The Human Papillomavirus Type 16 E6 and E7 Oncoproteins Independently Induce Numerical and Structural Chromosome Instability

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ABSTRACT

The development of genomic instability is a hallmark of high-risk human papillomavirus (HPV) associated cervical carcinogenesis. We have previously shown that the HPV-16 E7 oncoprotein rapidly subverts mitotic fidelity by inducing abnormal centrosome numbers and multipolar mitotic spindles. Here we report that expression of HPV-16 E6 and E7 independently results in various mitotic abnormalities. HPV-16 E6 and E7 were each associated with unaligned or lagging chromosomal material, indicating relaxation of spindle checkpoint control. Moreover, by overwhelming checkpoint control mechanisms that may prevent cells with multiple spindle poles to enter anaphase, expression of HPV-16 E6 and E7 leads to a small but significant number of cells with altered polarity at later stages of the cell division process. In addition to changes that have the potential to give rise to numerical chromosome imbalances, we discovered that expression of HPV-16 E7 could trigger anaphase bridge formation to an extent similar to that of high-risk HPV E6. Anaphase bridges typically develop after chromosomal breaks and alterations of chromosomal structure. Further investigation of mechanisms by which HPV-16 E6 and E7 contribute to the destabilization of the host cell genome revealed that both high-risk HPV oncoproteins induce DNA damage. Moreover, expression of HPV-16 E7 was associated with an increased number of cells exhibiting nuclear foci of phosphorylated histone H2AX as well as activation of cell cycle checkpoints triggered by DNA repair. Our results therefore suggest that HPV oncoproteins are a source for both numerical and structural chromosome instability during HPV-associated carcinogenesis.

INTRODUCTION

Cervical cancer is intimately associated with infection by certain high-risk HPV3 types such as HPV-16 or HPV-18. At least 90% of cervical carcinomas harbor high-risk HPV DNA integrated into the host cell genome and two viral oncoproteins, E6 and E7, are consistently expressed (1). The high-risk HPV E6 and E7 oncoproteins target host cell genome and two viral oncogenes, E6 and E7, are consistently expressed (1). The high-risk HPV E6 and E7 oncoproteins target suppressor signaling pathways that are critical for cellular growth control. Whereas high-risk HPV E6 induces accelerated pro-apoptotic degradation of p53 (2), high-risk HPV E7 binds and degrades the pRB retinoblastoma tumor suppressor protein (3, 4) and interacts with the cyclin-dependent kinase inhibitors p21WAF1/CIP1 (5, 6) and p27KIP1 (7).

The development of genomic instability is an early and crucial event during HPV-associated carcinogenesis (8). As in many other human cancers, the chromosomal changes observed in cervical carcinomas or high-risk HPV immortalized cells are complex (9, 10). They include numerical chromosomal imbalances such as gains and losses of whole chromosomes as well as structural chromosome changes e.g., gains of chromosome arm 3q (9). The relative roles of high-risk HPV E6 and E7 for the induction of complex patterns of chromosomal instability have not yet been defined in molecular detail. Previous studies have shown that expression of the HPV E6 oncoprotein is associated with structural chromosome alterations (11) and relaxation of mitotic checkpoint control (12, 13). Our previous studies showed that HPV-16 E7 rapidly induces primary centrosome duplication errors giving rise to abnormal centrosome numbers in otherwise normal cells. HPV-16 E7 thus functions as a mitotic mutator that drives formation of multipolar mitotic spindles, chromosome missegregation, and aneuploidy (14). In contrast, HPV-16 E6 expressing cells with diminished p53 levels, centrosome abnormalities accumulate in parallel with nuclear atypia, predominantly multinucleation (15). This is consistent with the model that in many cases inactivation of p53 may not be the primary trigger for centrosome abnormalities and genomic instability but has a permissive effect on genome disintegration (16–20).

Here, we show that the high-risk HPV-16 E6 and E7 oncoproteins can independently induce various mitotic abnormalities. HPV E6 and E7 were both associated with an increase of unaligned or lagging chromosomal material in cells undergoing mitosis. Our studies extend previous results by demonstrating that HPV E6 and E7 can overwhelm checkpoint control mechanisms in primary human keratinocytes that halt a large fraction of metaphase cells with multiple spindle poles to enter anaphase. These activities of the HPV E6 and E7 oncoproteins give rise to a significant number of multipolar anaphase cells in either population. Most remarkably, we found that expression of HPV-16 E7 can induce anaphase bridge formation to an extent similar to that of high-risk HPV E6. Anaphase bridges typically occur after structural chromosomal changes and chromosome breaks. To corroborate these results, we show that HPV-16 E6 and E7 can both independently induce DNA damage in primary human keratinocytes. In contrast to HPV-16 E6, keratinocyte populations expressing the HPV E7 oncoprotein exhibited an increased number of cells with enhanced nuclear PARP expression in combination with nuclear foci of phosphorylated histone H2AX. This observation further supports the notion that high-risk HPV E7 can induce DNA damage independent from the high-risk HPV E6 oncoprotein. We conclude that high-risk HPV-16 E6 and E7 oncoproteins each contribute to both numerical and structural chromosome instability and can therefore trigger the complex chromosomal changes that are observed in high-risk HPV-associated cancers.

MATERIALS AND METHODS

Cell Culture and Retroviral Infections. NHKs from neonatal foreskins were isolated as described previously (6) and cultured in keratinocyte growth medium (LifeTechnologies, Gaithersburg, MD) supplemented with bovine pituitary extract and epidermal growth factor (LifeTechnologies) at 37°C in 7% CO2. For retroviral infections, recombinant LXSNN-based retroviral vectors expressing low-risk HPV-6 E6 or E7 or high-risk HPV-16 E6 and/or E7 were used (21). Cells were selected in 200 μg/ml G418 (Gemini, Woodland, CA) for 48 h, and stable populations were expanded. In a subset of experiments, stable NHK populations expressing HPV-16 E6 and E7 were engineered by subsequent infection of LXSNN-HPV-16 E6-transduced cells with pBabe-HPV-16 E7 as described previously (15), followed by selection in 2 μg/ml
puromycin (Calbiochem, San Diego, CA) for 48 h and expansion of drug-resistant populations. Only cell populations at early passages were used for experiments, and age-matched cells expressing empty vector were included in all experiments as negative controls.

**Immunological Methods.** Cell lysates were made and analyzed by immunoblot as described previously (6). The antibodies used were: p53 [Ab-6 (Calbiochem) or anti-p53 (Cell Signaling, Beverly, MA)]; phospho-p53-Ser15, cdc2, phospho-cdc2-Tyr15 (all from Cell Signaling); PARP (C2–10; Trevigen, Gaithersburg, MD), HPV-16 E7 (ED17; Santa Cruz Biotechnology, Santa Cruz, CA); and actin (Chemicon, Temecula, CA).

For immunofluorescence analysis, cells were grown on coverslips, fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 1% Triton X-100 in PBS for 20 min at room temperature. Cells were then blocked with 10% normal donkey serum in water for 10 min at room temperature. A monoclonal antibody against γ-tubulin (GTU-88; Sigma-Aldrich, St. Louis, MO) was used at a 1:2000 dilution in PBS for at least 1 h followed by donkey antimouse secondary antibody labeled with Rhodamine Red (Jackson Immunoresearch, West Grove, PA) at a 1:100 dilution in PBS for at least 1 h at 37°C. For detection of PARP expression, cells were incubated with a monoclonal anti-PARP antibody (C2–10; Trevigen) at a 1:500 dilution followed by Rhodamine Red donkey antimuscope secondary antibody at a 1:200 dilution. Coexpression of PARP and γ-H2AX was assessed by sequential immunofluorescence staining for PARP followed by an anti-γ-H2AX antibody (Trevigen) at a 1:100 dilution followed by a FITC-labeled donkey antirabbit secondary antibody at a 1:200 dilution. Cells were washed in PBS, counter-stained with Hoechst 33258 DNA dye, and analyzed using a Leica DMLB epifluorescence microscope equipped with a multiband filter set (Omega Optical, Brattleboro, VT) and a Sony DRC5000 digital camera. Images were transferred to Adobe’s Photoshoop for printout.

**Telomere Length Assay.** To assess telomere length, the TeloTAGGE telomere length assay (Roche Molecular Biochemicals, Mannheim, Germany) was used according to the manufacturer’s instructions.

**Comet Assay.** DNA breaks were detected using the CometAssay kit (Trevigen, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, cells were harvested and plated in low melting point agarose on slides. After hardening of the agarose gel, cell were lysed and subjected to alkali electrophoresis for 30 min at 4°C. After air-drying, DNA was visualized using SYBR green. Cells were scored by epifluorescence microscopy for the distribution of DNA between the “tail” and the “head.” Whereas the DNA that remained in the nucleus (head) represents undamaged DNA, the tail contains fragmented DNA that was able to move out of the cell body during electrophoresis.

**Statistical Analysis.** Statistical significance was assessed using Student’s two-tailed t test for independent samples. In at least 3 independent experiments, more than 50 cell division figures were evaluated for each cell population studied, and mean + SE are given unless indicated otherwise.

**RESULTS**

**HPV-16 E6 and E7 Independently Induce Chromosome Misalignment and Lagging Chromosomes during Mitosis.** To characterize the spectrum of cell division abnormalities, we manipulated primary human foreskin keratinocytes to stably express HPV-16 E6 and/or E7. HPV-16 E7 expression was directly assessed by immunoblot analysis, whereas E6 expression was monitored by analyzing p53 steady-state levels that were decreased because of HPV E6 induced degradation (data not shown). Nuclei of dividing cells at early passages were studied morphologically using the Hoechst 33258 DNA dye to visualize unaligned or lagging chromosomal material. In addition, cells were costained for centrosomes by immunofluorescence for γ-tubulin (22).

We frequently observed un- or misaligned chromosomal material as characterized by Hoechst 33258-positive structures at a distance from the metaphase plate (Fig. 1, A–D). Un- or misaligned chromosomal material was found either in the presence of a bipolar (Fig. 1A) or multipolar (Fig. 1, B and D) mitotic spindle.

Lagging chromosomal material was observed during anaphase, often in combination with additional abnormalities such as anaphase bridges (Fig. 1C). A prominent feature in keratinocytes expressing both HPV-16 E6 and E7 was the formation of metaphases with a dramatic increase of the chromosomal material ("giant" metaphases). These metaphases regularly presented with multiple spindle poles and unaligned chromosomes (Fig. 1D).

We next quantified these abnormalities to investigate potential differences between HPV-16 E6 and/or E7 oncoprotein-expressing cell populations (Fig. 1E). The incidence of un- or misaligned chromosomal material during metaphase was at 0.7% of mitoses in LXSN-infected control cells. The fraction of metaphases with unaligned chromosomal material increased 10.7-fold to 7.5% in HPV-16 E6-expressing cells, whereas HPV-16 E7-expressing cells and cells expressing both HPV-16 E6 and E7 showed a 9- and 14.1-fold increase to 6.3 and 9.9%, respectively. Differences between LXSN-infected controls and NHKs with coexpression of HPV-16 E6 and E7 were statistically significant (P < 0.05; Fig. 1E). In a representative experiment, the incidence of unaligned chromosomal material in low-risk HPV-6 E6 or E7-expressing NHKs was not increased relative to controls.

We next studied the presence of lagging chromosomal material in anaphase/telophase cells (Fig. 1E). The percentage of anaphase/telophase cells with lagging chromosomal material was 1.3% in LXSN-infected control cells. This proportion was 1.6-fold increased to 2.1% in HPV-16 E6-expressing cells and 2.5-fold increased to 3.3% in HPV-16 E7-expressing cells. In HPV-16 E6 and E7-expressing cells, we observed a 3.9-fold increase to 5.1%. These results did not yield statistical significance. In a representative experiment, lagging chromosomal material was detected in 1% of anaphases in keratinocyte populations expressing low-risk HPV-6 E6 or E7, respectively. This value is similar to that in control cells.
Our findings show that HPV-16 E6 and E7 can independently induce cell division abnormalities that can lead to chromosomal gains and/or losses. These changes comprise un- or misalignment of chromosomal material during metaphase and lagging chromosomas at later stages of the cell division process. Moreover, coexpression of HPV E6 and E7 results in a further increase of these changes during metaphase and anaphase/telophase.

Expression of HPV-16 E6 and/or E7 Can Overcome Mitotic Checkpoint Control That Blocks Normal Human Keratinocytes to Enter Anaphase in the Presence of Multiple Spindle Poles. As reported previously, multipolar metaphases with supernumerary spindle poles are frequently observed in HPV-16 E6 and/or E7-expressing keratinocytes (14, 15). However, control mechanisms monitoring centrosome-related mitotic defects in primary human cells have not been studied in detail, and it is still unclear whether such checkpoints exist (23). We therefore compared the proportion of multipolar metaphase cells to the proportion of cells in anaphase or telophase that exhibited a multipolar configuration. During a normal cell division, a bipolar metaphase spindle (Fig. 2A) ensures symmetrical distribution of the chromosomes between the two daughter cells during anaphase and telophase (Fig. 2B). Failure to control centrosome duplication can result in monopolar spindle formation (if duplication was unsuccessful; Fig. 2C), or multipolar spindles (if centrosome duplication errors lead to supernumerary centrosomes; Fig. 1, B and D). In HPV oncoprotein-expressing NHKs, monopolar spindle formation hindering chromosome segregation was very rare (<0.1%) and occurred only in HPV-16 E6-expressing populations (Fig. 2C). Much more frequently, multipolar anaphases were observed (Fig. 2D). We quantified disturbances of polarity of the cell division process in metaphase and compared the results to anaphase/telophase cells in control and HPV oncoprotein-expressing primary human keratinocyte populations (Fig. 2E).

During metaphase, multipolar spindle pole formation was present in 1.3% of LXSN-infected control cells. We observed a 15.3-fold increase of multipolar mitoses in HPV-16 E6-expressing cells to 19.6% (P ≤ 0.05). In HPV-16 E7-expressing cells, a 10.4-fold increase to 13.5% of cells with abnormal spindle pole formation was observed. The most dramatic changes, however, were detected in NHKs expressing both HPV-16 E6 and E7, in which a 24.2-fold increase to 31.4% of dividing cells showing a multipolar spindle pole arrangement was found (P ≤ 0.005; Fig. 2E). These numbers include giant metaphases with multiple defects such as un- or misaligned chromosomal material and a disturbed polarity. No such abnormalities were detected in LXSN-infected control cells or in cells expressing the HPV-16 E7 oncoprotein only but were found in 4.2% of abnormal metaphases in keratinocyte populations expressing HPV-16 E6 and in 9.1% of abnormal metaphases expressing both HPV-16 E6 and HPV-17. In a representative experiment, multipolar metaphase configurations were observed in 2% of cells expressing low-risk HPV-6 E6 or E7 proteins.

We next addressed the question whether an increased proportion of multipolar metaphases in HPV oncoprotein-expressing cell populations are associated with a concomitant increase of multipolar anaphases or telophases. We found that multipolar anaphase/telophases occur at a much lower frequency in HPV-16 E6- and/or E7-expressing cells compared to metaphases (Fig. 2E). A multipolar anaphase/telophase arrangement (mostly tetrapolar) was found in 0.3% of LXSN-infected controls (Fig. 2E). In HPV-16 E6- or E7-expressing cells, the incidence of multipolar anaphase/telophases was 2.7 and 1.3%, respectively. In NHKs expressing both HPV-16 E6 and E7, we observed disturbances of anaphase/telophase polarity in 3.4% of dividing cells.

In conclusion, the differences between dividing cells exhibiting multipolarity in metaphase compared with anaphase/telophase were strikingly reduced in all HPV oncoprotein-expressing keratinocyte populations. These results suggest the presence of checkpoint mechanisms in primary human keratinocytes that inhibit entry into anaphase when multiple spindle poles are present during metaphase. However, in HPV-16 E6- and HPV-17-expressing cells, this control mechanism is not sufficiently active to prevent a small but significant number of cells from undergoing anaphase/telophase progression despite multipolar spindle arrangement.

Expression of HPV-16 E6 and E7 Is Associated with Anaphase Bridges. Stringlike chromatid connections between separating daughter cells have been referred to as anaphase bridges (Fig. 3, A and B). The formation of anaphase bridges has been shown previously to reflect structural chromosomal abnormalities, mostly as a result from chromosome breakage (24). Previous studies have shown that the high-risk HPV E6 oncoprotein can induce structural chromosomal abnormalities in fibroblasts (11). Here, we studied anaphase bridge formation using Hoechst 33258 DNA dye to detect chromatin strings in primary human keratinocyte populations expressing HPV-16 E6 and E7 oncoproteins and in controls (Fig. 3C).
лерные хромосомные аномалии (11), есть только непрямые доказательства, что экспрессия HPV E7 оncoproteina ассоциируется с структурными хромосомными изменениями (27). Мы, следовательно, исследовали вопрос, может ли экспрессия HPV-16 E7 приводить к нанесению повреждений ДНК и, следовательно, к хромосомным расстройствам (Fig. 4). Мы первоначально проанализировали индуцированные повреждения ДНК в человеческих кератиноцитах сегментов, экспрессирующих HPV-16 E6 и/or E7 оncoproteїнов, используя тест комет (Fig. 4, A и B). В этом опыте, хромосомные повреждения оценивались с помощью кинетического электрофореза, который приводит к миграции отрезков разрушенной ДНК из клетки в вытесняющую телосферу (электрическое поле). Мы обнаружили значительный (P = 0.05) 2.6-кратный увеличение доли клеток с равной или увеличенной пропорцией хвоста и головы ДНК сигналов (Fig. 4) в HPV-16 E6 оncoproteїнов-экспрессирующих клетках (5.9%) по сравнению с контролем (2.3%; Fig. 4C) и значительный (P < 0.05) 3.1-кратный увеличение доли клеток, когда экспрессия HPV-16 E7 (7.1%) была сравнима с контролем. NHKs экспрессирующих высокопроизводительный HPV E6 и E7 показали 3.5-кратное увеличение доли клеток с увеличенной пропорцией хвоста и головы ДНК сигналов (7%) по сравнению с контролем. Эти результаты демонстрируют, что высокопроизводительный HPV E6 и E7 оncoproteїны могут независимо индуцировать ДНК повреждения.

Для дальнейшего уточнения этих результатов, мы анализировали NHK популяции, экспрессирующие высокопроизводительный HPV E6 и/or E7 оncoproteїны для индукции ДНК репарационного фермента PARP, используя иммуноцитохимический анализ и иммунофлюоресцентный микроскоп. PARP - это ядерный белок, который срывает быстро ДНК разрывы, связанные с ДНК и ответственны за поддержание целостности ДНК и также за апоптоз и циклическую регуляцию (28–30). Мы обнаружили увеличение PARP белковых уровней в NHKs экспрессирующих HPV-16 E6 и/or E7, когда клетки проходили через нормоанапластический этап развития. Эти результаты подтверждают, что высокопроизводительный HPV E6 и E7 оncoproteїны могут независимо индуцировать ДНК повреждения.

Анафазные мостики не были обнаружены в >250 анафаз-телофазных фигурах, исследованных в LXSN-инфицированных контрольных кератиноцитах. В представительном эксперименте, экспрессия низокопроизводительного HPV-6 E6 и E7 белков в нормальных кератиноцитах не была обнаружена в 0 или 1% анафазных мостов, соответственно. В высокопроизводительном HPV-16 E6 или E7 экспрессирующих NHKs, 2.4 и 2%, соответственно, анафазных клеток показали хроматидные мостики между дочерними клетками. В клетках экспрессирующих HPV-16 E6 и E7, 4% анафазных клеток были представлены анафазными мостами.

Потому что анафазные мостики не были обнаружены после теломераттреции (25), мы определили теломерную длину в НК популяциях. Длины теломер в разных популяциях были стабильными и мигрировали с высоким молекулярным весом теломерической ДНК (Fig. 3D). Тем не менее, в низкопроизводительных HPV-16 E6 и E7 экспрессирующих NHKs, 2.4 и 2%, соответственно, анафазных клеток показаны хроматидные мостики между дочерними клетками. В клетках экспрессирующих HPV-16 E6 и E7, 4% анафазных клеток были представлены анафазными мостами.

Среди различных популяций NHK, экспрессирующих UVB-16 E6 и/or E7 оncoproteїны, мы не нашли значительных динамик теломерной длины (Fig. 3D). Тем не менее, в высокопроизводительных HPV-16 E6 и E7 экспрессирующих NHKs, 2.4 и 2%, соответственно, анафазных клеток показаны хроматидные мостики между дочерними клетками. В клетках экспрессирующих HPV-16 E6 и E7, 4% анафазных клеток были представлены анафазными мостами. Кроме того, этот процесс усиливается, когда клетки экспрессирующие UVB-16 E6 и/or E7 оncoproteїны, независимо индуцируют ДНК повреждения.

**Expression of HPV-16 E6 and E7 Oncoproteins Is Associated with DNA Damage.** The occurrence of anaphase bridges in HPV-16 E6- and/or E7-expressing keratinocytes independent of telomere attrition suggests that other mechanisms may be involved. Anaphase bridges can also result from chromosome breakage leading to the formation of chromosomes with more than one centromere that cannot adequately separate during mitosis (26). Whereas the high-risk HPV E6 oncoprotein has been previously implicated in generating structural chromosomal abnormalities (11), there is only indirect evidence that expression of the HPV E7 oncoprotein is associated with structural chromosomal changes (27). We therefore investigated whether expression of HPV-16 E7 may contribute to DNA damage and ultimately chromosomal breakage (Fig. 4). We first analyzed induction of DNA breaks in primary human keratinocyte populations expressing the HPV-16 E6 and/or E7 oncoproteins using a comet assay (Fig. 4, A and B). In this assay, chromosomal breakage is assessed by single-cell gel electrophoresis, which leads to the migration of broken DNA out of the cellular body giving rise to a tail of DNA fragments. Unbroken DNA remains in the cellular nucleus as head of the comet. Cells were assessed for the ratio between head and tail fluorescence signals. We found a significant (P = 0.05) 2.6-fold increase of the proportion of cells with an equal or increased ratio of head to tail DNA signals in HPV-16 E6 oncoprotein-expressing cells (5.9%) compared with controls (2.3%; Fig. 4C) and a significant (P = 0.05) 3.1-fold increase when cells expressing HPV-16 E7 (7.1%) were compared with controls. NHKs expressing high-risk HPV E6 and E7 showed a 3-fold increase of cells with enhanced tail:head ratio (7%) compared with controls. These findings demonstrate that the high-risk HPV E6 and E7 oncoproteins can each independently induce DNA breakage.

To further corroborate these findings, we analyzed NHK populations expressing the high-risk HPV E6 and/or E7 oncoproteins for the induction of the DNA repair enzyme PARP using immunoblotting and immunofluorescence microscopy. PARP is a nuclear protein that binds rapidly to DNA strand breaks and is involved in DNA repair and maintenance of genomic integrity but also in apoptosis and cell cycle regulation (28–30). We found an increase of PARP protein levels in NHKs expressing HPV-16 E6 and/or E7, when cells expressing HPV-16 E7 (7.1%) were compared with controls. These findings demonstrate that the high-risk HPV E6 and E7 oncoproteins can each independently induce DNA breakage.

**Anaphase bridges**

Anaphase bridges were absent in >250 anaphase/telophase figures studied in LXSN-infected control keratinocyte populations. In a representative experiment, expression of low-risk HPV-6 E6 and E7 proteins in primary human keratinocytes was found to be associated with no or 1% anaphase bridges, respectively. In high-risk HPV-16 E6 or E7 expressing NHKs, 2.4 and 2%, respectively, of anaphase cells showed chromatid bridges between the daughter cells. In cells expressing both HPV-16 E6 and E7, 4% of anaphase cells presented with anaphase bridge configuration.

Because anaphase bridge formation has been reported to occur after telomere attrition (25), we determined telomere length in the NHK populations studied. The telomere lengths of the different populations were indistinguishable and comigrated with high molecular weight telomeric DNA (Fig. 3D). Thus, we consider it unlikely that telomere attrition is the primary cause for the formation of anaphase bridges in the keratinocyte cultures that we used for this study, and we conclude that HPV-16 E6 and E7 can each induce anaphase bridge formation before a critical reduction of telomere length. Moreover, this process is enhanced when the high-risk HPV E6 and E7 oncoproteins are coexpressed.

Expression of HPV-16 E6 and E7 Oncoproteins Is Associated with DNA Damage. The occurrence of anaphase bridges in HPV-16 E6- and/or E7-expressing keratinocytes independent of telomere attrition suggests that other mechanisms may be involved. Anaphase bridges can also result from chromosome breakage leading to the formation of chromosomes with more than one centromere that cannot adequately separate during mitosis (26). Whereas the high-risk HPV E6 oncoprotein has been previously implicated in generating struc-
DNA breaks are known to activate cell cycle checkpoint control. DNA strand breaks, for example after ionizing radiation, induce phosphorylation of p53 at serine 15, thus stimulating trans-activation of p53 targets (34). Using immunoblot analysis and after normalization against background and protein loading differences, we detected a modest 1.45-fold increase of cellular p53 protein levels in HPV E7 oncoprotein-expressing NHKs and a similar 1.3-fold increase of serine 15-phosphorylated p53 in E7 oncoprotein-expressing cells compared with controls (Fig. 6, A and C). Moreover, inhibitory phosphorylation of cdc2 at tyrosine 15 has also been described to play a role to induce a G2 arrest after DNA damage (35). The overall cdc2 protein level was 3.5-fold increased in HPV-16 E7-expressing cells, and we observed a 2.2-fold increase of tyrosine 15-phosphorylated cdc2 compared with controls (Fig. 6, B and C). Similar results were obtained in multiple independent experiments. These experiments are consistent with the notion that expression of HPV-16 E7 triggers the activation of cell cycle checkpoints similar to what has been described after chromosomal breaks.

We conclude that both HPV-16 E6 and E7 can induce DNA damage. In particular, expression of HPV-16 E7 induces speckled nuclear foci of γ-H2AX together with overexpression of PARP. In addition, E7 expression may trigger DNA damage sensing cell cycle checkpoints. Taken together, these results suggest that high-

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**Fig. 5.** HPV-16 E7 induces PARP expression and nuclear γ-H2AX foci. A, immunoblot analysis of PARP protein levels in NHKs stably expressing HPV-16 E6 and/or E7 or empty LXSN vector. Actin was used as loading control. B, quantitation of the increase of PARP protein levels in HPV-16 oncoprotein-expressing cells after normalization against LXSN-infected NHKs used as control and loading (actin). Results from a representative experiment are shown. C, comimmunofluorescence microscopy for γ-H2AX (FITC) and PARP (Rhodamine Red) in NHKs stably expressing empty vector (Control) or HPV-16 E7. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Bar, 10 μm. D, quantitation of cells with high nuclear PARP expression and focal γ-H2AX staining in NHK populations expressing HPV-16 E6 and/or E7 compared with controls. Each bar indicates mean ± SE of triplicate quantitations for each cell population in a representative experiment.

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**Fig. 6.** HPV-16 E7 triggers DNA damage-sensing cell cycle checkpoints. A, immunoblot analysis and quantitation of phospho-p53 and p53 protein levels in HPV-16 E7-expressing cells compared with controls. B, immunoblot analysis and quantitation of phospho-cdc2 and cdc2 protein levels in HPV-16 E7-expressing cells compared with controls. Results from a representative experiment are shown. C, quantitation of increases of p53, phospho-p53, cdc2, and phospho-cdc2 protein levels in HPV-16 E7-expressing cells after normalization against LXSN-infected control NHKs and loading (actin).
risk HPV E7 can induce DNA damage independent from the cooperating E6 oncoprotein.

**DISCUSSION**

Cancer has been characterized as a “disease of genomic instability” (36). The majority of human tumors exhibit genomic instability on the chromosome level with various numerical and structural aberrations (16). These changes frequently occur concomitantly, giving rise to the complex cytogenetic abnormalities seen in many epithelial cancers including HPV-associated cervical carcinomas (9). The pathogenesis of complex chromosomal changes is largely unclear. In particular, it is unknown whether numerical and structural chromosome instability can have a common origin, which may have important implications for our understanding of cancer development and progression.

HPV-associated carcinogenesis is an excellent model to study the development of chromosomal instability because only two viral oncoproteins, E6 and E7, are consistently expressed in cervical carcinomas (8, 37). The majority of high-risk HPV-associated lesions become aneuploid at an early, preinvasive stage (38–40), which can be preceded by increased tetraploidy (41). In addition, complex cytogenetic aberrations with chromosomal gains or losses as well as deletions or amplifications of chromosome arms are detected in HPV-immortalized cells (10) and cervical cancers (9, 42). Given the observation that numerical and structural chromosomal abnormalities frequently develop together in HPV-immortalized cells and carcinomas, we asked whether there is common source for complex chromosomal aberrations during HPV-associated carcinogenesis. We report here that the high-risk HPV-16 E6 and E7 oncoproteins can independently induce various mitotic abnormalities and DNA damage. Mitotic aberrations found in high-risk HPV E6- or E7-expressing NHKs included changes that can give rise to numerical chromosomal imbalances, mostly centrosome-related disturbances of polarity and unaligned or lagging chromosomal material. However, we also observed anaphase bridges in both HPV E6- and E7-expressing cells, which typically arise as a consequence of structural chromosomal changes and chromosome breakage.

Unaligned or lagging chromosomal material during cell division can result from defects of the mitotic spindle checkpoint. This checkpoint normally monitors proper attachment of kinetochores to spindle microtubules and alignment of all chromosomes at the metaphase plate (43). It has been previously shown that the HPV-16 E6 and E7 oncoproteins abrogate mitotic checkpoint control (12, 13). Whereas p53, which is targeted by the high-risk HPV E6 oncoprotein, has been shown to be required for checkpoint control that prevents DNA rereplication after spindle disruption (44), the role of the HPV E7 oncoprotein remains unclear. We confirmed results by Thomas and Laimins (13) and showed by flow cytometry and fluorescence in situ hybridization that expression of high-risk HPV E7 alone allows for rereplication of chromosomes after spindle depolymerization by nocodazole (data not shown). Further support for this notion comes from the observation that giant metaphases with a large increase of chromosomal material were mainly observed in NIH populations coexpressing HPV-16 E6 and E7. The mechanisms by which the HPV E7 oncoprotein interferes with the spindle checkpoint remain to be elucidated in detail. A direct interaction of a viral oncoprotein with a mitotic spindle checkpoint protein has been described for the HTLV-1 oncoprotein Tax (45), but no similar interactions have been reported for HPV E6 or E7. Unaligned or lagging chromosomes may also result from the inability of a cell to coordinate different functions of the cell division process or from premature exit from metaphase. Interestingly, disturbances of spindle pole formation and un- or misalignment of chromosomes frequently occurred in parallel in our cell system as exemplified in Fig. 1, B and D.

We have previously shown that centrosome-related mitotic disturbances are a prominent finding in HPV oncoprotein-expressing cells and that these abnormalities are caused by expression of high-risk HPV E6 and E7. Previous studies by Sluder et al. (23) have suggested that there is no sufficient checkpoint control for the metaphase-anaphase transition in cells with multiple spindle poles. In our experiments, centrosome-related disturbances of spindle bipolarity were the most frequent microscopically detectable metaphase abnormalities in HPV-16 E6- and/or E7-expressing keratinocytes. However, when analyzing only cells in anaphase or telophase, we found a dramatically decreased number of cell division figures with altered polarity. In fact, the vast majority of cells showed a bipolar anaphase arrangement despite a high incidence of multipolar metaphases in the same cell population. Our findings therefore suggest that in primary human keratinocytes there are control mechanisms that restrict cells with multipolar metaphase configuration to progress into anaphase. However, it is conceivable that HPV-16 oncoproteins can override this control by producing large numbers of multipolar metaphases giving rise to a small but significant proportion of cells that exit into anaphase with a multipolar (mostly tripolar) spindle pole arrangement. Our experiments cannot rule out that additional biological consequences of E6 and E7 oncoprotein expression trigger checkpoint control mechanisms that block anaphase entry independent of abnormal spindle pole numbers. Whereas previous work has shown that multinucleated cells with multiple centrosomes which can result from mitotic dysfunction do not show an increased apoptotic rate but rather acquire a senescence-like phenotype (15), little is known about mitotic processes that may control polarity disturbances during cell division. Our finding that in high-risk HPV oncoprotein-expressing keratinocytes there is a discrepancy between the rate of multipolar metaphases and the proportion of cells progressing into anaphase with a disturbed polarity suggest that such mechanisms exist, but additional studies are necessary to track the fates of individual cells undergoing aberrant mitosis.

Structural abnormalities of chromosomes have been frequently reported in HPV oncoprotein-expressing cells (10). Previous studies have implicated the high-risk HPV E6 oncoprotein for the pathogenesis of structural chromosome instability (11). Human fibroblasts expressing HPV-16 E6 and selected for resistance against N-(phosphonacetyl)-L-aspartate were found to acquire drug resistance by expression of structural chromosome instability (11). Human fibroblasts expressing HPV-16 E6 and selected for resistance against N-(phosphonacetyl)-L-aspartate were found to acquire drug resistance by amplification of resistance gene, whereas HPV-16 E7-expressing cells became resistant through multiple copies of the chromosome that harbors the gene conferring drug resistance (11). We report here that anaphase bridges as a morphological correlate for structural chromosomal changes not only occur in HPV-16 E6 expressing cells but can also be found in HPV-16 E7-expressing keratinoyte populations (Fig. 3). Anaphase bridges have been observed in various malignancies and are believed to result from chromosomal breaks and formation of di- or multicentromeric chromosomes, hindering proper segregation during mitosis. If anaphase bridges break, chromosome fragments that are able to fuse with other chromosomes can be generated, and repeated cycles of breakage-bridge-fusion can occur (24, 46). The increased incidence of anaphase bridges in these HPV-16 E6- and/or E7 oncoprotein-expressing keratinocyte populations was not attributable to telomere attrition (Fig. 3D). However, both HPV-16 E6 and E7 independently induced DNA breakage as detected by a comet assay (Fig. 4). Moreover, γ-H2AX foci in the presence of increased nuclear PARP expression were found to be increased in HPV-16 E7-expressing cells, but not in cells expressing the HPV E6 oncoprotein. We cannot rule out that the latter observation may be related to inefficient histone H2AX phosphorylation in...
HPV-16 E6-expressing cells in response to DNA damage (47); however, it clearly shows that HPV-16 E7-expressing cells exhibit signs of DNA strand breaks and presumably also DNA repair (32). The absence of nuclear hallmarks of apoptosis, the low level or programmed cell death as defined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (<1% in all cell populations studied), and the absence of PARP cleavage products (Fig. 5A) strongly suggest that cells exhibiting high nuclear PARP and speckled γ-H2AX foci represent cells that have acquired DNA damage. Moreover, levels of serine 15-phosphorylated forms of p35 and tyrosine 15-phosphorylated cdc2 species, which are both induced by DNA damage (34, 35, 48), were increased in HPV-16 E7-expressing NHKs (Fig. 6). Taken together, these results provide direct and indirect evidence that expression of HPV-16 E7 may cause DNA damage in primary human keratinocytes. This finding is supported by previous observations that high-risk HPV E7 enhances integration of DNA into chromosomes (27). Although pRB has been shown to play a role in cell cycle arrest after DNA damage (49) and inactivation of pRB itself has been implicated in genomic instability (50), the precise mechanisms of the HPV E7 oncoprotein-associated increase of DNA breakage remains to be determined.

In summary, this study shows that the HPV-16 E6 and E7 oncoproteins can independently induce various mitotic abnormalities and DNA damage in primary human keratinocytes. The two oncoproteins cooperate as shown by an increase of cell division abnormalities when both oncopgenes are coexpressed. Future studies will determine whether this effect is based on a permissive effect of p53 inactivation on the growth of genomically unstable cells. Our results demonstrate that the high-risk HPV E6 and E7 oncoproteins can be the source for both numerical and structural chromosome instability. Although it is conceivable that the induction of genomic instability by HPV oncoproteins results mostly in nonviable cells, a sufficient number of events may ultimately allow the occurrence of numerical and/or structural genetic alterations that confer a growth advantage and facilitate traversing selection barriers (51). Our findings will help to better understand early steps of cancer development and may improve strategies to target genomic instability for preventive or therapeutic purposes.

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The Human Papillomavirus Type 16 E6 and E7 Oncoproteins Independently Induce Numerical and Structural Chromosome Instability

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