The Cytokines Tumor Necrosis Factor-α (TNF-α) and TNF-related Apoptosis-inducing Ligand Differentially Modulate Proliferation and Apoptotic Pathways in Human Keratinocytes Expressing the Human Papillomavirus-16 E7 Oncoprotein*

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Keratinocytes are the natural target cells for infection by human papillomaviruses (HPVs), most of which cause benign epithelial hyperplasias (warts). However, a subset of papillomaviruses, the “high risk” HPVs, cause lesions that can progress to carcinomas. Inflammatory mediators such as tumor necrosis factor-α (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL) are produced by cells in response to a viral infection. To determine the effects of TNF-α and TRAIL on keratinocytes expressing the high risk HPV-16 oncoprotein E7, human foreskin keratinocytes stably expressing E7 were treated with TNF-α and TRAIL. Treatment with TNF-α alone, but not TRAIL, induced growth arrest and differentiation in keratinocytes that was almost completely overcome by expression of HPV-16 E7. Both cytokines induced apoptosis when administered in combination with the protein synthesis inhibitor cycloheximide, but the apoptotic response to TRAIL was significantly more rapid and efficient compared with the response seen after TNF-α treatment. HPV-16 E7-expressing keratinocytes were more prone to both TNF-α- and TRAIL-mediated apoptosis compared with vector-infected controls.

The human papillomaviruses (HPVs)1 are a family of small DNA viruses with a pronounced tropism for epithelial cells (1). Infection with a member of this large family of viruses usually causes papillomatous hyperplasia (warts) but lesions caused by a subset of these viruses, the “high risk” HPVs (such as HPV-16, -18, -31, or -33) have a propensity for malignant progression (2). Infection with a member of this large family of viruses usually causes papillomatous hyperplasia (warts) but lesions caused by a subset of these viruses, the “high risk” HPVs (such as HPV-16, -18, -31, or -33) have a propensity for malignant progression (2). During malignant progression, the HPV genome frequently integrates into the host DNA resulting in the expression of only two viral proteins, E6 and E7, that bind and induce the degradation of the p53 and pRB tumor suppressor proteins, respectively (4–8). An important function of pRB is to modulate the activity of members of the E2F family of transcription factors that play important roles in regulating the G1/S-phase transition (9, 10). Degradation of pRB by E7 abrogates this regulatory circuit and relaxes G1/S checkpoint control (11). E6 promotes the proteasomal degradation of the tumor suppressor p53, rendering the cell unable to efficiently execute a program of growth arrest and apoptosis under conditions of cellular stress, DNA damage, or aberrant growth signals (10, 12). The loss of cellular surveillance function permits replication of damaged DNA, resulting in the accumulation of cells with genomic aberrations and increasing the likelihood of neoplastic changes (13).

Viral infection of an immunocompetent host induces the production and release of cytokines, powerful mediators of inflammation produced by fibroblasts, macrophages, lymphocytes, and keratinocytes themselves (14). TNF-α is one of the main mediators of inflammation in the skin and mucosa, the first barrier encountered by an epitheliotropic virus (14). TNF-α and its related cytokines bind to specific members of the TNF receptor superfamily initiating various signaling pathways that lead to growth arrest, proliferation, or cell death (15). The cellular response to a cytokine depends upon the specific ligand-receptor interaction, the cell type, and the immediate cellular microenvironment (16).

TNF-R1 contains four cysteine-rich extracellular domains that become trimerized upon TNF-α binding (16). Ligation of this receptor approximates and cross-links intracellular death domains that then bind the death-domain-containing adapter proteins TRADD and FADD. Oligomerization of these proteins results in caspase activation and apoptosis (17, 18). In addition, TNF-R1 can simultaneously activate protective responses via NF-κB-dependent and -independent gene transcription and protein synthesis (19, 20).

A recently identified member of the TNF family of cytokines, TRAIL, has not been as extensively studied, and its effects on keratinocytes are largely unknown. Found in a wide range of tissues, TRAIL (also known as Apo-2 ligand) is a type II transmembrane protein closely homologous to Fas ligand, TNF-α, and lymphotoxin-β (21–23). Four major receptors for TRAIL have been characterized. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) are type I transmembrane proteins with cytoplasmic death domains homologous to those found in TNF-R1 and Fas (17, 21, 23–25). TRAIL-R3 (also called TRID or DrE1) lacks a...
cytoplasmic domain and instead is anchored directly to the cell membrane via a glycosylphosphatidylinositol link (26–30). TRAIL-R4 (DcR2) contains an incomplete cytoplasmic death domain (31). TRAIL receptors 3 and 4 cannot initiate the caspase cascade and thereby may confer resistance to TRAIL-induced apoptosis by competing for ligand. A fifth and most recently described cell-type-specific receptor, osteoprotegerin, can be stimulated by TRAIL and is involved in the regulation of bone resorption (32, 33).

Like other members of the TNF receptor superfamily, the cytoplasmic domains of TRAIL receptors 1 and 2 are believed to interact with the death-domain-containing adapter molecules TRADD and FADD to transmit a death signal. To date, there is conflicting evidence as to whether adapters are actually required, the order of their assembly, and whether or not there exists as yet unidentified adapter proteins unique to the TRAIL system (34–38).

To determine the effects of the inflammatory cytokines TNF-α and TRAIL on the natural host cell of human papillomaviruses, the genital keratinocyte, retroviruses expressing the HPV-16 E6 and E7 oncoprotein were used to infect HFKs. These cells were treated with cytokines with and without the protein synthesis inhibitor cycloheximide. TNF-α alone induced growth arrest and differentiation in control HFKs but not in HFKs expressing HPV-16 E7. HFKs expressing E6 or dominant negative p53 also arrested in the presence of TNF-α, indicating that TNF-α-induced cytostasis is p53-independent and can be overcome by E7. In contrast, TRAIL alone did not inhibit proliferation, suggesting a basic difference in the way these cytokines and their corresponding receptors transmit intracellular signals. As in many other cell types, apoptosis could be induced in cytokine-treated HFKs only with the concurrent administration of the protein synthesis inhibitor cycloheximide. TRAIL treatment induced apoptosis more rapidly and efficiently than TNF-α and expression of the HPV-16 E7 protein potentiated the cytotoxicity of both TNF-α and TRAIL.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary HFKs were prepared from a pool of several neonatal foreskins obtained after routine circumcision, following the protocol of Rheinwald and Beckett (39). The cells were maintained in serum-free keratinocyte growth medium (Keratinocyte-SFM, Life Technologies, Inc.). IMR-90 fibroblasts were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Retroviral Infections—LXSN-based retroviral vectors for expressing HPV-16 E6 and E7 (40), dominant negative p53 (41), and SV40 large T antigen (42) were prepared using the protocols of Miller and Rosman (43). Cells were infected with viral supernatants for 4 h at 37 °C in the presence of 4 µg/ml Polybrene (hexadimethrine bromide, Sigma Chemical Co.). The viral supernatant was removed, and the plates were washed twice with phosphate-buffered saline. HFKs were maintained in normal keratinocyte growth medium for the first 24 h after infection and then selected for 2 days in keratinocyte growth medium containing 200 µg/ml G418 (Calbiochem).

Cytokine Treatment—Subconfluent plates of HFKs were treated with the indicated concentrations of TNF-α (R&D Systems), TRAIL (Alexis), or agonistic Fas antibody (Clone DX 2.1, R&D Systems) for the indicated periods of time. Fresh media containing the appropriate cytokines was added to the cultures for each day where the treatment extended past 24 h.

Reverse Transcriptase Polymerase Chain Reaction—Total RNA was extracted from a subconfluent plate of HFKs using the RNeasy Mini Kit (Qiagen). A 5-µg aliquot was reverse-transcribed with murine leukemia virus reverse transcriptase (New England BioLabs), and the resulting cDNA was diluted with 5 µl of each pair of PCR primers (Life Technologies, Inc.) in a final reaction volume of 50 µl. A polymerase chain reaction was carried out for 35 cycles (95 °C melting temperature for 1 min; 55 °C annealing temperature for 1 min; 72 °C extension temperature for 1 min). The nucleotide sequences for the PCR primers (Life Technologies, Inc.) have been described previously (44).

Immunological Methods—Cells were lysed in EBC lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 µl/ml aprotinin and leupeptin) and phosphatase inhibitors (2 mM NaF and 0.5 mM sodium orthovanadate) for 30 min at 4 °C. After centrifugation, protein concentrations were measured using the Lowry assay. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore Corp.). The membranes were then incubated with the appropriate antibodies. The antibodies used were as follows: TRAIL-R1 (Alexis, 210-730-C100); TRAIL-R2 (Imgenex, IMG-120); TRAIL-R3 (Alexis, 210-744-R100); TRAIL-R4 (Imgenex, IMG-121); p53 (Ab-6, Oncogene Science); p21 (Ab-1, Oncogene Research Products); IgG1 antibody to transglutaminase II, Neomarkers; actin (Ab-1, Oncogene Research Products); phospho-Akt (Thr-308, New England Biolabs). Proteins were detected using the ECL chemiluminescence system (Amersham Pharmacia Biotech), and quantitation was performed with NIH Image software.

Detection of Cell Proliferation—Equal numbers of HFKs, as determined by a cell counter (Coulter), were aliquoted into 35-mm plates and treated with the appropriate cytokines. At each time point, the cells were trypsinized in equal volumes of trypsin/EDTA and quantified.

Cell Cycle Analysis—Cells were trypsinized and stained with propidium iodide (Sigma), as described previously (45). The samples were analyzed by fluorescence-activated cell sorting (FACS) using Cell Quest software (Becton Dickinson).

Detection of Cell Death—Keratinocytes growing on 35-mm tissue culture plates were treated with 10 ng/ml TNF-α or TRAIL and 30 µg/ml cycloheximide (Sigma). The cells were stained with 1 µg/ml bisbenzimide (Hoechst 33258, Sigma) and apoptotic nuclei were visualized by fluorescence microscopy as described previously (41). 500 cells were counted per sample.

RESULTS

TNF-α, but Not TRAIL, Induces Growth Arrest in Keratinocytes—Although it has been previously reported that TNF-α treatment causes a cytostatic effect in HFKs (46–49), little is known about the effects of TRAIL on HFK growth. Therefore, HFKs were grown with or without 10, 25, or 50 ng/ml TNF-α or TRAIL, and the cells were counted at regular intervals. As previously reported, TNF-α treatment of control HFKs had a strong cytostatic effect (Fig. 1a). FACS analysis revealed that TNF-α-treated cells were mostly arrested in G1/G0 (Fig. 1a). In contrast, treatment with 10, 25, or 50 ng/ml TRAIL (Fig. 1a) or 1 or 10 µg/ml agonistic Fas antibody (data not shown) did not affect HFK growth. To eliminate the possibility that the lack of growth arrest in response to TRAIL treatment was due to the failure of TRAIL receptor signaling, an immunoblot for the phosphorylated, active form of c-Akt was performed. c-Akt is a cell survival factor that is phosphorylated by the phosphatidylinositol 3′-OH kinase in response to ligation of surface receptors such as those for platelet-derived growth factor, insulin-like growth factor, and TNF-α (50–53). HFKs incubated with 10 ng/ml TNF-α or TRAIL showed strong c-Akt phosphorylation 2 min after addition of the cytokine to the media, indicating that TRAIL signaling is intact (Fig. 1b). To determine whether the cytostatic effect is not only specific for TNF-α but also keratinocyte-specific, IMR-90 normal human diploid lung fibroblasts were treated with 10 or 25 ng/ml TNF-α. No growth inhibition was observed in these cells (Fig. 1c).

TNF-α-induced Cytostatic Effect Is Overcome by HPV-16 E7—HPV-16 E7 can interact with pRB and accelerate its degradation, an effect closely correlated with transformation (5, 41). We wanted to determine if HPV 16 E7 expression could interfere with TNF-α-mediated growth arrest. Therefore, HPV 16 E7-expressing HFKs were grown with or without 10, 25, or 50 ng/ml TNF-α or TRAIL, and the cells were counted at regular intervals. The growth of HPV-16 E7-expressing HFKs was not significantly inhibited at any concentration of TNF-α (Fig. 2a). HFKs stably expressing the transformation-defective HPV-16 E7 mutants 16E7 ΔpRbE10, which can bind but not degrade pRB, and 16E7 ΔD21-C24, which can neither bind nor...
degrade pRB (41), were unable to overcome TNF-α-induced growth arrest (Fig. 2b). To further test whether inactivation of pRB is linked to abrogation of TNF-α-mediated growth arrest, we tested SV40 large tumor antigen, which can also interact with and inactivate pRB (54). Like HPV-16 E7-expressing cells, HFKs stably expressing SV40 large tumor antigen were resistant to TNF-α-mediated growth arrest at all concentrations of TNF-α (Fig. 2c, left panel). In contrast, HFKs expressing a non-transforming pRB binding-deficient SV40 large tumor antigen mutant (T107) remained sensitive to TNF-α-mediated growth arrest (Fig. 2c, right panel). Hence, the ability of a viral oncoprotein to overcome the TNF-α-mediated growth arrest correlates with pRB inactivation and cellular transformation.

**TNF-α-induced Cytostatic Effect Is Independent of p53**—In HPV-infected cells the E7 protein is co-expressed with E6, which targets p53, an important mediator of growth arrest, for proteasomal degradation (7). To determine if p53 mediates the TNF-α cytostatic effect, HFKs were infected with a retrovirus expressing either the HPV-16 E6 oncoprotein or a dominant negative form of p53 (55). p53 protein levels were then analyzed by immunoblot. Cells expressing HPV-16 E6 contained very low p53 levels, presumably due to E6-mediated protein degradation (Fig. 3a). In contrast, p53 levels were increased in cells expressing the dominant negative mutant, which binds and stabilizes endogenous p53 (Fig. 3a). Cells expressing either E6 or dominant negative p53 underwent
growth arrest when incubated with TNF-α (Fig. 3b). This indicates that the TNF-α-induced cytotstatic effect is not mediated by p53.

Effects of TNF-α and TRAIL Treatment on Differentiation of LX and E7 HFKs—The TNF-α cytotstatic effect in keratinocytes may be related to differentiation (48, 49). We therefore examined HFKs treated with TNF-α for transglutaminase, a late marker of keratinocyte differentiation (56, 57). Transglutaminase expression increased in response to TNF-α in LXSN- and E6-expressing cells but not in E7-expressing cells (Fig. 4a). This demonstrates that the growth inhibitory activity of TNF-α correlates with induction of differentiation in human keratinocytes and can be resisted by cells expressing HPV-16 E7.

Because the cdk inhibitor p21Cip1/Waf1 plays an important role in coupling growth arrest and differentiation in human and murine keratinocytes in response to Ca²⁺ (40, 58, 59), we probed the same blot for p21Cip1/Waf1. There was an increase of p21Cip1/Waf1 protein in LXSN-, E7-, and E6-infected HFKs (Fig. 4a). Although p21Cip1/Waf1 levels increased in E7 populations, this may be due to the ability of E7 to bind and stabilize p21Cip1/Waf1 (60), which may contribute to the ability of E7-expressing cells to overcome arrest (40, 61).

To further examine whether growth arrest and differentiation were specific for TNF-α, we treated HFKs with TRAIL. As expected, TRAIL treatment, which does not cause growth arrest, did not increase transglutaminase levels (Fig. 4b).

Effects of TNF-α and TRAIL Treatment on the Apoptotic Response of LX and E7 HFKs—Because TNF-α and TRAIL differentially affected keratinocyte proliferation, we wanted to investigate the effects of these cytokines on apoptosis. In many cell types, TNF-α or TRAIL induces an apoptotic response only when new protein synthesis is suppressed by treatment with cycloheximide. HFKs were therefore treated with 10 ng/ml TNF-α or TRAIL, along with 30 μg/ml cycloheximide. Under these conditions, TRAIL caused a rapid and strong apoptotic response whereas the apoptotic response to TNF-α treatment of keratinocytes was less pronounced (Fig. 5a). Importantly, however, expression of E7 enhanced HFK apoptosis 2-fold for TNF-α treatment and almost 4-fold for TRAIL treatment (Fig. 5b).

Expression of TRAIL and TNF-R1 Receptors in Primary Hu-
Responses of HPV-16 E7-expressing HFKs to TNF-α and TRAIL

Fig. 5. a, TRAIL causes rapid and efficient apoptosis in HFKs upon inhibition of new protein synthesis. Normal HFKs were treated with 30 μg/ml cycloheximide alone or 30 μg/ml cycloheximide and 10 ng/ml TNF-α or TRAIL for the indicated periods of time. The cells were then fixed in methanol and stained with Hoechst 33258. Apoptotic nuclei were counted as a percentage of total nuclei, with the data points representing the averages of three experiments. b, expression of HPV 16 E7 enhances TNF-α- and TRAIL-mediated apoptosis in keratinocytes. LXSN and E7 keratinocytes were treated with 10 ng/ml TNF-α or TRAIL along with 30 μg/ml cycloheximide for 8 h. The cells were fixed in methanol, and the nuclei were visualized by Hoechst 33258. Apoptotic nuclei were counted as a percentage of total nuclei, with the data representing the averages of 12 experiments.

man Keratinocytes—It has been reported that the relative abundance of TRAIL receptors, in particular the anti-apoptotic decoy receptors TRAIL-R3 and TRAIL-R4, may contribute to the sensitivity of cells to TRAIL-mediated apoptosis (26, 27, 29, 31). To determine if TRAIL receptors R1 through R4 are present in HFKs, reverse transcriptase-PCR was performed using keratinocyte mRNA as a template. Signals for TRAIL receptors R1, R2, R3, and R4 were detected in HFKs (Fig. 6a). Next, we performed immunoblots on LXSN and E7 HFK extracts. These results corroborated the results of the reverse transcriptase-PCR experiments showing expression of these four receptors and demonstrated that they are present at similar levels in both LX- and E7-expressing cells (Fig. 6b). Similarly, levels of TNF-R1 were also unchanged in cells expressing E7 (Fig. 6b). Therefore, the observed differences do not reflect alterations in receptor expression.

DISCUSSION

Upon viral infection, the host initiates a cell-mediated immune response (62). Inflammatory cytokines are produced that modulate gene expression through receptor-mediated induction of phosphorylation and protein interaction cascades (63). Previous studies on the effects of the inflammatory cytokine TNF-α have shown that it induces growth arrest in keratinocytes, possibly through differentiation (46–49). Less is known of the effects of the cytokine TRAIL in the skin and mucosa.

Growth arrest is an effective defense against viral infection, because HPV can reproduce only if the host cell’s DNA synthesis machinery is active. The human papillomaviruses overcome this defense by producing oncoproteins that abrogate the function of p53 and pRB, proteins that regulate progression through the cell cycle (11). Histologically, this is seen as cell division within the upper levels of stratified epithelium normally reserved for quiescent, differentiating cells (64, 65).

Here we show that TNF-α treatment of normal HFKs causes growth arrest predominantly in the G0/G1 phase of the cell cycle (Fig. 1a). This effect is not observed even following treatment with high concentrations of TRAIL or incubation with an agonistic Fas antibody (Fig. 1a and data not shown, respectively). Because deficient receptor signaling in keratinocytes could explain the lack of a cytostatic effect in TRAIL treatment, an immunoblot was performed for phosphorylated, activated Akt. It is known that many extracellular stimuli such as growth factors and TNF-α can influence cell proliferation and survival through receptor-mediated activation of the mitogen-activate protein kinase and phosphatidylinositol 3′-OH-kinase/Akt pathways (50–53). Here we show that TRAIL signaling is intact and functional in keratinocytes by demonstrating the ability of TRAIL to induce phosphorylation of Akt at levels equal to that seen in TNF-α treatment (Fig. 1b). Therefore, the cytostatic effect appears to be specific for TNF-α. In addition, this effect is likely specific for keratinocytes, because treatment of normal human fibroblasts with TNF-α has no effect on their growth (Fig. 1c).

It has been previously reported that HPV-immortalized cell lines are less sensitive to TNF-α-induced growth arrest (66). Our experiments show that cells expressing the HPV-16 E7 oncoprotein overcome the growth inhibitory effects of TNF-α, even at concentrations of 50 ng/ml (Fig. 2a). This correlates with the ability of HPV-16 E7 to bind and degrade pRB and induce cellular transformation, because mutants that cannot degrade pRB undergo growth arrest in response to TNF-α (Fig. 2b). The importance of pRB inactivation and cellular transformation in overcoming the TNF-α cytostatic effect is further emphasized by the ability of keratinocytes expressing the SV40 large T antigen to continue to grow in high concentrations of TNF-α (Fig. 2c, left panel). Similar to E7, HFKs expressing a transformation-deficient SV40 large T antigen that does not interact with pRB remain sensitive to TNF-α-mediated growth arrest (Fig. 2c, right panel). Interestingly, although pRB status appears critical in the mechanism of growth arrest, TNF-α-induced cytostasis is not mediated by p53, because HFKs expressing E6 or dominant negative p53 were still arrested by TNF-α (Fig. 3b).

Our results suggest that E7 attenuates both cell cycle arrest and differentiation in TNF-α-treated HFKs. p21Cip1/Waf1 is a cyclin-dependent kinase inhibitor that regulates progression into S-phase by preventing cyclin E/cdk2 from phosphorylating and inactivating pRB or by directly inhibiting E2F-1 itself (67). Growth arrest and differentiation caused by Ca2+ treatment of keratinocytes demonstrates a corresponding increase in p21Cip1/Waf1 protein levels (40, 58, 59). In our experiments, similar increases in p21Cip1/Waf1 levels are seen following TNF-α treatment in HFKs (Fig. 4a). Significantly, a gradual increase in p21Cip1/Waf1 levels is also observed in TNF-α-treated E6-expressing keratinocytes, again suggesting p53-independent p21Cip1/Waf1 regulation during cytokine-mediated keratinocyte growth arrest and differentiation.

E7 has been shown to bind and stabilize p21Cip1/Waf1 and...
abrogate its function during Ca\(^{2+}\)-induced differentiation (40, 60, 61). This could explain why levels of p21\(^{Cip1/Waf1}\) are generally higher in E7-expressing cells throughout the course of TNF-\(\alpha\) treatment (Fig. 4a). E7-expressing cells may therefore be able to resist TNF-\(\alpha\)-mediated growth arrest not only through the ability of HPV-16 E7 to degrade pRB but also through direct inhibition of p21\(^{Cip1/Waf1}\). Inhibition of p21\(^{Cip1/Waf1}\) by antisense mRNA or intracellular injection of \(\alpha\)-p21\(^{Cip1/Waf1}\) antibodies has in fact been shown to force senesced or differentiated cells back into the cell cycle (68, 69).

Levels of transglutaminase, a marker of keratinocyte differentiation, increase in LXSN and E6 cells grown in TNF-\(\alpha\) but not in identically treated E7 HFKs (Fig. 4a). These findings are consistent with previous studies of E7- and E6/E7-expressing HFKs that have shown delayed differentiation and continued expression of cyclin A, an S-phase cyclin, when grown in semisolid media or Ca\(^{2+}\), conditions that normally favor differentiation (40, 70, 71). Treatment of LXSN-infected HFKs with TRAIL alone, which has no effect on the growth of keratinocytes, does not cause an increase in levels of transglutaminase (Fig. 4b). Therefore, unlike TNF-\(\alpha\), TRAIL cannot activate a differentiation program.

Apoptotic signaling in response to TRAIL has not been well characterized. There is conflicting evidence regarding the recruitment of adapter proteins to the receptor and the mechanism of caspase activation. In our study, treatment of keratinocytes with cycloheximide and TNF-\(\alpha\) causes relatively low levels of apoptosis, whereas treatment with TRAIL and cycloheximide causes more rapid and efficient apoptosis (Fig. 5a).

HFKs expressing the E7 oncoprotein are more prone to both TNF-\(\alpha\)- and TRAIL-mediated apoptosis (Fig. 5b). This is not due to up-regulation of expression of TNF or TRAIL receptors by E7, because receptors R1 through R4 are present in both cell types in equal amounts (Fig. 6b). Instead, the ability of E7 to enhance E2F transcriptional activity through degradation of pRB is likely responsible for this effect. Increased E2F-1 activity has been shown in other cell types to promote not only progression through S-phase but also apoptosis (72, 73). Previous studies have shown that E7-expressing keratinocytes exhibited not only enhanced growth, but also spontaneous apoptosis (74). In cytokine-treated HFKs, free E2F-1 may further enhance cell death by directly inhibiting anti-apoptotic signaling from TNF receptor-associated factor 2 (TRAF2) at the level of the receptor (75).

E7-mediated p21\(^{Cip1/Waf1}\) inactivation, which helps overcome TNF-\(\alpha\)-mediated growth arrest, may also enhance apoptosis. In general, cyclin-dependent kinase inhibitor expression associated with senescence or differentiation has been shown to confer resistance to cell death (76, 77). TNF-\(\alpha\)-induced increases in p21\(^{Cip1/Waf1}\) delay cell death in MCF-7 cells (78). Indeed, it has been suggested that p21\(^{Cip1/Waf1}\) may need to be degraded before a cell can even commence apoptosis (79). One possible mechanism for this could be through the ability of p21\(^{Cip1/Waf1}\) to directly inhibit caspase 3 (80).

Here we report that HPV-16 E7 can modulate the responses of its natural host cell to the closely related cytokines TNF-\(\alpha\) and TRAIL. Future studies will try to decipher the nature of TRAIL signaling in keratinocytes. Dissection and analysis of cytokine signaling pathways and understanding how this virus disrupts intracellular signaling and cell cycle control could lead to a better understanding of how HFKs infected with HPV-16 avoid the immune system and progress to malignancy.

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**Fig. 6.** Expression of TRAIL and TNF receptors in HFKs. a, reverse transcriptase-PCR analysis of TRAIL receptor mRNA expression in primary human keratinocytes. b, control (LXSN) and HPV-16 E7-expressing HFKs were lysed, and steady-state levels of TRAIL-R1, -R2, -R3, and -R4 were determined by SDS-PAGE and immunoblot analysis.
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