Transformation by Human Papillomavirus 16 E6 and E7: Role of the Insulin-like Growth Factor 1 Receptor

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ABSTRACT

Human papillomavirus-16 E6 and E7 inactivate the tumor suppressors p53 and pRB, respectively, and cooperate during malignant transformation, but the downstream molecular events remain incompletely understood. Using fibroblast cell lines derived from mice with a homozygous disruption of the insulin-like growth factor-1 receptor (IGF-1R) gene (R- cells) and their wild-type (WT) littermates, we have stably transfected plasmids encoding E6 and E7 proteins and examined their transforming potential in these cells. Consistent with previous studies using NIH3T3 cells, pooled cultures of E7-transfected WT cells readily formed colonies after suspension in soft agar. In contrast, R- cells were not transformed by E7. E6 had little transforming activity in WT (WT/E6) or R- (R-/E6) cells. However, transfection of R- cells with E6 plus E7 resulted in extensive colony formation. Because IGF-1R and E6 appear to be functionally equivalent in this transformation assay and both have been implicated in antiapoptotic responses, we investigated the apoptotic responses of the cells after exposure to the potent protein kinase C inhibitor, staurosporine. Compared to WT cells, R- cells were relatively resistant to staurosporine-induced apoptosis, but susceptibility to staurosporine was decreased in both WT/E6 and R-/E6 cells relative to WT and R- cells transfected with mock vector, respectively. In fibroblast cells from p53 gene knockout mice, transfection with E6 also conferred relative resistance to staurosporine-induced apoptosis. Our data suggest that transformation by E7 requires the participation of the IGF-1R and that E6 may assist E7 in transforming R- cells by functionally substituting for the IGF-1R. Because IGF-1R activated by its ligands (IGF-1 and IGF-2) protects cells from apoptosis, the role of the IGF-1R and E6 in transformation by E7 is probably related to the recruitment of survival pathways. In addition, because E6 suppressed apoptosis in p53 knockout cells, our data also suggest that E6 may participate in a p53-independent process that protects cells from apoptosis.

INTRODUCTION

Emerging evidence indicates that control of cellular proliferation is intimately connected with programmed cell death or apoptosis. Inactivation of the tumor suppressor genes p53 and pRB are associated with a wide variety of malignancies (1), and recent studies indicate that they play important roles in inducing apoptosis (2). High-risk HPV* -transforming proteins E6 and E7 are thought to participate in HPV-associated carcinogenesis, at least in part, through their interactions with p53 and pRB, respectively, and each can affect cell cycle checkpoint function (3, 4). However, the molecular events responsible for the functional consequences of HPV infection remain incompletely understood.

Rapidly accumulating evidence indicates that IGF-1R can play a major role in transformation and tumorigenesis (5). For example, it has been shown that overexpression of IGF-1R induces ligand-dependent transformation and tumorigenesis in rodent fibroblasts (6), whereas the overexpression of a dominant negative mutant IGF-1R inhibits this effect (7); that WTI, the Wilms' tumor suppressor gene, represses the expression of both IGF-1 and one of its ligands, IGF-2 (8); and that activation of IGF-1R has been linked to cell survival (9, 10). In addition, using embryonic fibroblasts from normal and R- mice, Sell et al. (11) demonstrated that the SV40 T-Ag requires a functional IGF-1R gene for transformation.

Because HPV-16 E6 and E7 share certain properties with T-Ag, such as inactivation of the tumor suppressors p53 and pRB, respectively, we investigated whether a functional IGF-1R is also a prerequisite for the transforming effects of HPV E6 and E7. We show that in contrast to T-Ag, HPV E6 and E7 together can transform murine fibroblasts in the absence of a functional IGF-1R gene, whereas E6 or E7 alone cannot. Using staurosporine, a potent protein kinase-C inhibitor, to induce apoptosis (12, 13), we demonstrated that E6 appears to suppress apoptotic responses by a p53-independent mechanism. These experiments suggest that IGF-1R and E6 are functionally equivalent in assisting E7 in transformation and may each participate in survival pathways that protect from apoptosis.

MATERIALS AND METHODS

Cells and Cell Culture. Embryonic fibroblasts from normal littermates (WT) and R- mice, as well as the conditions for culturing them, have been described previously (11). Fibroblasts from p53-/-- cells [generously provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA)] were cultured as described by Livingstone et al. (14). The cells were cultured in DMEM (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 2 mM l-glutamine and 100 units/ml penicillin-streptomycin. Incubations were carried out at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Plasmids. The expression plasmids pLTR, pE6, pE7, and pE6E7 have been described previously (15). Briefly, pLTR is a pUC19-based plasmid containing the Ha LTR inserted into pUC19 at the EcoRI site. pE6 contains the Ha LTR and HPV-16 nts 24–654 such that HPV-16 sequences are transcribed from the LTR when the plasmid is transfected into mammalian cells. pE6 also contains a 2-nt substitution of the WT sequence that inactivates the splice donor site at nt 226 and thereby increases the translation of the full-length E6 product. pE7 contains nts 505–1176, and pE6E7 contains nts 24–1176 of HPV-16 downstream of the Ha LTR. The plasmids that contain the full-length coding sequence of the human IGF-1R gene cDNA with (mutated IGF-1R; Ref. 16) or without (WT IGF-1R; Ref. 17) a point mutation in the ATP binding site have been described previously.

Transfection. Cells were seeded overnight at 1 X 106 per 10-cm dish, and then 10 μg test plasmid plus 0.5 μg of vector DNA containing the puromycin resistance gene were introduced into cells using the calcium phosphate precipitation method. After selection under puromycin (1.5 μg/ml; Sigma Chemical Co., St. Louis, MO) for 2 weeks, the colonies were pooled and analyzed for the presence of each transfected gene by PCR.

PCR. High molecular weight DNA was extracted from the transfected cells using the QIAamp Blood Kit (Qiagen, Inc., Chatsworth, CA). The PCR was performed in a 100-ml reaction volume containing 0.5–1.0 μg DNA, 200 μM dNTP, 1 μg sense and 1 μg antisense primers, and 2.5 units Taq polymerase (Life Technologies, Inc., Gaithersburg, MD). To detect HPV-16 E6 DNA, a sense primer spanning nts 139–158 and an antisense primer spanning nts...
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RESULTS

Analysis of Viral DNAs in Transfected Cells. To determine if the transfected cells contained HPV-16 DNA, cellular DNA was isolated and subjected to PCR. The primers used for the analysis should give rise to a 274-nt fragment corresponding to E6 and a 185-nt fragment corresponding to E7. As expected, the cells transfected with pE6 produced only the predicted 274-nt product, whereas pE7-transfected cells expressed only the 185-nt product; for cells transfected with pE6E7, both products were detected (data not shown).

Transformation Assay. pE6, pE7, and pE6E7 were transfected into R− or WT cells that are 3T3-like cells derived from mouse embryos with a targeted disruption of the IGF-1R genes and their WT littermates, respectively. To assess for the presence or absence of a transformed phenotype, bulk cultures of puromycin-resistant cells were scored for colony formation in soft agar. The cells were maintained in 10% serum for at least 3 weeks, which is a more than sufficient amount of time for establishment of colonies in soft agar, even at reduced growth rates. The results of a typical soft agar assay are shown in Fig. 1, and colony counts are depicted in Fig. 2. Consistent with previous studies using IGF-1R-positive NIH3T3 cells (20), pooled cultures of pE7 transfected WT (WT/E7) cells readily formed colonies after suspension in soft agar (15). In contrast, R− cells were not transformed by pE7 (R−/E7). Neither pLTR or pE6 had significant transforming activity in either WT or R− cells. However, transfection of R− cells with pE6E7 resulted in extensive colony formation. Therefore, E6 appears to assist E7 in transforming IGF-1R-null (R−) cells, whereas E7 alone can transform cells with intact IGF-1R signaling, suggesting that E6 is functionally equivalent to the IGF-1R for the transformation of mouse embryo fibroblasts.

An additional experiment was performed to investigate whether the inability of E7 to transform R− cells was a direct consequence of the absence of the IGF-1R or an indirect result of an irreversibly altered phenotype of mutant cells that have undergone crisis. For this purpose, pE7 was cotransfected with a selectable marker (puromycin resistance) and either the full-length coding sequence of the human IGF-1R cDNA (16) or an IGF-1R cDNA with a point mutation in the ATP binding site (17). This latter plasmid encodes for a nonfunctional IGF-1R that cannot be autophosphorylated but retains the ability to bind to its ligands. As shown in Fig. 3, when R− cells were cotransfected with E7 and the WT IGF-1R, colonies efficiently formed in soft agar. In contrast, when the mutated IGF-1R was coexpressed with E7, the R− cells remained resistant to transformation. Therefore, E7 realizes its transforming potential in R− cells if the IGF-1R is again expressed. E6E7 and E7 plus IGF-1R induced similar numbers of colonies, reinforcing the notion that E6 and IGF-1R are functionally equivalent in this assay.

Fig. 1. Transformation of WT and R− cells by HPV-16 E6, E7, or E6 and E7 as measured by growth in soft agar. Cells were cotransfected with a puromycin resistance gene and plasmids encoding E6, E7, or E6 and E7. Cells were selected with puromycin, and pooled cultures were plated in agar. Data are representative results from three independent experiments.
Apoptotic Responses of WT and R− Cells to Staurosporine. The apoptotic responses to the potent protein kinase C inhibitor staurosporine were examined using a quantitative ELISA that measures cytoplasmic DNA histone complexes generated during apoptotic DNA fragmentation. The results using this technique were corroborated by morphological inspection of apoptotic nuclei using Hoechst’s 33342 dye (data not shown). At each tested dose, WT cells were consistently more sensitive to staurosporine than R− cells as measured by ELISA (Fig. 4). When compared with untreated cells, the cytoplasmic DNA in WT cells increased by more than 6-fold after treatment with 1 μm of staurosporine, as compared to less than 4-fold in R− cells, and at each dose, R− cells were 40−50% less sensitive to staurosporine than WT cells. These results suggest that the R− cells may have compensated for the absence of IGF-1R signaling by recruiting an alternate survival pathway that is less sensitive to the effects of staurosporine.

Effect of pE6 on Staurosporine-induced Apoptosis. Because E6 has been implicated in suppressing apoptotic responses (21), we investigated the effects of its expression on the susceptibility of cells to staurosporine. To assess the consequences of E6 expression, cells transfected with either pLTR (vector) or pE6 were cultured in the presence or absence of 1 μM staurosporine, and the apoptotic responses were measured as above. The results are shown in Fig. 5 and expressed as a percentage of the amount of cytoplasmic DNA enrichment relative to mock-transfected control cells. These results have been normalized to permit proportional comparison of the effect of E6 between cell types, but statistical analysis was performed prior to normalization to directly examine the effect of transfection with either mock vector (pLTR) or pE6 within each cell type. Cells expressing pE6 consistently demonstrated less susceptibility to staurosporine relative to the pLTR counterpart. The inhibition of WT and R− cell death was 46 and 51%, respectively, comparing each E6-expressing cell with its pLTR-transfected counterpart. This outcome occurred independent of IGF-1R since both WT and R− cells gave equivalent results.

In previous investigations with R− cells, SV40 T-Ag could not induce colony formation in soft agar (11, 22). Since T-Ag binds and inactivates both p53 and pRb, our results with pE6E7 raised the possibility that E6 might assist E7 in transformation by a p53-independent mechanism. To assess whether E6 could inhibit apoptotic responses in the absence of p53, p53−/− cells were transfected with either pLTR or pE6 and tested for susceptibility to staurosporine-induced apoptosis. Similar to both WT and R− cells, the same apoptotic-inhibitory effect was observed in p53−/− cells expressing E6 (54% inhibition), which suggests that at least part of the death-suppressive effect of E6 is independent of an interaction with p53 (Fig. 5).

DISCUSSION

Using embryonic mouse fibroblasts with a targeted disruption of the IGF-1R gene (23, 24), we have investigated the role of IGF-1R in HPV-16 E6- and E7-induced transformation. Because WT mouse fibroblast transfected with E7 readily formed colonies in soft agar but R− cells did not, our results indicate that a functional IGF-1R is normally required for E7-induced transformation. However, since R− cells formed similar numbers of colonies after cotransfection of E7 with either IGF-1R or E6, E6 was able to functionally substitute for the IGF-1R requirement in this assay.

A role for the IGF-1R pathway in cell survival has been recognized in both normal and neoplastic processes. For instance, IGFs function as survival factors for newly formed oligodendrocytes and their pre-
cooperate in the transformation of cells lacking the IGF-1R, in contrast to the inability of SV4O T-Ag to do so (11), is one of the first cancers. It is therefore of interest to recognize in vitro activities of E6 regulators of cell proliferation, but they are not associated with human DNA tumor viruses, since they could identify unique HPV functions also encode transforming proteins that inactivate these two negative members of the adenoviruses and polyoma/SV4O families of viruses carcinogenesis have focused on the molecular interactions between other hand, it is obvious that the IGF-1R pathway is not indispensable these viral and tumor suppressor proteins. However, HPV-induced anogenital cancers (31). Because E6 and E7 of high-risk HPVs are high-risk HPV has been strongly associated with cervical and other phenotype.

Based on substantial epidemiological and molecular evidence, high-risk HPV has been strongly associated with cervical and other anogenital cancers (31). Because E6 and E7 of high-risk HPVs are selectively retained and expressed in cervical cancers (32) and inactivate p53 and pRB, respectively, many investigations of cervical carcinogenesis have focused on the molecular interactions between these viral and tumor suppressor proteins. However, HPV-induced carcinogenesis is likely to involve more than these interactions since members of the adenoviruses and polyoma/SV4O families of viruses also encode transforming proteins that inactivate these two negative regulators of cell proliferation, but they are not associated with human cancers. It is therefore of interest to recognize in vitro activities of E6 and/or E7 that are not shared by the transforming genes of other DNA tumor viruses.

In other studies using R– cells, Morroni et al. (33) have recently demonstrated that, like HPV-16 E7, the E5-transforming protein of BPV is also unable to induce transformation in these cells, but the reintroduction of a functional IGF-1R restores its susceptibility to transformation (33). Nilson and DiMaio (34) and Petti et al. (35) have shown that transformation by BPV E5 requires a functional PDGF receptor and that E5 directly interacts with the PDGF receptor and phosphorylates it (34, 35). The concept of a growth factor signaling cascade was introduced several years ago, where competence growth factors such as PDGF and EGF initiate signals that are "upstream" from progression factors including IGF-1, IGF-2, and insulin (36, 37). For example, the IGF-1-mediated mitogenesis of murine fibroblasts requires costimulation with PDGF, and PDGF has been shown to induce transcription of the IGF-1R gene promotor, thereby enhancing IGF-1 binding sites (38). However, no direct association has yet been described for HPV-16 E7 and any growth factor receptor. Therefore, the shared requirement of a functional IGF-1R for transformation by both BPV E5 and HPV-16 E7 suggests that these oncoproteins may act within the same mitogenic signaling pathway but at distinctly different points.

The protective effects of activated IGF-1R have been previously demonstrated using R– cells and their WT counterparts: etoposide-induced apoptosis was inhibited when WT cells were stimulated with IGF-1, but no such protective effect was observed in R– cells (10). In the current study, R– cells were relatively resistant to staurosporine-induced cell death compared to WT cells, suggesting that staurosporine may interfere with survival signals involving the IGF-1R. R– cells may be relatively resistant to staurosporine since in the absence of the IGF-1R, these cells have adapted by activating survival pathways that are less sensitive to staurosporine.

E6 inactivates p53 by inducing its ubiquitin-dependent degradation, whereas T-Ag forms a stable inactive complex with p53 (39). Because p53 can induce apoptosis (40), it is possible that the IGF-1R-independent transformation exhibited by the HPV genes but not by T-Ag is the consequence of these different mechanisms of p53 inactivation. However, E6 was able to inhibit staurosporine-induced apoptosis in p53-negative as well as normal fibroblasts. Therefore, it seems more likely that the difference between T-Ag and E6 lies in the ability of E6 to inhibit the apoptosis that results from inactivation of pRb through examples of an E6 or E7 activity that is clearly not shared by the transforming genes of other DNA tumor viruses.

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A p53-independent mechanism not shared by T-Ag. p53-independent transformation and transactivation by E6 have also been described (41–43). Whether these E6 activities and the antiapoptotic activity described here are the result of a common or distinct mechanism remains to be determined.

The ability of E6 to cooperate with E7 by suppressing apoptosis may be relevant to human carcinogenesis in cells containing functional IGF-1R, although it is not required for in vitro transformation, since the availability of IGF-I and other growth factors are likely to be more limiting in vivo than they are under cell culture conditions. However, the role of E6 in cervical carcinogenesis may not solely be to functionally substitute for IGF-1R activity since overexpression of IGF-1R has been observed in HPV-positive as well as HPV-negative cervical tumors (30). Further studies are clearly needed to elucidate the interactions between the HPV oncoproteins and other growth regulatory pathways that are critical for the induction of HPV-associated human cancers.

REFERENCES

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