Short Communication

Cervical Epithelial Cells Transduced with the Papillomavirus E6/E7 Oncogenes Maintain Stable Levels of Oncoprotein Expression but Exhibit Progressive, Major Increases in hTERT Gene Expression and Telomerase Activity

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Cervical carcinoma cells display high telomerase activity and usually contain and express integrated copies of the human papillomavirus (HPV) genome. Recent studies have demonstrated that the E6 oncogene of malignancy-associated HPVs increases cellular telomerase activity, predominantly via transcriptional activation of the catalytic subunit of telomerase, hTERT. To examine the relationship between E6 oncoprotein expression and telomerase expression during cellular immortalization, we transduced primary human cervical epithelial cells with the HPV E6/E7 genes and monitored temporal changes in viral oncoprotein expression, cellular hTERT RNA expression, and cellular telomerase activity. Quantitation of the individual E6 and E7 proteins, using a newly developed immunoprecipitation/immunoblotting technique, demonstrated that both oncoproteins were expressed at stable levels during successive passages of cervical cells. In contrast, the levels of hTERT mRNA and telomerase activity increased progressively and dramatically during passaging. Late-passage immortalized cells (passage 30) showed a 25-fold increase in hTERT mRNA and a 300-fold increase in telomerase activity compared to early-passage (passage 4) cells. Thus, neither hTERT mRNA expression nor telomerase activity are directly proportional to the level of E6 oncoprotein, indicating that E6 is not the sole determinant of the high levels of telomerase in cervical

cells during immortalization. (Am J Pathol 2002, 160:1251-1257)

Infection with specific high-risk types of human papillomaviruses (HPVs) such as HPV-16 is a critical etiological factor in the development of cervical cancer.¹ The high-risk group of HPVs synthesizes two viral oncoproteins, E6 and E7, that alter the function of cellular regulatory proteins.² The E7 protein interacts with pRb and abrogates its tumor-suppressive activity^{3,4} whereas the E6 protein cooperates with E6AP, a ubiquitin E3 ligase, to degrade p53 via a ubiquitin-dependent pathway.^{5,6} As a consequence of these and other activities of the E6/E7 proteins, keratinocytes reactivate DNA synthesis and exhibit altered growth and differentiation properties.⁷ Genital keratinocytes expressing the high-risk E6/E7 proteins also develop into cell lines *in vitro* (immortalization), a property not observed with the E6/E7 proteins of low-risk HPVs such as HPV-6 and HPV-11.^{8,9}

HPV-induced immortalization is closely linked to the increased activity of cellular telomerase, a ribonucleoprotein enzyme that synthesizes telomeric DNA.¹⁰⁻¹² Indeed, overexpression of the hTERT component of this enzyme complex can induce immortalization of many human cell types,^{13,14} apparently by preventing the progressive loss of telomere length during DNA replication. The telomerase activity of HPV-immortalized cells seems to result at least in part from the expression of the HPV E6 protein, because the HPV E6 protein can induce telomerase in keratinocytes that lack detectable activity.¹⁵

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More recent studies demonstrate that the induction of telomerase activity correlates directly with the E6-enhanced transcription of the hTERT promoter.^{16,17} E6-mediated activation of telomerase has also been shown to be independent of p53 degradation,¹⁵ eliminating the possibility that this transcription factor is a determinant of hTERT induction.

Although E6 can induce telomerase activity, it is clear that there are distinct cellular mechanisms operative during cell immortalization and transformation because E6-negative cervical carcinomas (as well as the vast majority of non-HPV-related tumors) also overexpress telomerase.^{18,19} To determine whether events in addition to E6 expression contribute to hTERT induction during HPV-mediated cellular immortalization, we performed the following experiments. Primary cervical cells were transduced with retroviruses containing the HPV-16 E6/E7 genes and then passaged in serum-free medium until cell immortalization had been achieved. At selected passages, the cervical cells were analyzed by quantitative techniques to correlate the expression of the E6 and E7 oncogenes with the level of hTERT mRNA and telomerase activity. Our results confirm that E6 does induce the rapid appearance of hTERT mRNA and telomerase in early-passage cervical cells, but that there is a subsequent progressive and profound increase in hTERT and telomerase expression during cell passaging that is independent of E6 protein levels.

Materials and Methods

Cell Culture

Primary human ectocervical keratinocytes were derived from fresh cervical tissue obtained after hysterectomy for benign uterine diseases. Standard trypsinization procedures were used to isolate the keratinocytes, which were cultured in serum-free keratinocyte medium supplemented with 50 μ g/ml of bovine pituitary extract and 26 ng/ml of recombinant epidermal growth factor (Life Technologies, Inc., Grand Island, NY).²⁰ Primary cultures were infected at passage 4 with high-titer LXS_N retroviruses expressing either the HPV-6 or HPV-16 E6/E7 genes, with the HPV-16 E6 gene containing an AU1 epitope at the 3'-terminus to facilitate immunodetection.¹⁶ Control LXS_N retroviruses expressed only the neomycin resistance gene. After infection, cells were selected with 50 μ g/ml of G418 for 5 days and were subcultured at least once before extraction of RNA. All subsequent passages were performed at a split ratio of 1:4.

Immunoprecipitation and Western Blotting

Cell lysate (700 μ g) was reacted with AU1 monoclonal antibody (BabCO) to immunoprecipitate the epitope-tagged HPV-16 E6 protein, or with ED17 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to immunoprecipitate the HPV-16 E7 protein. The immunoprecipitated proteins were separated in 1.5-mm polyacrylamide Tris-glycine minigels (Invitrogen, Carlsbad,

CA) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). For E6 immunoblot analysis, the membrane was labeled in washing buffer (0.5% Triton X-100, 140 mmol/L NaCl, 10 mmol/L Na₃PO₄) in the presence of 2% bovine serum albumin with 0.4 μ g/ml of HPV-16 E6 goat polyclonal antibody (N-17, Santa Cruz Biotechnology) for 90 minutes. For E7 analysis, the membrane was reacted with HPV-16 E7 mouse monoclonal antibody (ED17, Santa Cruz Biotechnology) in phosphate-buffered saline (PBS) containing 2% bovine serum albumin. After reaction with either alkaline phosphatase-conjugated mouse anti-goat IgG (GT34) antibody (Sigma Chemical Co., St. Louis, MO) or goat anti-mouse IgG antibody (Tropix, Bedford, MA) for 90 minutes, the E6 and E7 proteins were visualized with a chemiluminescent substrate (Tropix).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from ectocervical keratinocytes grown in 150-mm tissue culture dishes to 80% confluence using Trizol reagent according to instructions of the manufacturer (Life Technologies, Inc.). HeLa cells were used as a positive control. First-strand cDNA was synthesized as described previously²¹ with some modifications using 5 μ g of RNA and 0.5 μ g of oligo-dT by following the instructions of Superscript First-Strand Synthesis System for RT-PCR (Life Technologies, Inc.). Ten percent of the RNase H-treated cDNA product was subjected to PCR reaction for hTERT determination in a total volume of 50 μ l containing 10 μ mol/L sense primer (5'-CGGAAGAGTCTGGAGCAA-3') and 10 μ mol/L anti-sense primer (5'-GGATGAAGCGAGTCTGGA-3') as described in the manufacturer's protocol. Initial denaturation for 3 minutes at 94°C was followed by 30 cycles of PCR amplification (94°C for 45 seconds, 57°C for 60 seconds, 72°C for 60 seconds). The same cDNA-samples were applied to PCR amplification for 36B4, a ribosomal phosphoprotein selected as a riboprobe for endogenous RNA control.²² To demonstrate purity of extracted RNA, PCR was performed without adding reverse transcriptase to samples. PCR products were separated by electrophoresis in 10% polyacrylamide gels.

Quantitative RT-PCR

DNase-treated total RNA (1 μ g) was reverse-transcribed using random hexamers and oligo-dT from the Superscript First-Strand Synthesis System for RT-PCR (Life Technologies, Inc.). A control reaction with no reverse transcriptase was performed to check for contamination by genomic DNA. Multiplex PCR reactions contained first-strand cDNA, recommended amounts of 18S rRNA Predeveloped Taqman Assay Reagent and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nmol/L of sense primer (5'-AAAGCATTGGAATCAGACAGCA-3') 900 nmol/L of anti-sense primer (5'-TTCACAATCGGCCGAG-3'), and 250 nmol/L FAM-labeled probe (5'-CCTGCTGACGTCCAGACTCCGCTT-

3'). PCR reactions were performed in duplicates on the ABI Prism 7700 sequence detector (50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute). Data were analyzed with Sequence Detector v1.6.3. software (Applied Biosystems). 18S RNA was used as a housekeeping gene to normalize amounts of RNA. For each reaction, expression was calculated as $2^{-\Delta C_t}$, where ΔC_t is the difference between the C_t for the gene of interest and the C_t for 18S RNA. The average expression for the duplicates is reported with the SD.

Telomerase Activity Assay

Human cervical keratinocytes were grown in 100-mm tissue-culture dishes to 80% confluence, harvested by trypsinization, washed in cold PBS, and transferred to a microfuge tube. Cell pellets were lysed for 30 minutes on ice in 400 μ l of telomeric repeat amplification protocol (TRAP) buffer (0.5% Chaps, 10 mmol/L Tris, pH 7.5, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 5 mmol/L β -ME, 10% glycerol, 0.1 mmol/L 4-(2-amino-ethyl)benzene-sulfonyl fluoride hydrochloride (AEBSF). Lysates were centrifuged at $14,000 \times g$ for 5 minutes at 4°C, the supernatant was transferred to a new tube, and protein concentration was determined (Bio-Rad, Richmond, CA). A TRAP assay¹⁸ was performed on 5 μ g, 1.5 μ g, 0.15 μ g, and 0.015 μ g of protein lysates as described, with some modifications. Lysates were incubated for 30 minutes at room temperature in a 50- μ l reaction volume containing 1 $\times$ PCR buffer (20 mmol/L Tris, pH 8.4, 50 mmol/L KCl), 100 ng telomerase substrate primer (5'-AATCCGTCGAGCA-GAGTT-3'), 50 μ mol/L each deoxynucleoside triphosphate (dATP, dTTP, dGTP, dCTP), 1.5 mmol/L $MgCl_2$, and 0.5 μ g T4 gene protein (Boehringer Mannheim, Indianapolis, IN). After initial denaturation for 4 minutes at 94°C 100 ng of downstream primer (5'-CCCTTACCCT-TACCCTTACCCTAA-3') and 2.5 U of *Taq* DNA polymerase were added, followed by 31 cycles of PCR amplification (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 45 seconds). HeLa cells (HPV-18-positive cervical cancer cell line) served as a positive control and IMR90 cells (normal embryonic lung fibroblast cell strain) as a negative control for telomerase activity, respectively. Twenty percent of the PCR products were separated on 10% nondenaturing polyacrylamide gels and visualized using the Gelcode color silver-staining kit (Pierce, Rockford, IL).

Results

Effect of Low-Risk and High-Risk HPV E6/E7 Oncogenes on the Life Span of Ectocervical Epithelial Cells

Primary cultures of human ectocervical keratinocytes were established from fresh cervical tissue as in Materials and Methods. After the fourth passage, cells were infected with three different LXSNS: an empty

Figure 1. Immunoblot analysis of E6 and E7 oncoproteins in transduced cervical epithelial cells. At the indicated passage number, protein extracts were prepared from cervical cells that had been transduced with the HPV-16 E6/E7 oncogenes. The lysates were immunoprecipitated and immunoblotted with the respective antibodies for epitope-tagged E6 (**A**) and E7 (**B**) as described in Materials and Methods. E6 and E7 proteins were detected only in cells transduced with the viral oncogenes and appeared stable throughout passing.

vector LXSNS virus (control), LXSNS containing low-risk HPV-6 E6/E7, and LXSNS containing high-risk HPV-16 E6/E7. Expression of the HPV-6 and HPV-16 E6/E7 oncogenes by these retroviruses was confirmed by quantitative PCR and Northern blot analysis (data not shown). As expected, the LXSNS control cells exhibited a very short life span, an average of eight population doublings. After transduction with low-risk HPV-6 E6/E7, cell proliferation was slightly extended to an average of 10 population doublings. In contrast, cells transduced with the high-risk HPV-16 E6/E7 genes proliferated indefinitely and have been maintained *in vitro* for more than 200 population doublings without evidence of a detectable crisis period. Two additional, independent experiments performed with this same protocol have given similar results.

High-Risk E6/E7 Oncoprotein Levels Remain Stable during *In Vitro* Cell Passaging

Generating immortalized cervical cells via repeated cell passaging (as above) could potentially result in the selective outgrowth of a subset of E6/E7-transduced cells. It was possible, therefore, that late-passage cultures might consist of cells with a selective growth advantage because of higher levels of viral oncoprotein expression. To evaluate this possibility, we developed an immunoprecipitation/immunoblotting technique that allowed us to measure the amount of epitope-tagged E6 as well as E7 oncoprotein in cervical cells and to determine whether there were indeed variations in oncoprotein levels that were dependent on passage number (Figure 1). Our results were somewhat unexpected. The levels of the E6 and E7 proteins showed virtually no change from early passage (passage 4) through immortalization (passage 30). Titration of cell lysates used in the immunoprecipitation demonstrated that the detected levels of E6 and E7 proteins were within the linear range for the immunoprecipitation/Western technique (data not shown). These results are consistent with the hypothesis that the transduced, selected cells were uniformly expressing E6/E7

Figure 2. Telomerase hTERT mRNA expression in E6/E7-transduced cervical cells as determined by qualitative and quantitative RT-PCR. **A:** Qualitative RT-PCR. Levels of hTERT mRNA were evaluated in the transduced cells via RT-PCR as described in Materials and Methods. Primers were also used to detect control ribosomal 36B4 mRNA to ensure cDNA integrity and to control for sample loading. hTERT RNA expression was observed in high-risk HPV-16 E6/E7-transduced cells but not in low-risk HPV-6 E6/E7 cells or in control LXSN cells. The HPV-16 E6/E7 cells showed a progressive increase in hTERT mRNA during cell passaging (passage 4 to passage 30). **B:** Quantitative RT-PCR. To verify the above results, temporal changes in the expression of telomerase hTERT mRNA were also determined by real-time quantitative RT-PCR. Levels of hTERT mRNA were evaluated in ectocervical cells transduced with retrovirus LXSN (control), HPV-6 E6/E7, or HPV-16 E6/E7. The primers and methods for this analysis are described in detail in Materials and Methods. Error bars indicate the SD of duplicate PCR reactions. High-risk HPV-16 E6/E7 induced an acute 20-fold increase in hTERT expression (LXSN versus HPV-16 E6/E7 passage 1) that further increased up to 500-fold at the stage of immortalization (LXSN versus HPV-16 E6/E7 passage 20).

and that these levels of expression did not change at later times. In addition, our data suggest that there is no apparent growth advantage for cells containing higher levels of E6/E7 than detected at early times.

hTERT mRNA Expression Increases during in Vitro Passaging

If the induction of hTERT mRNA (catalytic subunit of telomerase) and telomerase activity required only E6 expression, then it would be anticipated that they, like E6, would also remain constant throughout cell passaging. To examine this possibility, we monitored hTERT mRNA expression by reverse transcriptase-PCR amplification as well as quantitative PCR amplification. RNA was isolated from control, low-risk, and high-risk E6/E7-transduced cervical cells, reverse-transcribed to generate cDNAs, and subjected to PCR (30 cycles) with the hTERT primers indicated in Materials and Methods (Figure 2A). The ex-

pression of hTERT increased progressively with cell passage, with the highest levels being detected at passage 30. Analysis of the samples with a 36B4-specific riboprobe confirmed the uniformity of the cDNA reactions. Thus, the apparent level of hTERT mRNA increased with successive passages. To accurately measure this progressive increase in hTERT mRNA, we used a quantitative PCR methodology. Cellular RNA was reverse-transcribed as described in Materials and Methods to generate a first-strand cDNA preparation. Subsequent multiplex amplifications were performed with the Taqman assay system and analyzed with the ABI Prism 7700 sequence detector (Figure 2B). 18S RNA was also amplified as a control housekeeping gene and used to normalize the hTERT data. Similar to the initial PCR results shown in Figure 2A, the quantitative PCR experiments demonstrated that cells transduced with the HPV-16 E6/E7 genes exhibited a 25-fold increase in hTERT mRNA at late passages (passage 20) when compared to the same cells at passage 1. Intermediate passage cells (passage 6) showed an intermediate level of hTERT increase (threefold increase compared to passage 1). Thus, two independent assays have verified that hTERT expression increases progressively with cell passaging. Similar results were obtained from a second series of transduced cervical cells.

TRAP Assays Demonstrate Increasing Telomerase Activity during Cell Passaging

To evaluate whether the increased levels of hTERT mRNA were reflected in corresponding increases in telomerase activity, we performed a highly sensitive PCR-based assay referred to as the telomeric repeat amplification protocol (TRAP assay). Five μg of protein was used to measure telomerase at the same time points at which we measured hTERT mRNA (Figure 3A). Telomerase activity was clearly not detectable at any time in LXSN control cells or in ectocervical cell lines transduced with low-risk HPV-6 E6/E7. These findings parallel the lack of hTERT mRNA levels in these cells. In contrast, viral transduction with high-risk HPV-16 E6/E7 oncogenes immediately induced telomerase activity in ectocervical cells. This activity increased progressively throughout cell passaging, similar to the increases in hTERT mRNA.

To estimate the relative telomerase activity of the various cell extracts, we performed a series of TRAP assays on sequential dilutions of cellular protein: 5.0 μg (Figure 3A), 1.5 μg (Figure 3B), 0.15 μg (Figure 3C), 0.015 μg (Figure 3D). Cells with the highest telomerase activity will exhibit a positive TRAP assay even at the higher dilutions. Because we observed similar TRAP assay signals for early-passage cells (passage 4) at high protein concentration (5 μg /reaction) compared to late-passage cells (passage 30) at low protein concentration (0.015 μg /reaction), we can estimate that late-passage cells have ~300-fold more telomerase activity than early-passage cells. These assays have been repeated at least three times for each cell line and passage number with virtually identical results.

Figure 3. Telomerase activity in E6/E7-transduced cells. To verify that the increased hTERT expression observed in Figure 2 resulted in increased telomerase activity, the same cells were extracted and 5 µg of total protein was assayed by TRAP assay. Telomerase activity was detected in HPV-16 E6/E7 cells but not in control LXSN cells or cells transduced with HPV-6 E6/E7. HeLa cells (HPV-18-positive cervical cancer cell line) and IMR-90 cells (normal embryonic lung fibroblasts) served as telomerase-positive and telomerase-negative controls, respectively. Serial dilutions of lysates from HPV-16 E6/E7-expressing cells were assayed to provide semiquantitative estimates of telomerase activity (**A**, 5 µg; **B**, 1.5 µg; **C**, 0.15 µg; and **D**, 0.015 µg). HPV-16 E6/E7 cells showed a 300-fold increase in telomerase activity between passages 4 and 30 (compare similar activities of **lane 4** of **A** with **lane 7** of **D**).

Discussion

The experiments outlined in this study addressed the question about whether cellular telomerase activity in cervical epithelial cells is directly proportional to the expression of the HPV-16 E6 and E7 oncoproteins. We investigated this relationship during the process of cell passaging and immortalization after the retroviral transduction of the HPV-16 E6/E7 oncogenes. However, before correlating E6/E7 protein expression with hTERT mRNA and telomerase activity, we first had to develop an immunoprecipitation/immunoblotting technique that allowed us to monitor the E6/E7 proteins in stable cell strains and cell lines. Although these proteins can be detected readily when overexpressed,⁷ they have been

extremely difficult to observe in immortalized cells, and most studies have resorted to measuring respective mRNA levels as an indirect indicator of expression.² The combined immunoprecipitation/immunoblotting assay described in this study provides the required enhanced sensitivity to monitor these low-abundance proteins and to our knowledge it is the first time that E6/E7 protein expression has been detected in human cervical cells.

As anticipated from previous studies,^{15,16,23} our results show that the E6 oncoprotein rapidly induces hTERT expression and telomerase activity. In this investigation, we have used real-time PCR to obtain reliable quantitation of hTERT mRNA levels. Our studies demonstrate clearly that, despite stable expression of the E6 and E7 proteins, there is a progressive and dramatic increase in hTERT mRNA expression and telomerase activity during cell passaging *in vitro*. The level of hTERT and telomerase activity in cervical cells, therefore, is not directly proportional to the level of E6 expression. This observation could result from either of three distinct mechanisms:

First, telomerase expression, induced uniformly in all cells by E6 at early times, might be further amplified by cellular mechanisms during the process of immortalization. In the current *in vitro* system in which we do not observe a distinct crisis phase of cell proliferation, this would suggest that cellular immortalization is a nonclonal event with many different cells becoming immortal, and with all exhibiting higher telomerase activity at late times. The increase of telomerase activity observed in late-passage or immortalized cells would therefore be a composite of E6-dependent and E6-independent mechanisms.

Second, telomerase activity might be imprinted or established at an early time by E6, with some cells expressing very high levels of telomerase and other cells expressing lower levels. Cell passaging would then select for the subpopulation of transduced cells that had high telomerase activity. This second proposed mechanism is consistent with the experimental findings of Kiyono and colleagues¹² who found that clones of E6/E7-transduced cells varied considerably in their telomerase activity. They hypothesized that only cells with higher telomerase activity, and consequently longer average telomeres, were selected for continuous proliferation.

Third, early-passage keratinocytes represent a mixed population of stem cells, replicative cells, and differentiating cells. The stem cells, although representing only a very small percentage of the total number of cervical cells, are believed to be responsible for the detectable levels of telomerase observed in early-passage cervical cells.²⁴ Later passage cells lack this activity. Thus, it is possible that E6 induces telomerase in all cervical cells, but that the induction in stem cells results in higher levels of telomerase (because of the combined action of E6 and existing cellular factors) and the selective outgrowth of these cells produces the progressive increase in hTERT and telomerase.

Although one study has reported that telomerase activity can only be detected in late-passage HPV-immortalized keratinocytes,²⁵ several laboratories, including our own, have shown that telomerase is rapidly induced in nonimmortalized keratinocytes by E6.^{15–17,26} Our anal-

ysis of hTERT expression by real-time PCR further substantiates this early induction in nonimmortal cells. Indeed, this sensitive quantitative PCR technique can detect low-level hTERT mRNA expression at the earliest time after transduction with E6.

Although hTERT mRNA and telomerase activity are increased in the large majority of cervical cancers,^{10,11,27,28} telomerase activity is probably not applicable as a prognostic factor in early-stage cervical cancer.²⁸ For example, telomerase activity has also been detected in normal cervical tissues^{29,30} as well as in benign cervical lesions^{30–32} and is independent of the presence of HPV.³³ It is possible, however, that quantitative detection of hTERT mRNA by real-time PCR could be useful diagnostically. The absolute levels of hTERT mRNA in normal cervical tissue and low-risk HPV-induced neoplasms would be expected to be much lower than those found in high-risk HPV-expressing lesions.

In summary, the mechanism responsible for the progressive increase of hTERT gene expression (and concomitant telomerase activity) during cellular immortalization is unknown. Identifying the relevant cellular changes involved in this progression should assist in understanding and diagnosing the tumorigenic conversion of HPV-induced lesions.

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