HPV-16 E6/7 Immortalization Sensitizes Human Keratinocytes to Ultraviolet B by Altering the Pathway from Caspase-8 to Caspase-9-dependent Apoptosis*

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UVB from solar radiation is both an initiating and promoting agent for skin cancer. We have found that primary human keratinocytes undergo an apoptotic response to UVB. To determine whether these responses are altered during the course of immortalization, we examined markers of apoptosis in primary human foreskin keratinocytes (HFK) transduced with either a retroviral vector expressing the E6 and E7 genes of HPV-16 or with empty vector alone (LXSN-HFK). Whereas LXSN-HFK as well as early passage keratinocytes expressing HPV-16 E6 and E7 (p7 E6/7-HFK) were both moderately sensitive to UVB irradiation, late passage-immortalized keratinocytes (p27 E6/7-HFK) were exquisitely sensitive to UVB-induced apoptosis. After exposure to UVB, enhanced annexin V-positivity and internucleosomal DNA fragmentation were observed in p27 E6/7-HFK compared with either LXSN- or p7 E6/7-HFK. Caspase-3 fluorometric activity assays as well as immunoblot analysis with antibodies to caspase-3 and poly(ADP-ribose) polymerase revealed elevated caspase-3 activity and processing at lower UVB doses in p27 E6/7-HFK compared with LXSN- or p7 E6/7-HFK. In addition, the caspase inhibitor DEVD-CHO reduced the apoptotic response and increased survival of all three HFK types. Immunoblot analysis revealed that caspase-8 was activated in all three cell types, but caspase-9 was only activated in p27 E6/7-HFK. Cell cycle analysis further showed that only p27 E6/7-HFK exhibit G2/M accumulation that is enhanced by UVB treatment. This accumulation was associated with a rapid down-regulation of Bcl-2 in these cells. The immortalization process subsequent to the expression of HPV E6 and E7 may therefore determine UVB sensitivity by switching the mode of apoptosis from a caspase-8 to a Bcl-2-caspase-9-mediated pathway of apoptosis.

The most common malignancy in humans is skin cancer. The incidences of basal cell carcinoma, squamous cell carcinoma, and melanoma continue to rise and approach those of all other cancer subtypes combined (1). UV1 irradiation causes skin cancer through a series of cellular changes that are not all identified. However, since UV acts as a promoting (selective) as well as an initiating (mutating) agent (2), it is clear that in addition to genetic alterations, inappropriate or altered growth, differentiation, and/or apoptotic responses to UV play key roles in this process.

The connection between UV-induced apoptosis and skin cancer has been well studied in the context of p53. UV induces a signature pattern of p53 mutations in squamous cell carcinoma and basal cell carcinoma (3, 4) as well as in normal sun-exposed skin (5). Keratinocytes harboring these p53-inactivating mutations are resistant to senescence in culture, and it has been further proposed that such keratinocytes are also resistant to growth inhibition or cytotoxicity from subsequent UV exposure (6). In support of this idea, clones of p53-mutated keratinocytes can be found within normal sun-exposed human epidermis (2, 5) and can be generated in mice in which clonal expansion of p53-mutant keratinocytes is continually driven by UVB (7).

Although mucosal HPV types have been long implicated in anogenital cancer, a variety HPV types have been identified in a high percentage of basal cell carcinoma and squamous cell carcinoma in immunosuppressed patients (8) as well as in actinic keratoses and squamous cell carcinoma in those with the inherited disorder epidermodysplasia verruciformis (EV; Ref. 9). HPV has also been postulated to play a role in basal cell carcinoma and squamous cell carcinoma from immunocompetent non-EV patients as well (8, 10). In most cases, carcinomas occur in sun-exposed sites, indicating cooperation between UV and HPV. Because the HPV E6 gene product inactivates p53 (for review, see Ref. 11), E6 presumably serves some of the same functions as UV-induced p53-inactivating mutations in skin carcinogenesis (12).

HPV E6 and E7 oncoproteins likely play roles in the early immortalization stage of carcinogenesis, resulting ultimately in the stable activation of the telomerase catalytic subunit (hTERT) and inactivation of the p16/Rb pathway (13, 14). In cell culture, spontaneous immortalization of keratinocytes is a rare event that can be enhanced by HPV-16/18 E6 and E7. Although HPV E6 has been shown to transiently up-regulate the gene and promoter of hTERT (15, 16) and E7 inactivates Rb, other genetic events are apparently required for immortalization. These other genes may be related to the stable expression of hTERT (17), since loss of a region of chromosome 6.

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¶ The abbreviations used are: UV, ultraviolet (UV) B; EV, epidermodysplasia verruciformis; HFK, human foreskin keratinocytes; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PI, propidium iodide; FACS, fluorescence-activated cell sorter.
were irradiated with 480 J/m² UVB, and 16 h later, cells were fixed and stained with Hoechst (A). B, cells were irradiated with 480 J/m² UVB, and after the indicated times, whole cell extracts were prepared and subjected to immunoblot analysis using antibodies specific for the active form (p17) of caspase-3 or PARP.

MATERIALS AND METHODS

Cells—Primary human keratinocytes were derived from neonatal foreskins and grown in KSF medium supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Invitrogen). The primary cells were infected with an amphotropic LXSN retrovirus expressing the HPV-16 open reading frames of the E6 and E7 genes. The retrovirus was generated as described (20) with the use of existing recombinant vectors (21). Retrovirus-infected cells were selected in G418 (100 µg/ml) for 10 days, and G418-resistant colonies were pooled from each transduction and passed every 3–4 days.

Keratinocytes (from different passages after infection with HPV-16 E6/7) were grown under identical conditions to 70–80% confluency, trypsinized before UVB exposure, and passaged at equal cell densities. Cells were allowed to recover, and after replacement of the KSF medium with phosphate-buffered saline, cells were irradiated with ultraviolet light using a UVB source with a peak wavelength of 312 nm (FS40 sunlamp (Philips) with a Kodacel filter (Eastman Kodak Co.)) at various doses. At different time points after UVB irradiation or 16 h after exposure to different doses of UVB cells were derived for further analyses.

Fluorometric Assay of Caspase-3 Activity—Cells were resuspended in lysis buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 0.5% Nonidet P-40 (Nonidet P-40), 10 µg/ml aprotinin, 20 µg/ml leupeptin, 10 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride, incubated for 10 min on ice, and freeze-thawed 3 times. The cell lysate was centrifuged at 14,000 × g for 5 min, and the protein concentration of cytosolic extract was determined with the Bio-Rad DC protein assay kit. For the fluorometric caspase-3 activity assay, 25 µg of cytosolic extract was initially resuspended up to a volume of 50 µl with Nonidet P-40 lysis buffer, to which 50 µl of caspase assay buffer (10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol) was added. The aliquots were then mixed with an equal amount (100 µl) of 40 µM fluorescent tetrapeptide substrate specific for caspase-3 (Ac-DEVD-AMC; Bachem) in caspase assay buffer and transferred to 96-well plates. Free aminomethylcoumarin (AMC), generated from the substrate, was measured by the Bio-Rad Fluoroskan Lumi plate reader using an excitation wavelength of 390 nm and an emission wavelength of 460 nm.
plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

Immunoblot Analysis—SDS-PAGE and transfer of separated proteins to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to verify equal loading and transfer of proteins. They were then incubated with antibodies to the p17 subunit of caspase-3 (1:200; Santa Cruz Biotechnology), PARP (1:200; Santa Cruz Biotechnology), caspase-8 (1:500; BD PharMingen), caspase-9 (1:100; Calbiochem), Bcl-2 (1:250; Transduction Labs), p53 (1:500; Calbiochem), or E7 (1:100; Santa Cruz Biotechnology). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase-conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce).

Cell Cycle Analysis—Nuclei were prepared for flow cytometric analysis as described (22). Cells were exposed to trypsin, resuspended in 100 μl of a solution containing 250 mM sucrose, 40 mM sodium citrate (pH 7.6), and 5% Me2SO, and subsequently lysed in a solution containing 3.4 mM sodium citrate, 0.1% Nonidet P-40, 1.5 mM spermine tetrahydrochloride, and 0.5 mM Tris–HCl (pH 7.6). Lysates were incubated for 10 min with RNase A (0.1 mg/ml), after which nuclei were stained for 15 min with propidium iodide (0.42 mg/ml), filtered through a 37-μm nylon mesh, and analyzed with a dual-laser flow cytometer (FACScan, BD PharMingen).

Analysis of DNA Fragmentation—Cells were harvested and lysed in 0.5 ml of 7 M guanidine hydrochloride. The lysate was mixed with 1 ml of Wizard Miniprep resin (Promega, Madison, WI), incubated at room temperature for 15 min with occasional mixing, and then centrifuged at 10,000 × g for 5 min. The resulting pellet was suspended in 2 ml of washing solution (90 mM NaCl, 9 mM Tris–HCl (pH 7.4), 2.25 mM EDTA, 55% (v/v) ethanol) and drawn by vacuum through a Wizard Minicolumn...
(Promega) mounted onto a vacuum manifold. The column was washed twice with 4 ml of washing solution and dried by centrifugation at 10,000 g for 2 min. DNA was eluted from the column by the addition of 50 ml of deionized H2O, incubation at room temperature for 15 min, and then centrifugation at 10,000 g for 5 min. Residual RNA in the eluate was removed by incubation with 10 g of RNase A (5 Prime, Inc., Boulder, CO) at 37 °C for 30 min. DNA samples were loaded onto a 1.5% agarose gel in Tris borate-EDTA buffer and subjected to electrophoresis at 4 V/cm. DNA ladders were visualized by staining with ethidium bromide (0.5 g/ml), and images were captured with the Kodak EDAS 120 gel documentation system.

RESULTS

UVB Induces Caspase-3-mediated Apoptosis in HFK—We have previously shown that the DNA alkylating agent sulfur mustard induces markers of terminal differentiation and apoptosis in normal human epidermal keratinocytes, including the early activation and late cleavage of PARP (23). To determine whether UVB induces the apoptotic response in HFK, cells were exposed to UVB, and markers of apoptosis were examined. Nuclear fragmentation (Fig. 1A) as well as the proteolytic processing of caspase-3 to its active form (p17; Fig. 1B) occurs 16 h after exposure to 480 J/m2 UVB. PARP is also catalytically cleaved by caspases-3 from a 116-kDa full-length into an 89-kDa fragment that contains the C-terminal catalytic and auto-modification domains, a hallmark of apoptosis (Fig. 1B).

To determine the effects of HPV-16 E6 and E7 as well as immortalization on the response of HFK to UVB, we transduced HFK with a LXSN retroviral vector expressing the E6 and E7 genes of HPV-16 or with empty retroviral vector alone. Although E6 levels are technically difficult to detect, we examined the levels of p53 by Western blot analysis, since E6 induces the degradation of p53. Fig. 2A, top, shows that p53 is detectable in LXSN-HFK but not in p7 or p27 E6/7-HFK. In addition, comparison with HFK shows no effect of transfection with vector alone. The expression of E7 was directly detected by Western analysis in both p7 and p27 E6/7 HFK but not in control LXSN-HFK (Fig. 2A, bottom).

Phosphatidylserine is exposed on the surface of apoptotic cells (24), and the presence of these residues can be detected by their ability to bind to annexin V (25). To further examine the
sensitivity to UVB-induced apoptosis in control and HFK E6/7 transfectants, we analyzed the cells for annexin V binding by FACS analysis 16 h after irradiation. Fig. 2 shows that p27 E6/7-HFK are much more sensitive to UVB-induced apoptosis at all doses tested than either p7 E6/7- or LXSN-HFK. A plot of the survival rates (PI negative, annexin V-negative) also confirms that p27 cells are more sensitive to UVB-mediated killing at all doses (Fig. 2C), thus indicating that the additional step(s) in immortalization plays a role in the sensitization to UVB.

Apoptosis is also characterized by the internucleosomal cleavage of DNA. We irradiated the three HFK cell types with the indicated doses of UVB, after which DNA was extracted and resolved by agarose gel electrophoresis. Fig. 3 shows that although nonspecific smearing is observed only at higher doses in p7- and LXSN-HFK (superimposed on two diffuse white bands that are the negative images of bromphenol blue and xylene cyanol dyes), a dose-dependent increase in internucleosomal DNA fragmentation is only observed in p27-HFK, with DNA ladders observed at the lowest dose of UVB used (60 J/m²).

To determine the mode of apoptotic cell death, we analyzed the amount of caspase-3 activity using a quantitative fluorometric DEVDase assay. The three cell types were irradiated with increasing doses of UVB, after which the cytosolic extracts were assayed for caspase-3 activity. Fig. 4 shows that caspase-3 is activated in all three cell types, but caspase-3 is activated at lower UVB doses in p27 E6/7-HFK than in either p7 E6/7- or LXSN-HFK.

Caspase-3, responsible for the cleavage of PARP during apoptosis, is composed of two subunits of 17 and 12 kDa that are derived from a common proenzyme (26). To further analyze the proteolytic processing of caspase-3 and its substrate PARP, we performed immunoblot analysis of extracts from cells treated with different doses of UVB using an antibody specific for the p17 subunit of caspase-3 or to PARP. Fig. 5 shows that caspase-3 is proteolytically processed to its active form (p17), and PARP is specifically cleaved at lower UVB doses in p27 E6/7-HFK than either LXSN- or p7 E6/7-HFK.

To examine whether caspase-3 was in fact responsible for UVB-induced apoptosis, we preincubated the three cell types with an inhibitor of caspase-3 (DEVD-CHO) for 30 min before and during UVB exposure. The caspase-3 inhibitor decreased the proteolytic processing of PARP (Fig. 6C), reduced the number of cells undergoing apoptosis after 480 J/m² UVB exposure, as determined by annexin V plus PI staining (Fig. 6A), and reduced internucleosomal fragmentation in all three cell types (Fig. 3). In addition, survival was increased (Fig. 6B). Thus UVB induces an apoptotic mode of death that is partially dependent upon caspase-3.

Immortalization of HFK Switches the Mode of Apoptosis from a Caspase-8- to a Caspase-9-dependent Pathway—Various reports indicate that UVB activates either a death receptor or mitochondrial pathway of apoptosis. The former pathway results in the rapid activation of caspase-8, whereas the latter pathway activates caspase-9. We performed immunoblot analysis of extracts derived from the three cell types treated with different doses of UVB or with a single dose of UVB to deter-
mine a time course. Fig. 7B shows that caspase-8 is activated in all three cell types, although at a lower dose in p27 E6/7-HFK. In contrast, caspase-9 (Fig. 7A) is only proteolytically activated in p27 E6/7-HFK, as revealed by the loss of the inactive caspase-9 precursor. To determine which caspase is activated first, a time course was performed. Fig. 8 shows that although caspase-9 is activated almost immediately after UVB irradiation in p27 E6/7-HFK, caspase-8 is not activated until 16 h after exposure. Consistent with the results of the dose-response experiments, caspase-9 is not proteolytically processed at any time after UVB exposure in p7 E6/E7- or LXSN-HFK.

To confirm that these results are representative of responses of E6/E7-immortalized keratinocytes, we derived two additional batches of pooled cell clones from two different E6/E7 retroviral infections of different mixed foreskins. Comparison of LXSN, p7 E6/E7-HFK, and p27 E6/7-HFK from matched sets confirmed our earlier findings that p27 cells are more sensitive to apoptosis than either LXSN or p7 E6/E7-HFK as a result of the preferential activation of caspase-9 in the later passage cells (Fig. 9).

To determine the possible reason for the switch in the apoptotic pathway in p27 E6/7-HFK, we examined the cell cycle before and after UVB irradiation in the three cell types. Fig. 10A shows that LXSN-HFK displays a strong G1 arrest after all doses of UVB irradiation. As might be predicted from the reduced levels of p53, neither p7 nor p27 E6/7-HFK exhibit an appreciable G1 arrest at any dose of UVB. However, only p27 E6/7-HFK showed a marked increase in the population of cells in G2/M after UVB treatment (Fig. 10C). Thus, although the elimination of the G1 arrest is associated with expression of E6/7, the UVB-induced G2 arrest appears to be attributable to the immortalization process.

Immortalization Results in a Bcl-2-dependent Pathway for UVB-induced Apoptosis—Although a causal link has not been unequivocally demonstrated, a strong correlation has been observed between G2 arrest and apoptosis. This link is also associated with the phosphorylation and degradation of Bcl-2, associated with a mitochondrial pathway of apoptosis (27). To determine whether this was a possible mechanism that re-
sulted in the switch to a caspase-9-mediated pathway for apoptosis in immortalized HFK, we performed an immunoblot analysis of UVB-irradiated cells using a Bcl-2-specific antibody. Fig. 11 shows that the lowest dose of UVB results in the disappearance of immunodetectable Bcl-2 in p27 E6/7-HFK. In contrast, Bcl-2 levels persist at higher doses of UVB in p7 E6/7- and LXSN-HFK. In fact, Bcl-2 persists in the latter cell types until it is specifically cleaved into a 23-kDa fragment, most likely by caspase-3 or -7 (28), at higher UVB doses.

**DISCUSSION**

Both HPV and UV have been shown to be etiologic agents for skin cancer. Immortalization is thought to be an early step in this process that involves the selection of a population of cells that progresses to the next stage of cancer. Based on these earlier observations, we separately tested the effects of E6/7 expression and immortalization on UVB apoptosis using early (p7) and immortalized late (p27) passage E6/7-transduced HFK. We found that UVB induces a caspase-3-mediated apoptotic death in all three cell types, but that p27 E6/7-HFK cells were more sensitive than either p7 E6/7-HFK cells or cells transduced with empty vector alone (LXSN-HFK).

Caspase-3 is activated in all three cell-types, but caspase-3 is activated at lower UVB doses in p27 E6/7-HFK than in either p7 E6/7- or LXSN-HFK, although a drop in caspase-3 activity is seen in p27 E6/7-HFK at higher doses. The mechanism for this phenomenon remains to be elucidated. An important point is that although the activity at 16 h is decreased, caspase-3 is processed into its active form at the higher doses (Fig. 5). Clearly, however, the sum total of caspase-3 activity expressed within the first 16 h after higher doses of UV treatment of late passage E6/7 is sufficient to completely proteolyze PARP (Fig. 5) and activate internucleosomal cleavage (Fig. 3). Likewise, all other apoptotic markers are higher in the late passage cells.

One potential explanation for our findings is that E6 and E7 are expressed at low levels in p7 E6/7-HFK. However, four lines of evidence argue against this possibility. First, HFK were infected at a high multiplicity of infection with the E6/7 retroviral construct and subsequently selected for 10 days in G418 (see “Materials and Methods”). Second, p53 levels were reduced in p7 E6/7-HFK as well as p27 E6/7-HFK, indicating that E6 was present and functional in both cell types (Fig. 2A). Third, E7 levels were similar in both p7 E6/7-HFK and p27 E6/7-HFK (Fig. 2B). Fourth, FACS analysis revealed that the GI arrest induced by UV was not observed in either p7 E6/7- or p27 E6/7-HFK (Fig. 10). Thus, it appears that events subsequent to expression of E6 and E7 are critical to the increased sensitivity to UVB. The idea that immortalization requires genetic events in addition to the expression of E6 and E7 is not a new one.
Sensitization of HFK to UVB Apoptosis by Immortalization

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