CDK, cyclin-dependent kinase; ERK 1, extracellular-signal related kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPV, human papillomavirus; hTERT, human telomerase catalytic subunit; MSP, methylation-specific PCR; ppRb, hyperphosphorylated pRb; pRb, retinoblastoma gene product; RT–PCR, reverse transcription–PCR.
expression status of p21WAF1/CIP1 in the immortal cell lines, because p21WAF1/CIP1 inhibits cyclin-dependent kinase activity (for a review, see ref. 20), is highly expressed in senescent normal human fibroblasts (7), and induces senescence when expressed in immortal cells (10). Brown et al. (21) also report that inactivation of WAF1/CIP1 gene by two sequential rounds of targeted homologous recombination is sufficient to bypass senescence in normal human fibroblasts.

Materials and methods

Cell lines

The cell lines used are shown in Table I. WHE-7 E6 Cl 6 is a cell line immortalized by infection of WHE-7 cells (human fetal fibroblasts) with a retrovirus vector encoding the HPV-16 E6 genes, generously provided by D.A. Galloway. This cell line is clonally derived, and expresses mRNA for the infected oncogene. In addition, it shows loss of p53 and pRb functions. KMST-6 (22) immortalized by irradiation of KMST-6 (human fetal fibroblasts) with 60Co, and OUMS-24F (24) immortalized by treatment of OUMS-24 (human fetal fibroblasts) with 4-NQO were generously provided by M. Namba (Okayama University, Okayama, Japan). LCS-ST is a spontaneously immortalized cell line (25,26) derived from skin fibroblasts (MDAH 087) obtained from a Li–Fraumeni syndrome patient with a mutated p53 allele. LCS-AF-1, LCS-AF-1-3 and LCS-AF-1-2 (21) is a cell line immortalized by irradiation of MDAH 087 with X-ray. Epi E6 is a cell line immortalized by infection of normal human gingival keratinocytes (AK) obtained from the donor AK with a retrovirus vector encoding the HPV-16 E6 gene by two sequential rounds of targeted homologous recombination is sufficient to bypass senescence in normal human fibroblasts.

Western blot analysis

Western blots were performed by using the method described by Ikeda et al. (31) with a minor modification. Cells were lysed in buffer [0.1% Nonidet P-40/250 mM KCl/50 mM HEPES, pH 7.9/10% (v/v) glycerol/4 mM NaF/4 mM sodium orthovanadate/0.2 mM EDTA/0.2 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/1 aprotinin, pepstatin, and leupeptin each at 1 µg/ml]. Then, 50 µg of total protein per lane were run on a 6% SDS–PAGE gel for pRb and a 12% SDS–PAGE gel for p16INK4a and p21WAF1/CIP1 analyses and then transferred to nitrocellulose (Millipore, Tokyo, Japan). p16INK4a/WAF1/CIP1 analyses was observed with the p16INK4a monoclonal antibody (PharMingen, San Diego, CA), a horseradish peroxidase conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and detection by enhanced chemiluminescence (Amersham Pharmacia Biotech). The blot was exposed to X-ray films (RX-U, Fuji Film, Tokyo, Japan). pRb was observed with the pRb polyclonal antibody (C-15, Santa Cruz Biotechnology), while p21WAF1/CIP1 was observed with the p21WAF1/CIP1 polyclonal antibody (C-19, Santa Cruz Biotechnology). Both pRb and p21WAF1/CIP1 blotted using a horseradish peroxidase conjugated goat anti-donkey antibody (Santa Cruz Biotechnology), followed by detection with enhanced chemiluminescence (Amersham Pharmacia Biotech). The probe for extracellular-signal related kinase 1 (ERK1) was used as an internal control.

Telomerase assay

Telomerase activity in cells was detected by the telomeric repeat amplification protocol (TRAP) developed and modified by Shay’s group (32). Cells (106) in the logarithmic growth phase were suspended in 100 µl of ice-cold lysis buffer [0.5% CHAPS, 10 mM Tris–HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM AEBSF (a protease inhibitor) and 10% glycerol], and kept on ice for 30 min with occasional tapping. The lysate was centrifuged at 16 000 g for 20 min at 4°C. The supernatant (1 µl: equivalent to 104 cells) was assayed in 50 µl of reaction mixture containing 50 µM of each deoxynucleoside triphosphate, 344 nM of the deoxylucosine-nucleotide primer TS(5′-AATCCGTCGGACAGATG-3′), 0.5 µM T4 gene 32 protein (Boehringer Mannheim), 4 µCi [32P]deoxycytidine triphosphate (sp. act. ~3000 Ci/mmol, Amersham Pharmacia Biotech), 2 U of Taq DNA polymerase (Takara) and 5 µl of 10× PCR buffer in a 0.5 ml tube that contained 344 nM of the deoxylucosine-nucleotide primer CX(5′-CTTACCCCTACCTCTACCTAAA-3′) sequenced from the other reaction components by a DNA sequencer. After 20 min incubation at room temperature, the reaction mixture was amplified by PCR for 31 cycles in the presence of

Table I. Human immortal cell lines used in the present study

<table>
<thead>
<tr>
<th>Parental cells</th>
<th>Immortal cell lines</th>
<th>Cell type</th>
<th>Agents used for immortalization</th>
<th>Telomere maintenance mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHE-7 E6 Cl 6</td>
<td>WHE-7 E6 Cl 6</td>
<td>Fibroblast</td>
<td>E6α</td>
<td>Telomerase</td>
<td>17,18</td>
</tr>
<tr>
<td>KMS-6</td>
<td>KMST-6</td>
<td>Fibroblast</td>
<td>60Co</td>
<td>ALTα</td>
<td>23,24</td>
</tr>
<tr>
<td>OUMS-24</td>
<td>OUMS-24F</td>
<td>Fibroblast</td>
<td>4-NQOβ</td>
<td>ALT</td>
<td>24,25</td>
</tr>
<tr>
<td>MDAH 087</td>
<td>LCS-ST</td>
<td>Fibroblast</td>
<td>None</td>
<td>ALT</td>
<td>18,26,27</td>
</tr>
<tr>
<td>LCS-AF-1-3</td>
<td>LCS-AF-1-3</td>
<td>Fibroblast</td>
<td>Aftaxiton B1</td>
<td>ALT</td>
<td>18,27</td>
</tr>
<tr>
<td>LCS-4x2</td>
<td>LCS-4x2</td>
<td>Fibroblast</td>
<td>X-ray</td>
<td>ALT</td>
<td>18,22</td>
</tr>
<tr>
<td>Ay Ayt</td>
<td>Fibroblast</td>
<td>hTERT cDNA</td>
<td>Telomerase</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Pel Pelt</td>
<td>Fibroblast</td>
<td>hTERT cDNA</td>
<td>Telomerase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK Epi E6</td>
<td>Keratinocyte</td>
<td>E6</td>
<td>Telomerase</td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

*Human papillomavirus (HPV)-16 E6 genes; b4-nitroquinoline 1-oxide; cimmortalized spontaneously; dhuman telomerase catalytic subunit; ealternative lengthening of telomeres; f telomerase activity in this cell line was demonstrated in the present study.

Methylation-specific PCR (MSP)

CpG island promoter methylation of INK4a in the immortal cell lines was studied by MSP. DNA was extracted from the immortal cell lines as described previously (21). The extracted DNA (1 µg) underwent a bisulfite modification by using a CpGenome™ DNA modification kit (Intergen, Purchase, NY), and subsequently amplified with a CpG Wiz™ p16 amplification kit (Intergen) according to the manufacturer’s instructions.

Reverse transcription–PCR (RT–PCR)

Total RNA in cells was isolated by the RNAzol™ B extraction according to the manufacturer’s instructions (Tel-Test, Friendswood, TX). Total RNA (5 µg) was subjected to RT using the RTG You Prime First-Strand Beads™ (Amersham Pharmacia Biotech, Tokyo, Japan). PCR was performed in the reaction mixture (100 µl) containing 1 µl of the RT reaction product, 25 µM dNTP, 0.1 µl (10–3P)pICTP (Amersham Pharmacia Biotec), 0.025 U of Taq DNA polymerase (Takara, Tokyo, Japan) and 50 µM of sense and antisense primers for INK4a (exon 1x) (29) or Rb (exon 22–27) (30). RT–PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Each cycle consisted of denaturation at 94°C for 1 min, annealing for 1 min at either 56°C for INK4a or 55°C for Rb, amplification at 72°C for 2 min followed by a final elongation at 72°C for 5 min. After 35 cycles, a 20 µl aliquot of each reaction was subjected to polyacrylamide (6%) gel electrophoresis, stained with ethidium bromide, and visualized under UV illumination.
5 attograms of an internal TRAP assay standard (ITAS: generously provided by E. Hiyama, Hiroshima University, Hiroshima, Japan). The PCR product was subjected to electrophoresis on 10% polyacrylamide gels.

Results

First, we confirmed our previous findings on the involvement of inactivation of p16\(^{\text{INK4a}}\) and pRb in immortalization of the E6-expressing fibroblast cell line WHE-7 E6 Cl 6. We also examined the expression status of p16\(^{\text{INK4a}}\) and pRb in the human gingival keratinocyte cell line Epi E6 immortalized by HPV-E6 alone, because the requirement of p16\(^{\text{INK4a}}\)/pRb inactivation was suggested for immortalization of E6-expressing human mammary epithelial cells (15). As shown in Figure 1, the CpG island within the promoter region of INK4a was unmethylated in the parental WHE-7 cells (PD 10), while it was methylated in WHE-7 E6 Cl 6. INK4a CpG island was unmethylated in both Epi E6 and the parental AK cells. INK4a mRNA was expressed in WHE-7 and AK, but not in WHE-7 E6 Cl 6 and Epi E6 (Figure 2). Rb mRNA was expressed in all these cells (Figure 2). Although WHE-7 and AK expressed p16\(^{\text{INK4a}}\) protein, WHE-7 E6 Cl 6 and Epi E6 showed no detectable p16\(^{\text{INK4a}}\). Both E6-immortalized cell lines had a hyperphosphorylated pRb. Expression levels of p21\(^{\text{WAF1/CIP1}}\) were down-regulated in these immortal cell lines compared with the parental cells (Figure 3).

We next examined the expression status of p16\(^{\text{INK4a}}\), pRb, and p21\(^{\text{WAF1/CIP1}}\) in physical or chemical carcinogen-induced immortal fibroblasts. Although detected in the parental KMS-6, DNA sequences in the INK4a promoter region in the \(^{60}\)Co-immortalized cell line KMST-6 were undetectable with PCR analysis (Figure 1). The same results were obtained even when we used 10-fold (1 \(\mu\)g) of cellular DNA for PCR analysis (Figure 4A). KMST-6 contained a normal DNA sequence in the INK4a exon 1 because the size of PCR product obtained with KMST-6 (190 bp) was the same as those obtained with the parental KMS-6 cells or with the other normal fibroblasts WHE-7 (Figure 4B). The INK4a CpG island was unmethylated in both OUMS-24 and its immortal cell line OUMS-24F induced by 4-NQO (Figure 1). Although minor or major signals exhibiting methylated or unmethylated DNA, respectively, within the promoter region of INK4a were observed in MDAH 087 skin fibroblasts derived from a patient with Li-Fraumeni syndrome, the INK4a promoter was methylated in all of their five cell lines immortalized either spontaneously (LCS-ST) or by aflatoxin B\(_1\) (LCS-AF.1-2, LCS-AF.1-3 and LCS-AF.3-1) or X-rays (LCS-4x2) (Figure 1).

As shown in Figure 2, KMST-6 displayed loss of mRNA expression for INK4a. OUMS-24F retained the same level of INK4a mRNA expression as the parental OUMS-24 cells. INK4a mRNA expression was observed in the parental MDAH 087 cells, but not in any of their five LCS cell lines. All these immortal cell lines expressed the same levels of Rb mRNA as their parental cells.

Although p16\(^{\text{INK4a}}\) expression was observed in KMS-6, OUMS-24 and MDAH 087, their immortal cell lines expressed undetectable p16\(^{\text{INK4a}}\) (Figure 3). pRb proteins of these immortal cell lines were in a hyperphosphorylated state (Figure 3). The expression of p21\(^{\text{WAF1/CIP1}}\) was up-regulated in OUMS-24F and LCS-ST, but down-regulated in KMST-6 and the other four LCS cell lines compared with their parental cells (Figure 3).

It was previously demonstrated that WHE-7 E6 Cl 6 is telomerase-positive, and the other immortal fibroblast cell lines are ALT-positive (17, 23). As shown in Figure 5, TRAP assay showed that Epi E6 was positive for telomerase activity. When compared the expression status of p16\(^{\text{INK4a}}\) and pRb between the telomerase-positive immortal cell lines and the ALT-positive immortal cell lines, as well as between immortal cell lines consisting of fibroblasts or keratinocytes, there were no significant differences (Figure 3).
Inactivation of p16\(^{\text{INK4a}}\)/pRb pathway is necessary but not sufficient for human cell immortalization (15). Activation of the telomere maintenance mechanisms is considered as an additional genetic event (15). Thus, we examined the requirement of telomerase activity for immortalization of human fibroblasts. In this experiment, we studied the expression status of p16\(^{\text{INK4a}}\) and pRb in the following three human fibroblasts infected with a retrovirus vector encoding hTERT cDNA, i.e. WHEt, Ayt and Pelt, each of which was obtained by introduction of hTERT cDNA into WHE-7 cells at 9 PD, Ay cells at 8 PD or Pel cells at 9 PD, respectively. The cumulative growth of these three cells after infection is shown in Figure 6. WHE-7 cells infected with a retrovirus vector encoding neo gene alone senesced at 56 PD. Ay cells or Pel cells introduced neo alone senesced at 10 PD or 12 PD, respectively. Although WHEt exhibited a considerable extension of lifespan, it eventually senesced at 207 PD. On the other hand, Ayt and Pelt grew slowly for the first 340 days compared with WHEt, then maintained a vigorous growth with no signs of senescence. They are currently at \(\geq 400\) PD. All the hTERT-infected cells at early and late passages after infection displayed both hTERT mRNA expression and telomerase activity (Figures 7 and 8). In particular, WHEt at 190 PD that decreased in growth rate due to approaching senescence still expressed both phenotypes. Although very weak in Ayt at 16 PD, the expression of p16\(^{\text{INK4a}}\) was observed in all the parental cells and their hTERT-infected cells, except, Ayt at 192 PD and Pelt at 202 PD (Figure 9). As shown in Figure 10, INK4a mRNA was expressed in the three parental cells, WHEt at 26 PD and 190 PD, Ayt at 16 PD and Pelt at 17 PD but not in Ayt at 192 PD and Pelt at 202 PD. The CpG island within the promoter region of INK4a in the three parental cells and WHEt at 190 PD was unmethylated but similar levels of two signals showing methylation or unmethylation of the INK4a CpG islands were observed in Ayt at 192 PD and Pelt at 202 PD (Figure 11), suggesting that the absence of INK4a mRNA expression in both Ayt and Pelt might be due to either methylation of some other region of the CpG island that is more important in these cells or overexpression of the Polycomb-group gene bmi-1 identified in mouse lymphomas (32). Out of all cells examined, only Ayt at 192 PD and Pelt at 202 PD that became immortalized expressed a hyperphosphorylated pRb. WHEt that failed to immortalize expressed the same status of pRb as the parental WHE-7 cells (Figure 9). Even WHEt at 167 PD with no signs of senescence and in the exponential growth phase did not express a hyperphosphorylated pRb (data not shown). These results clearly demonstrate that acquisition of telomerase activity alone is not sufficient for immortalizing human fibroblasts, and that inactivation of p16\(^{\text{INK4a}}\) and pRb pathways is required for immortalization of human fibroblasts with telomerase activity.

**Discussion**

The status of p16\(^{\text{INK4a}}\), pRb, or p21\(^{\text{WAF1/CIP1}}\) expressions was examined using \textit{in vitro} immortalized human cell lines including fibroblasts and keratinocytes, in which telomere lengths were maintained either by activation of telomerase or by activation of the ALT mechanism (17). The results are summarized in Table II. Two E6-expressing cell lines with...
p16INK4a/pRb inactivation and human cell immortalization

Fig. 8. TRAP assay for detecting telomerase activity in human fibroblasts. Normal human fibroblasts (WHE-7, Ay and Pel) were infected with a retrovirus vector encoding hTERT cDNA, resulting in the creation of WHEt, Ayt and Pelt. Cells were harvested at the indicated PDs. The equivalent of 104 cells was analyzed with the internal TRAP assay standard (ITAS). The position of the ITAS is indicated.

Fig. 9. Western blotting for detecting p16INK4a and pRb in human fibroblasts. Normal human fibroblasts (WHE-7, Ay and Pel) were infected with a retrovirus vector encoding hTERT cDNA, resulting in the creation of WHEt, Ayt and Pelt. Cells were harvested at the indicated PDs. ERK1, used as an internal control.

Fig. 10. RT–PCR analysis for detecting mRNA expression for INK4a in human fibroblasts. Normal human fibroblasts (WHE-7, Ay and Pel) were infected with a retrovirus vector encoding hTERT cDNA, resulting in the creation of WHEt, Ayt and Pelt. Cells were harvested at the indicated PDs. GAPDH, used as an internal control.

Table II. Summary of the results in the present study

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Agents used for immortalization</th>
<th>Telomere maintenance mechanism</th>
<th>Protein expression</th>
<th>p16INK4a</th>
<th>pRb</th>
<th>p21WAF1/CIP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>Noneb</td>
<td>Telomerase (–)</td>
<td>ppRbg ±</td>
<td>ND j</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60Co ALT</td>
<td>Telomerase (–)</td>
<td>ppRb ▼</td>
<td>ND j</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-NQOc ALT</td>
<td>Telomerase (–)</td>
<td>ppRb ▼</td>
<td>ND j</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B1 ALT</td>
<td>Telomerase (–)</td>
<td>ppRb ▼</td>
<td>ND j</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X-ray ALT</td>
<td>Telomerase (–)</td>
<td>ppRb ▼</td>
<td>ND j</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>hTERT d cDNA</td>
<td>Telomerase (–)</td>
<td>ppRb ▼</td>
<td>ND j</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aHPV-16 E6 genes; bimmortalized spontaneously; c4-nitroquinoline 1-oxide; dhuman telomerase catalytic subunit; ealternative lengthening of telomeres; flittle or no expression; fhyperphosphorylated pRb; ghdecreased expression; ienhanced expression; jnot done.

maintaining their telomeres by the ALT pathway, lost expression of p16INK4a and displayed hyperphosphorylation of pRb. Levels of p21WAF1/CIP1 expression varied among the cell lines. Taken together, all the cell lines that were immortalized by E6 alone, or either spontaneously or by physical or chemical carcinogens showed both loss of p16INK4a expression and hyperphosphorylation of pRb, which demonstrates the inactivation of both p16INK4a and pRb pathways in the cell lines. Although the number of cell lines examined were limited, the results obtained from the present experiments also indicate no significant differences in the status of p16INK4a and pRb expressions between fibroblast cell lines and keratinocyte cell lines and between telomerase-positive cell lines and ALT-positive cell lines.

p16INK4a is commonly deleted in human cancers and cancer cell lines through mutation, deletion or methylation of the gene (for a review, see ref. 8). Most non-virally immortalized cell lines have no detectable expression of p16INK4a and displayed hyperphosphorylation of pRb. Levels of p21WAF1/CIP1 expression varied among the cell lines. Taken together, all the cell lines that were immortalized by E6 alone, or either spontaneously or by physical or chemical carcinogens showed both loss of p16INK4a expression and hyperphosphorylation of pRb, which demonstrates the inactivation of both p16INK4a and pRb pathways in the cell lines. Although the number of cell lines examined were limited, the results obtained from the present experiments also indicate no significant differences in the status of p16INK4a and pRb expressions between fibroblast cell lines and keratinocyte cell lines and between telomerase-positive cell lines and ALT-positive cell lines.
vector encoding INK4a cDNA induces the expression of p16INK4a protein and senescence in the immortal cells (10). These suggest the involvement of INK4a methylation in spontaneous immortalization of mammalian fibroblasts.

The CpG island within the promoter region of INK4a was methylated in the E6-immortalized fibroblast cell line, WHE-7 E6 Cl 6, which exhibited loss of mRNA and protein expressions for INK4a. Although INK4a CpG island was unmethylated, expressions of INK4a mRNA and protein were not observed in the E6-immortalized keratinocyte cell line, Epi E6. As the PCR method for detection of methylation examines only a limited subset of the CpG dinucleotides in this island, the loss of INK4a expression may be due to methylation that the primers do not detect. Alternatively, some mutation in the INK4a may reflect the loss of INK4a expression because Epi E6 shows loss of heterozygosity when assayed for the genomic region D9S161 or D9S171 adjacent to the INK4a. The cell line, KMST-6, immortalized by 60Co showed deletion of DNA sequences within the promoter region of INK4a, but contained a normal exon 1 INK4a. Loss of heterozygosity is observed at D9S171 locus near the genomic region of the INK4a in KMST-6. Deletion of the INK4a promoter may be a causal mechanism of the loss of p16INK4a expression in this cell line. Although expressed INK4a mRNA, the 4-NQO-immortalized cell line OUMS-24F failed to express its translated protein, which may be due to harborage of nonsense or frameshift mutations in the INK4a gene, or due to either defects of INK4a mRNA at the level of processing and translation or enhanced degradation of the p16INK4a protein, as shown in many types of human carcinoma cell lines (6). Loss of p16INK4a expression in the five LCS cell lines immortalized either spontaneously or by aflatoxin B1 or X-rays can be attributed to methylation of the promoter region of INK4a.

Introduction of hTERT cDNA into normal human fibroblasts results in a considerable extension of replicative lifespan (for a review, see ref. 8). However, MacKenzie et al. (36) report that introduction of hTERT is not sufficient for immortalization of normal human fibroblasts. Telomerase activity induced by hTERT cDNA alone is not sufficient for immortalization of normal human keratinocytes as well, and telomerase activity accompanied by inactivation of the p16INK4a/pRb pathway induces immortalization of the cells (15). In the present study using human fibroblasts infected with a retrovirus vector encoding hTERT cDNA, we demonstrated that activation of telomerase activity alone was not sufficient for immortalization of human fibroblasts. Inactivation of both p16INK4a and pRb pathways was required for immortalization of human fibroblasts with telomerase activity. This is a novel finding which differs from the results of others (37,38) who report the absence of cancer- or transformation-associated changes in human fibroblasts by introduction of hTERT cDNA. We have previously shown that loss of p16INK4a and pRb functions accompanied by activation of the ALT mechanism is also required for immortalization of human fibroblasts (17). These findings suggest that inactivation of p16INK4a and pRb pathway together with activation of the telomer maintenance mechanism is required for immortalization of human fibroblasts. The expression of p21WAF1/CIP1 was reduced or absent in the cell lines immortalized by HPV-E6, but it varied among the cell lines immortalized either spontaneously or by physical or chemical carcinogens, indicating the lack of common alterations in the p21WAF1/CIP1 expression. Spontaneously immortalized human fibroblasts following treatment with 5-aza-2'-deoxycytidine which elevates the level of p16INK4a undergo senescence with no detectable change in the expression of p21WAF1/CIP1 (10). These results suggest the level of p21WAF1/CIP1 expression itself is of little significance for immortalization of human cells.

In summary, we demonstrated in the present study that all the immortal cell lines including fibroblasts and keratinocytes exhibited inactivation of p16INK4a and pRb functions. Loss of p16INK4a and pRb functions alone, or activation of telomer maintenance mechanism alone was not sufficient for immortalizing normal human cells. These findings indicate the requirement of inactivation of p16INK4a and pRb pathways, accompanied by activation of telomer maintenance mechanism, for immortalization of normal human cells.

References

p16^{INK4a}/pRb inactivation and human cell immortalization


Received April 24, 2002; revised August 30, 2002; accepted September 5, 2002