Human papillomavirus type 16 (HPV-16) E6 has been shown to prevent or enhance apoptosis depending on the stimulus and cell type. Here we present evidence that HPV-16 E6 sensitized murine fibrosarcoma L929 cells to tumor necrosis factor α (TNF)-induced cytolysis. The E6-enhanced cytolysis correlated with a precedent increase in reactive oxygen species (ROS) level and antioxidant treatment could completely block the E6-dependent sensitization. These findings represent the first demonstration of a link between a viral oncogene-sensitized cytolysis and ROS. Previous studies have shown conflicting results regarding whether TNF-induced cytolysis of L929 cells is through necrosis or apoptosis. Here we report that, although L929 cells underwent DNA fragmentation after exposure to TNF, they retained the morphology of intact nuclei while gaining permeability to propidium iodide, features characteristic of necrosis rather than apoptosis. We confirmed that the broad spectrum caspase inhibitor benzoylcarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone markedly increased the susceptibility of L929 cells to TNF, and further demonstrated that E6 enhanced this susceptibility, which again correlated with increased ROS accumulation. We showed that the expression of E6 in L929 cells did not alter the stability of p53, and the cells retained a p53 response to actinomycin D. Furthermore, two E6 mutants defective for p53 degradation in other systems exhibited differential effects on TNF sensitization. These results suggest that the enhancement of TNF-induced L929 cytolysis by E6 is independent of p53 degradation. We also found that TNF-induced activation of NF-κB did not account for the enhanced TNF susceptibility by E6.

Among the over 90 human papillomavirus (HPV) types, HPV type 16 is the most prevalent type associated with cervical cancer. The E6 oncoprotein from the “high risk” HPV genital types, including HPV-16, along with the other major oncoprotein E7, is selectively retained and expressed in HPV-positive cervical carcinomas and derived cell lines (for review, see Ref. 1). E6 has multiple functions including cellular transformation, immortalization, telomerase activation, and induction of resistance to calcium/serum-induced terminal differentiation (for review, see Ref. 2). Most recently, E6 has been reported to suppress or enhance apoptosis depending on the cell type and stimulus (3–8).

The murine fibrosarcoma cell line L929 is widely used to study responses to tumor necrosis factor α (TNF). TNF, produced mainly by activated macrophages during immune and inflammatory responses, exerts pleiotropic effects on a wide range of cells. In addition to the inflammatory and immunomodulatory activities, TNF exerts cytolytic activity on a variety of tumor cell lines in vitro (9), including L929. Depending on the cell type, TNF-mediated cytolysis may occur either through apoptosis or necrosis. TNF cytolysis of most cell types is through apoptosis (10), and is characterized by morphological and biochemical changes including cellular shrinkage, membrane blebbing, chromatin condensation, mitochondrial depolarization, activation of caspases, and internucleosomal DNA fragmentation (for review, see Ref. 11). However, there have been conflicting observations regarding TNF-induced cytolysis of L929. One group (12) reported that TNF-treated L929 cells rapidly died in a necrotic manner, characterized by swelling of the cytoplasm followed by disruption of the plasma membrane accompanied by cellular collapse without breakdown of the DNA. The recent finding that caspase inhibitors increase rather than decrease the susceptibility of L929 cells to TNF further supports the necrotic mode of cell death in TNF-treated L929 cells (13, 14). In contrast, another group (15) reported that treatment of L929 cells with TNF plus actinomycin D induced DNA fragmentation before significant cytolysis occurred, indicating that L929 cells underwent apoptosis. Other investigators also reported DNA fragmentation of L929 cells in response to TNF (16–19). Regardless of the discrepancy on the mode of cell death, both groups have shown that TNF-induced cytolysis of L929 cells is mediated by mitochondrial formation of reactive oxygen species (ROS) (15, 20).

Alterations in the structure and function of mitochondria are implicated in apoptosis and necrosis. Mitochondria generate oxygen species (ROS) that regulate and control a broad range of cellular processes including apoptosis and necrosis (21, 22). ROS can cause the peroxidation of lipids, denaturation of proteins, and fragmentation of DNA (23, 24). In addition, ROS can be involved in the induction of tumor necrosis factor (TNF) receptor-mediated death (25, 26). ROS have been suggested to play a role in the induction of apoptosis, either as mediators or effectors (27, 28). ROS generated by mitochondria and nuclear factors are important in the regulation of apoptosis (29). ROS can induce apoptosis by inducing mitochondrial depolarization and the release of cytochrome c (30, 31). However, ROS can also inhibit apoptosis by neutralizing the proapoptotic effect of ROS (32, 33). The balance between pro- and anti-apoptotic ROS is critical in regulating apoptosis and necrosis. The role of ROS in regulating apoptosis and necrosis is complex and depends on the cell type, stimulus, and conditions. The role of ROS in regulating apoptosis and necrosis is complex and depends on the cell type, stimulus, and conditions.
ROS as by-products of molecular oxygen consumption in the electron transport chain (for reviews, see Refs. 21 and 22). The role of ROS in cell death has been intensively examined (for reviews, see Refs. 23 and 24). Through indirect approaches, earlier studies suggested involvement of mitochondrial production of ROS in TNF-induced cytolysis of L929 cells (25, 26). Recently, direct evidence indicated that TNF-induced necrosis in L929 cells was due to increased ROS formation in mitochondria (20). Using dihydorhodamine 123 (DHR123), a cell-permeable ROS-specific fluorogenic marker, in combination with confocal laser scanning microscopy and flow cytometry, it was demonstrated that TNF-induced ROS formation occurred shortly before the commencement of irreversible cell damage (20). Furthermore, the TNF-induced ROS formation occurred exclusively under conditions where cells were sensitive to the cytotoxic activity of TNF. Therefore, TNF-induced ROS formation is thought to be causally related to TNF cytotoxicity in L929 cells (20). Earlier studies suggested that the TNF-induced mitochondrial production of ROS was mainly generated at the ubiquinone site, as implied by the different effects observed with the mitochondrial respiratory chain inhibitors (25, 26).

In the present study, we examined the effect of HPV-16 E6 expression on necrosis or apoptosis using the TNF sensitive L929 cell line as a model. We found that E6-expressing cells were more susceptible to TNF-induced cytolysis. Following the lead of increased ROS formation in TNF-mediated cytotoxicity (20), we investigated the possibility that the enhanced susceptibility to TNF by E6 was due to its effect on ROS. Our results indicate that HPV-16 E6-enhanced susceptibility to TNF correlates with increased ROS accumulation in mitochondria.

EXPERIMENTAL PROCEDURES

Reagents—Dihydorhodamine 123 (DHR123) was supplied by Molecular Probes (Eugene, OR), prepared in MeSO as 5 mM and used at 1 μM. The peptide caspase inhibitor zVAD-fmk was from Bachem (Torrance, CA). Recombinant murine TNF, propidium iodide (PI), butylated hydroxyanisole (BHA), cycloheximide, and actinomycin D were purchased from Sigma.

Cell Culture and Retroviral Infection—Amphotropic packaging cell line PA317 and murine fibrosarcoma L929 cell line (kindly provided by Honglin Li, Harvard Medical School) were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. The pLXSN-based retroviral constructs were introduced into the PA317 cells by calcium phosphate precipitation (27). G418-resistant colonies were pooled, and the supernatant containing roughly equivalent titer of virus was used to infect L929 cells. Colonies selected in 1.3 mg/ml G418 were pooled and established as stable cell lines for further experiments.

Cell Viability Assays—Cells were seeded in a 96-well plate at a density of 4,000 cells/well. The next day, cells were changed to media containing no TNF or various concentrations of TNF and incubated for 24 h. Cell viability was then assessed using the quantitative colorimetric MTT assay kit (Chemicon International Inc., Temecula, CA) as described (28). MTT conversion was measured by an ELISA plate reader at 570 nm with a reference wavelength of 655 nm. Percentage of cell survival was calculated as

\[
\frac{A_{570nm_{untreated\ cells}} - A_{570nm_{treated\ cells}}}{A_{570nm_{medium}}} \times 100.
\]

For flow cytometric measurement of cell viability, cells were seeded in six-well plates at 2 × 10^5 cells/well. The next day cells were changed to media containing no TNF or various concentrations of TNF and incubated for the indicated time. Both floating and adherent cells were harvested, stained with PI, and analyzed on a FACScan flow cytometer. The numbers shown in the histograms represent the percentage of dead or dying cells with damaged cellular membranes.
periods. Plasmidic histone-DNA complexes were detected using Cell Death Detection ELISA kit (Roche Molecular Biochemicals) as described (28), and the absorbance was measured on an ELISA plate reader at 405 nm with a reference of 550 nm.

For flow cytometric measurement of DNA fragmentation, cells were seeded in six-well plates at 2 x 10^5 cells/well and changed the next day to media containing DHR123 at 1 μM in the absence or presence of varying concentrations of TNF. At the indicated time intervals, both detached and adherent cells were harvested and resuspended in PBS containing 1 μg/ml PI. The cell suspensions were then analyzed simultaneously for DHR123-derived fluorescence and cell death by flow cytometry as follows. Red fluorescence (FL3) was measured with the strong fluorescence representing dead or dying cells (PI-positive) and the weak basal fluorescence representing viable cells (PI-negative). Rhodamine 123 fluorescence (Rh123) resulting from DHR123 oxidation by ROS was analyzed on PI-negative cells by setting a gate on weak FL3 cells, and detected as green fluorescence (FL1). Relative increase in Rh123 fluorescence is defined as the ratio between the increment of mean Rh123 fluorescence intensity and the initial fluorescence intensity for the same condition.

Flow Cytometric Detection of Nuclear Morphology—Cells were seeded in six-well plates at 2 x 10^5 cells/well and changed the next day to media containing 5% FBS and 3 parts 0.2N NaPO₄, 0.1 N sodium citrate, 0.5% Triton X-100, and 10 μg/ml PI. The cell suspensions were then left on ice, and the DNA content subsequently analyzed on the FACScan.

Measurement of Oxygen Radical Formation and Cell Death by Flow Cytometry—Cells were seeded in six-well plates at 2 x 10^5 cells/well and changed the next day to media containing DHR123 at 1 μM in the absence or presence of varying concentrations of TNF. At the indicated time intervals, both detached and adherent cells were harvested and resuspended in PBS containing 1 μg/ml PI. The cell suspensions were then analyzed simultaneously for DHR123-derived fluorescence and cell death by flow cytometry as follows. Red fluorescence (FL3) was measured with the strong fluorescence representing dead or dying cells (PI-positive) and the weak basal fluorescence representing viable cells (PI-negative). Rhodamine 123 fluorescence (Rh123) resulting from DHR123 oxidation by ROS was analyzed on PI-negative cells by setting a gate on weak FL3 cells, and detected as green fluorescence (FL1). Relative increase in Rh123 fluorescence is defined as the ratio between the increment of mean Rh123 fluorescence intensity and the initial fluorescence intensity for the same condition.

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RESULTS

Expression of HPV-16 E6 Sensitizes L929 Cells to TNF-induced Cytolysis—To examine the influence of HPV-16 E6 expression on TNF-induced cytosis, we introduced E6 into mouse L929 cells by retroviral infection. G418-resistant colonies were pooled and cultured to establish a stable cell line (L16E6). The control cell line (LXXSN) was established in a parallel manner by pooling approximately equal number of G418-resistant colonies derived from retroviral infection with the LXSN vector. Both L16E6 and LXXSN cells were treated with various concentrations of murine TNF, and cell viability was quantitatively determined by analysis of MTT conversion (33). This colorimetric assay reflects the ability of live cells to yield a dark blue formazan product by cleaving MTT. As shown in Fig. 1A, L929 cells exhibited decreased viability in response to TNF in a dose-dependent manner, and the susceptibility to TNF was enhanced in L16E6 cells at each TNF concentration. Next, we confirmed this observation by using the fluorescent exclusion dye PI and flow cytometric analysis as described previously.2 This assay is based on the fact that viable cells with intact plasma membranes exclude PI, while dead or dying cells with disrupted cell membrane take up PI and fluoresce when PI intercalates into DNA. Following TNF treatment, both detached and adherent cells were harvested, incubated with PI, and analyzed by flow cytometry. Fig. 1B shows an example of PI fluorescence histograms in which the PI-positive populations represent the dead cells and the PI-negative populations represent the viable cells. Clearly, L16E6 cells showed higher percentage of PI-positive cells in response to a range of TNF concentrations than did the LXXSN control cells. In addition, the cell lines established by retroviral infection, we established stable cell lines by transfection. We found that the transfection-derived cell lines behaved similarly to the retrovirally established cell lines (data not shown). Therefore, we chose the retrovirally established cell lines L16E6 and LXXSN for further studies.

E6-potentiated Cytolysis Correlates with Increased Accumulation of ROS—Direct, compelling evidence has suggested that TNF-induced cytotoxicity in L929 cells is due to the induced ROS production in mitochondria (20). Therefore, we decided to examine whether the sensitization by E6 was through alteration of ROS levels. For this purpose, we measured ROS levels using DHR123 as the fluorogenic ROS probe according to previous studies (14, 20). Oxidation of the non-fluorescent DHR123 by ROS yields the fluorescent Rh123, which is subsequently measured by activated mitochondria (34). To monitor ROS levels, cells were treated with TNF in the presence of DHR123 and stained with PI immediately before flow cytometric analysis. The percentage of TNF-induced cell death was monitored in the same way as shown in Fig. 1B. Following previously established methodology (14), the Rh123 green fluorescence due to the oxidation of DHR123 by ROS was measured exclusively in the viable cell population that exhibited

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basal red fluorescence (PI-negative) for the following reasons. First, it has been shown that, following TNF treatment, ROS was induced shortly before the occurrence of irreversible cell damage and was followed by a sharp drop of Rh123 fluorescence as a consequence of plasma membrane disruption and subsequent loss of mitochondria transmembrane potential. Therefore, it is unnecessary to measure ROS in dead cells. Second, by measuring ROS-derived fluorescence in the viable cell population that exhibits low, basal PI fluorescence, the influence of PI fluorescence on Rh123 fluorescence can be ruled out. Based on the individual red and green fluorescence histograms, we calculated, respectively, the relative increase in the percentage of cell death and in the percentage of mean fluorescence intensity derived from DHR123 oxidation. The result of a representative experiment is shown in Fig. 2. Relative to the untreated cultures, incubation of either LLXSN or L16E6 cells with TNF increased the mean DHR123-derived fluorescence as well as cell death in a time-dependent (Fig. 2A) and a dose-dependent (Fig. 2B) manner. The basal mean fluorescence intensities derived from DHR123 and spontaneous cell death were not changed by E6 (data not shown). However, the relative increases were consistently higher in L16E6 than in LLXSN cells. Importantly, as shown in Fig. 2A, after 2 h of TNF treatment, both LLXSN and L16E6 cells showed a small increase in DHR-derived fluorescence intensity (3% and 8%, respectively), whereas the percentage of collapsed cells barely increased (<1%). After 4 h of TNF treatment, the difference of cytolysis between LLXSN and L16E6 became notable (Fig. 2A). This was in agreement with the previous observation that TNF-induced ROS accumulation preceded cytolysis in L929 cells (14, 20). Furthermore, since the DHR123 oxidation by ROS was measured in viable cell populations with intact plasma membranes, ROS accumulation preceded cell death. Treatment with the antioxidant BHA, a ROS scavenger, resulted in a slight decrease in the basal level of DHR123-derived fluorescence (data not shown). Interestingly, when BHA was added at the same time as TNF, both ROS induction (Fig. 2, top panels) and cell death (Fig. 2, bottom panels) were blocked in both L16E6 and LLXSN cells. These data suggest that TNF-induced ROS formation is causally related to TNF-mediated cytolysis, consistent with previous findings (20). Likewise, the enhanced cytolysis by E6 appears to result from its positive impact on ROS accumulation.

TNF-treated L929 Cells Undergo Necrosis although DNA Fragmentation Occurs—Contradictory reports exist regarding TNF-induced necrosis lacking DNA fragmentation in L929 cells (12, 35) versus apoptosis exhibiting DNA fragmentation (15–19). To resolve this discrepancy, we first conducted several DNA fragmentation assays. Our Cell Death Detection ELISA® 

assay indicated that exposure to TNF resulted in a slight increase of cytoplasmic DNA-histone complexes before visible cell killing occurred (data not shown). Flow cytometric analysis also revealed slight increases in the sub-G1 population in TNF-treated LLXSN cells (Fig. 3). Again, an enhanced effect was observed in L16E6 cells (Fig. 3) in correlation with increased cytolysis (Fig. 1B). However, the DNA fragmentation-based approach failed to discriminate between apoptosis and necrosis (for review, see Ref. 22). To ascertain whether TNF-treated L929 cells underwent apoptosis, we examined nuclear morphology and plasma membrane integrity simultaneously by Hoechst and PI double staining (30). Hoechst is permeable to the cellular membrane and stains all nuclei blue. Only cells with damaged cell membrane are PI-permeable, and thus stain pink in the presence of PI and Hoechst. Jurkat cells that undergo extensive apoptotic cell death in response to serum starvation were used as a positive control for apoptosis. As shown in Fig. 4B, Jurkat cells starved of serum showed blue as well as pink condensed and fragmented nuclei. The blue condensed/fragmented nuclei are a hallmark of apoptosis lacking cell membrane damage, whereas the pink condensed/fragmented nuclei indicate the late stage of apoptosis with the plasma membrane being damaged. In contrast, although TNF treatment of L929 cells (LLXSN or L16E6) resulted in an increased number of cells with pink nuclei, which was more pronounced in L16E6 cells (Fig. 4D), the nuclei morphology was relatively maintained as compared with that of the untreated cells (Fig. 4C). Various concentrations of TNF and time points were examined, and none of the cells showed blue condensed/fragmented nuclei (data not shown). Addition of cycloheximide, the frequently used cytolysis co-inducer of TNF, markedly enhanced the percentage of pink nuclei yet failed to result in blue nuclei showing condensation/fragmentation (data not shown). These observations suggest that TNF-treated L929 cells undergo necrotic death, although some DNA fragmentation can be detected.

Enhanced Cytolysis by zVAD-fmk Is Increased in E6-expressing Cells in the Presence of TNF—It has been reported that the broad-spectrum caspase inhibitor zVAD-fmk enhanced the susceptibility of L929 cells to TNF, suggesting that caspases are negative rather than positive regulators of TNF-induced ROS formation and consequent cytolysis (14). In order to examine whether E6 affects the zVAD-fmk effect, we simultaneously measured ROS levels, loss of cell membrane integrity, and DNA fragmentation in TNF-treated or -untreated cells with or without pretreatment of zVAD-fmk. Data derived from flow cytometric analyses are depicted in Fig. 5. Treatment of LLXSN and L16E6 cells with 5 or 10 pg/ml TNF for 5 h and 30 min did not result in significant cytolysis and DNA fragmentation (Fig. 5, A and B). However, significant increases in ROS levels were observed at both TNF doses and were more pronounced in L16E6 cells (Fig. 5C), again demonstrating that ROS induction by TNF precedes cytolysis and DNA fragmentation. Notably, zVAD-fmk alone exhibited certain cytotoxicity and greatly increased the susceptibility of L929 cells to TNF, particularly in L16E6 cells. We observed a good correlation between cytolysis and DNA fragmentation, both of which were preceded by ROS accumulation (Fig. 5). In the experiment shown in Fig. 5, following a 2-h pretreatment with zVAD-fmk, TNF was added in the presence of zVAD-fmk. We also assessed the effect of zVAD-fmk by removing zVAD-fmk from cells after a 2-h pretreatment, then refed cells with media containing or lacking TNF. The removal of zVAD-fmk resulted in a delay of approximately 18 h in the onset of cytolysis. Except for this delay, a correlation of cytolysis, DNA fragmentation, and ROS accumulation similar to what was obtained in the continual presence of zVAD-fmk (Fig. 5) was observed (data not shown).

Sensitization by E6 Is Independent of Its Ability to Promote p53 Degradation—Expression of HPV-16 E6 has been shown to promote p53 degradation in the in vitro reticulocyte lysate system (36–38) and in various cell types in tissue culture (39–41) including mouse cells (42). As p53 plays a positive role in ROS generation (43–45), one would intuit that E6 expression should reduce ROS production. Although this rationale is opposite to what we observed, we investigated the possible involvement of E6 interaction with p53 in the increased ROS generation in E6-expressing L929 cells. First, we compared p53 turnover rate in LLXSN and L16E6 cells and found no significant difference between these cell lines (Fig. 6A), indicating that HPV-16 E6 did not promote p53 degradation in the stable L16E6 cell line. This failure of HPV-16 E6 to degrade mouse p53 has been previously reported in NIH 3T3 cells (31). L929 is a widely used mouse cell line that may contain wild-type p53. Consistent with this, LLXSN cells treated with ActD showed
 HPV-16 E6 Sensitizes TNF-induced Cytolysis

Fig. 1. E6 sensitization of TNF-induced ROS accumulation and cytolysis. A, time course. Cells plated in 6-well plates as in Fig. 1B were treated with DHR123 (1 μM) alone or DHR123 plus 1 ng/ml TNF for indicated times. Both detached and adherent cells were harvested, stained with PI, and subjected to flow cytometric analyses for DHR123-derived fluorescence (top panel) and cell death (bottom panel). Note that the DHR123-derived fluorescence was measured exclusively in the viable cell population (PI-negative). Relative increase in Rh123 fluorescence is defined as the relative increase of mean Rh123 fluorescence in TNF/DHR123-treated culture to that in DHR123-treated culture for the same time period. TNF-induced cytolysis represents the percentage of dead/dying cells in TNF/DHR123-treated cultures subtracted by spontaneous cell death in the DHR123-treated culture. B, dose curve. Cells were treated and analyzed as above except that 50 μM BHA was added as indicated at the same time as DHR123 and/or TNF and incubated for 6 h. TNF-induced relative change in Rh123 fluorescence or cytolysis is the value relative to that obtained for the same condition except TNF.

Fig. 2. E6 enhances TNF-induced DNA fragmentation. Cells plated as described in Fig. 1B were treated with indicated concentrations of TNF for 23 h. Cells were then harvested, processed as described under “Experimental Procedures,” and subjected to flow cytometric analysis. Numbers represent the percentage of cells with a sub-G1 population. Data obtained in this figure and in Fig. 1B are from the same experiment.

Fig. 3. E6 enhances TNF-induced ROS formation and consequent cytolysis are independent of the ability of E6 to promote p53 degradation. To further examine this inference, we tested two E6 mutants defective for p53 degradation as characterized in reticulocyte lysate and in human mammary epithelial cells (Ref. 46, and data not shown). While mutant F2V failed to enhance TNF-induced cytolysis and ROS generation as did the control cells, mutant S82D/L83W showed similar enhancement to that seen in L16E6 cells (Fig. 6B). Notably, the ActD-treated L16E6 cells displayed a similar extent of p53 induction (Fig. 6B). The retained p53 induction ability of E6-expressing L929 cells suggests two points. First, it is in agreement with the unaltered stability of p53 in L16E6 cells. Second, the p53 protein in our L929 cells is unlikely a loss-of-function mutant that resists E6-promoted degradation. Taken together, these results suggest that E6-enhanced ROS formation and consequent cytolysis are independent of the ability of E6 to promote p53 degradation.

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in the wild-type E6-expressing cells (Fig. 6C). The cells derived from both E6 mutations showed increased p53 levels in response to actinomycin D-induced DNA damage (Fig. 6B) and showed similar p53 turnover rates to LLXSN and L16E6 cells (data not shown). To exclude the possibility that the inability of mutant F2V to enhance TNF-induced ROS accumulation and cytolysis was due to a lack of expression, we examined E6 levels in the stable cell lines. Since it is difficult to detect E6 protein due to its extremely low levels in cells (for review, see Ref. 2), we confirmed the expression of E6 mRNA by RT-PCR. As shown in Fig. 7, RT-PCR revealed the presence of E6 transcripts that were expressed at comparable levels in L16E6 (lanes 3 and 8) and the mutant-derived cell lines (lanes 4 and 9, and lanes 5 and 10), and the absence of E6 in the LLXSN control cell line (lanes 2 and 7). This result further supports our conclusion that E6-enhanced TNF susceptibility of L929 cells is p53-independent.

**E6-enhanced Susceptibility Is Not through the Alteration of TNF-induced NF-κB Activation**—TNF activates a number of mitogen-activated protein kinase cascades upstream of the activation of the transcription factor NF-κB (for review, see Ref. 47). TNF-induced NF-κB activation has been shown to counteract TNF-induced cytolysis in various cell types (for review, see Ref. 48) or to be associated with the cytolysis in L929 cells (26). We tested the possibility that E6 might alter TNF-induced NF-κB activation. LLXSN and L16E6 cells were transfected with a reporter construct consisting of three NF-κB sites and a minimal promoter linked to the luciferase gene (32). Treatment of the transiently transfected cells with TNF for 3 h resulted in a small induction of luciferase activity (Fig. 8) as compared with that observed with the L929 cells stably transfected with an NF-κB luciferase reporter (14). As shown in Fig. 8, whereas 100 pg/ml TNF resulted in a slightly higher induction of luciferase activity in L16E6 cells as compared with LLXSN, 200 pg/ml TNF exhibited the opposite effect. This indicates a lack of correlation between E6-enhanced cytolysis and alteration of NF-κB activation in response to TNF.

**DISCUSSION**

In this report we show that expression of HPV-16 E6 sensitizes TNF-induced cytolysis of fibrosarcoma L929 cells. Similarly, HPV-16 E6 was recently shown to exert moderate sensitization of human primary keratinocytes to TNF-induced apoptosis (59). TNF plays an important role in host defenses against viral infection by selectively killing cells infected with viruses (49–56). Of particular relevance, TNF has been demonstrated to down-regulate transcription of the HPV-16 E6 and E7 genes by repressing the p97 promoter from which these genes are expressed (57). Keratinocytes, the natural target of HPV infection, secrete TNF as well as other cytokines that regulate host responses to infection and growth/differentiation. Interestingly, cervical cells immortalized by HPV-16 or -18 exhibited significantly reduced expression of specific cytokines including TNF, suggesting that the down-regulation of cytokine secretion may contribute to persistence of HPV-
infected cells (58).

Similar to the sensitization effect observed for HPV-16 E6, polyomavirus middle T antigen also sensitized L929 cells and C127 cells to TNF-induced cytolysis (61) and adenovirus E1A sensitized NIH 3T3 cells (38, 55, 62–64). To our knowledge, E6 is the first viral oncoprotein demonstrated to exert its sensitizing effect through ROS. However, E6 alone does not seem to affect the basal level of ROS in L929 cells. Whether similar E6-enhanced TNF-induced cytolysis via ROS also occurs in human keratinocytes remains to be determined. Various classes of viruses manifest the ability to increase ROS levels upon infection, which is thought to play a role in the pathogenesis of viral infections (for review, see Ref. 65). It has recently been argued that ROS may contribute to the carcinogenic process because tissue destruction resulting from ROS leads to compensatory cell proliferation (for review, see Ref. 66). Thus, it should be of great interest to investigate whether the previously observed sensitization effect by E6 of human keratino-

FIG. 6. Sensitization by E6 is independent of its ability to promote p53 degradation. A, E6 did not result in appreciably decreased stability of p53 in L929 cells. L16E6 and LLXSN cells were treated with 20 μg/ml cycloheximide for 0, 15, 30, and 60 min, respectively. Cell lysates containing approximately 150 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis and Western blotted with mouse monoclonal anti-p53 antibody Ab-3 (Calbiochem). The blot was reprobed with mouse monoclonal anti-tubulin μ (Sigma) as a loading control. B, E6 did not affect p53 induction in response to ActD. Parallel cultures of each indicated cell line were left untreated or treated with 12.5 nM ActD for 24 h. Cell lysates containing 400 μg of protein were subjected to immunoprecipitation of p53 followed by Western blot analysis. C, E6 mutants defective for p53 degradation show different phenotypes in TNF-induced cytolysis and ROS formation. The indicated cell lines plated as described in Fig. 1B were treated with 1 ng/ml TNF for 6 h followed by analysis as described in Fig. 2. Data shown represent the mean of three experiments derived from flow cytometric analysis.

FIG. 7. Expression of E6 mRNA. RT-PCR for the indicated cell lines was performed using two sets of E6-specific primers and the products run on a 2% agarose gel. Primer set 1 detected a 477-nt fragment from the unspliced mRNA, and a 292-nt fragment from the major spliced mRNA (lanes 3–5). Primer set 2 detected a 199-nt fragment derived only from the unspliced mRNA (lanes 8–10). Lanes 6 and 11 serve as the negative control for genomic DNA contamination, where the total RNA from L16E6 without reverse transcription was used as the template for PCR.

FIG. 8. Effect of E6 on TNF-induced NF-κB activation. LLXSN and L16E6 cells were transfected with an NF-κB luciferase reporter construct along with a β-galactosidase reporter. Twenty-four hours later, cells were untreated or treated with indicated concentrations of TNF for 3 h. Cell lysates were subjected in parallel to a luciferase assay and a β-galactosidase assay. NF-κB activation was expressed as -fold activation in response to TNF of luciferase activity after normalization with β-galactosidase activity. Data represent the mean ± S.D. of three experiments, each performed in duplicate. Black bars, LLXSN; striped bars, 16E6.
cytes to TNF-induced apoptosis (59) is also through ROS and its possible relevance to E6’s oncogenic potential.

Previous studies have shown conflicting results regarding whether TNF-induced cytosis of L929 cells is through necrosis or apoptosis. In the present study we were able to detect DNA fragmentation in TNF-challenged L929 cells. However, double staining with Hoechst and PI failed to reveal blue nuclei with condensed/fragmented nuclear morphology in our TNF-treated L929 cells. One study reported that TNF-treated L929 cells exhibited condensed/fragmented nuclei (18). However, whether plasma membrane disruption occurred at the time was not addressed. The discrepancy regarding the presence or absence of TNF-induced nuclear condensation/fragmentation may reflect subtle differences in the strains of L929 cells being investigated. From our data, necrosis rather than apoptosis accounted for the cytosis of the L929 cells. Moreover, we confirmed the recent finding that the broad-spectrum caspase inhibitor zVAD-fmk markedly increased susceptibility of L929 cells to TNF (14), and demonstrated that E6 enhanced this susceptibility. Caspase inhibitors normally protect against caspase-mediated apoptosis, although the inability of zVAD-fmk to prevent cytosis provoked by multiple different apoptosis inducers is not unprecedented (67, 68). However, the sensitization effect by the caspase inhibitor would argue against apoptosis. E6 is the first oncoprotein shown to act like zVAD-fmk in that both increased ROS and cytosis, although it differed from zVAD-fmk by doing so in an TNF-dependent manner.

How HPV-16 E6 increases TNF-induced ROS accumulation and cytosis is at present unclear. The increased ROS levels may result from an enhanced production of ROS or an impaired mitochondrial scavenging system. We found that the E6-expressing cells retained competence for scavenging ROS by glutathione, which has been suggested as the major mitochondrial scavenger of TNF-induced ROS (20). However, E6 did not affect exogenous \( \text{H}_2\text{O}_2 \)-induced ROS accumulation, indicating that species of ROS other than \( \text{H}_2\text{O}_2 \) may be responsible for the E6-enhanced TNF-induced ROS accumulation. One such ROS species may be superoxide \( \text{O}_2^\cdot \), which can be converted to \( \text{H}_2\text{O}_2 \) by manganese superoxide dismutase (MnSOD), a mitochondrial antioxidant enzyme. TNF has been well documented to induce the synthesis of MnSOD (69), and overexpression of MnSOD has been shown to protect against TNF-mediated cell death (70, 71). Human immunodeficiency virus-infected cells failed to show TNF-induced MnSOD expression, which may account for the increased sensitivity of the infected cells to heat and radiation (53, 71). It has also been reported that the AIDS-related Kaposi's sarcoma cells possess impaired ROS scavenging capacities, establishing conditions permissive for the intracellular retention of ROS (72, 73). Since E6 possesses transcriptional repression and protein degradation-promoting activities (for review, see Ref. 2), an intriguing possible mechanism for E6-enhanced ROS accumulation and cytosis is down-regulation of antioxidant enzymes such as MnSOD. On the other hand, since E6 also exhibits transcriptional activation activities (for review, see Ref. 2), it is possible that E6 up-regulates the expression of TNF-RI, the TNF receptor, or a downstream effector(s), thereby amplifying the signals leading to ROS formation. Experiments are under way to address these possibilities.

Given that p53 plays a positive role in ROS generation (43–45), although in a cell-type specific manner (44), and that HPV-16 E6 promotes p53 degradation, as demonstrated in rabbit reticulocyte lysate and in various cell types (36, 37, 39–41), we were surprised to find that E6 enhanced ROS accumulation. Our data strongly imply that E6-enhanced ROS accumulation is a p53-independent event. Our data also suggest that the enhanced effect by E6 is not through the modulation of NF-\( \kappa \)B activation, compatible with the recent demonstration that the signaling pathways leading to cytosis and to NF-\( \kappa \)B activation are segregated in L929 cells (35, 74). Recently it was reported that an activated form of oncoprotein c-Myc induced mouse Rat1 fibroblasts to produce ROS in response to TNF treatment (75). Interestingly, HPV-16 E6 has been shown to activate the c-Myc promoter (76) and to increase c-Myc protein in human mammary epithelial cells (77). Moreover, expression of the c-Myc gene was increased in HPV-positive cervical cancer cells and HPV-16-immortalized cervical cells (78, 79). These observations suggest that E6 may enhance ROS accumulation by up-regulating Myc. Paradoxically, Myc has recently been reported as a degradation target of E6 (80). Several other potential cellular targets of E6 have been identified recently (reviewed in Ref. 2; see Refs. 81 and 82), including E6TP1 (83) and Bak (4). Analogous to p53, a subset of these proteins have been reported to be targeted for degradation by E6 via the ubiquitin-proteasome system (4, 80, 83). Depletion of the pro-apoptotic protein Bak by E6 again seems to be contradictory to E6-enhanced cytosis. Interestingly, E6TP1 has been suggested as a potential GTPase-activating protein for small G proteins (83) and thus may inactivate G proteins. Given that the activated forms of the small GTP-binding proteins Ras or Rac1 play a positive role in TNF-induced ROS accumulation (84), the E6-targeted depletion of E6TP1 seems to be in concordance with our present finding of E6 sensitization. Other E6-binding proteins, such as E6BP/ERC-55 (29, 85, 86), a calcium-binding protein, may also be involved in enhancing ROS production under certain conditions by regulating calcium signaling. It has been well documented that calcium stimulates ROS production in mitochondria by the respiratory chain (for review, see Ref. 21). Further experimentation will be needed to delineate the mechanism by which E6 enhances TNF-induced ROS accumulation and the subsequent cytosis.

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