The Effect of Proteasome Inhibition on p53 Degradation and Proliferation in Tonsil Epithelial Cells

George F. Harris IV, MD; Mary E. Anderson; John H. Lee, MD

Objective: To determine whether proteasome inhibition could reverse E6-mediated p53 degradation, cause selective growth inhibition, and induce apoptosis in human papillomavirus E6-transformed primary tonsil epithelial cells.

Design: Primary human and mouse tonsil epithelial cell lines were transformed with a retrovirus containing human papillomavirus 16 oncogenes. MG132 was used to inhibit proteasome degradation in vitro and in vivo, and biochemical assays regarding p53 and apoptosis were performed.

Results: In cells that express E6, proteasome inhibition with MG132 restored p53 protein levels and decreased proliferation in a dose-dependent fashion that was significantly more pronounced compared with controls. However, inhibition of proliferation occurred at a lower concentration than restoration of p53 protein expression. Also, wild-type and p53 knockout mouse tonsil epithelial cells that express E6 had near-identical inhibition of growth, suggesting that growth inhibition was p53 independent. In vivo studies did not demonstrate any growth inhibition.

Conclusion: The findings suggest that proteasome inhibition preferentially inhibits proliferation in cells expressing E6 through a p53-independent mechanism.


Head and neck cancer is the ninth leading cause of new cancers in the United States and the 12th leading cause of death from cancer. Despite improved surgical and radiation techniques, overall survival related to head and neck squamous cell carcinoma (HNSCC) has not improved in 30 years. Most failure results from extensive local invasion or distant metastasis. To improve survival in HNSCC, targeted medical therapies that offer a substantial advantage over the current treatments for HNSCC need to be developed.

Targeted therapy is complicated by the fact that HNSCC may reach metastatic growth by many different cellular pathways. Achieving the goal of targeted therapy will require a method that will identify the biomarkers that a particular tumor relies on for growth and survival. Although pathways and biomarkers for these altered mechanisms are poorly defined for most HNSCCs, there is a particular subset, human papillomavirus–positive (HPV+) HNSCC, in which many of the altered mechanisms are predictable. Human papillomavirus type 16 (HPV-16) causes approximately 25% of HNSCCs. There are several factors that make HPV mechanistic-based therapies feasible. It is currently possible to identify these tumors easily and quickly with a polymerase chain reaction test. Some of the HPV-related mechanisms that lead to metastatic growth are known. Therefore, an HPV-related treatment would currently be possible to implement and would likely be beneficial for a significant number of HNSCCs.

Of the reported mechanisms of HPV that lead to carcinogenic growth, degradation of cellular p53 by HPV E6 is perhaps the best described. Inactivation of p53 is associated with loss of cell cycle arrest and evasion of apoptosis, which are thought to be requirements for malignant transformation of epithelial cells. Reversing E6-mediated p53 degradation could potentially stop malignant or metastatic disease. During normal cellular function, p53 undergoes ubiquitination followed by controlled degradation at the 26S proteasome. In HPV-associated disease, this process occurs more rapidly because HPV-16 E6 and the cellular component E6-AP, a ubiquitin-conjugating enzyme, act in concert to ubiquitinate and

Author Affiliations:
Department of Otolaryngology—Head and Neck Surgery, The University of Iowa, Iowa City (Drs Harris and Lee); and Department of Otolaryngology, US Department of Veterans Affairs, Veterans Health Administration, Iowa City (Ms Anderson and Dr Lee).
degrade p53 by targeting it to the 26S proteasome more quickly than normal. The proteasome is a cytosolic organelle consisting of one core 20S particle and two 19S complexes. The 20S particle consists of multiple α and β stacked rings. The α rings serve as gatekeepers to the proteolytic activity of the β subunits. The β rings each contain several proteolytic sites, including chymotrypsinlike, trypsinlike, and caspase-like sites. A new class of anticancer agents that take advantage of the dependence of rapidly proliferating cells on proteasome activity is now available. MG132 is a peptide aldehyde proteasome inhibitor. It is known to reversibly inhibit all proteolytically active subunits of the β rings as a transition state analogue. Because E6-mediated loss relies on increased proteasomal degradation, proteasome inhibitors should reverse the E6-mediated p53 degradation, potentially arresting tumor growth.

We are finding that currently available HPV+ HNSCC cell lines are of limited value in studying viral carcinogenic mechanisms due to mutations in key viral genes. To better understand specific viral mechanisms in HPV-associated tonsil cancer, we have chosen to develop cell lines with specific properties of virus-infected tonsil epithelium. We developed a technique for isolating and culturing primary tonsil epithelial cells (TECs) in both humans and mice. We then inserted the HPV oncogenes into these cells to study the mechanism of virus-associated transformation. The resulting cells allowed us to test a mechanism not only in vitro but also in immune-competent mice. Using these methods, we investigated whether E6-mediated p53 degradation could be inhibited by MG132, whether MG132 preferentially inhibited growth of E6-transformed keratinocytes in vitro and in vivo, and whether this growth inhibition was related to p53 restoration.

**METHODS**

**CELL CULTURE AND RETROVIRAL INFECTIONS**

C57BL/6 mice were euthanized, and oral epithelium overlying the tonsillar fossa was harvested. The epithelium was dissociated from the underlying dermis overnight with grade II collagenase (Dispase II; Roche Applied Science, Indianapolis, Indiana), manual tissue separation, brief trypsinization with 0.25% trypsin (Invitrogen Corp, Carlsbad, California), and subsequent outgrowth in keratinocyte serum-free media (Invitrogen Corp) containing 0.2-ng/mL epidermal growth factor and 2-µg/mL bovine pituitary extract (Invitrogen Corp), 1% penicillin-streptomycin solution, and 25-µg/mL amphotericin B (Fungizone; Gibco, Grand Island, New York). After the first passages, the cells were grown in Dulbecco modified Eagle medium containing 15% fetal bovine serum, 2% Ham F12, 1% penicillin-streptomycin solution, 25-µg/mL hydrocortisone, 8.4-ng/mL chola toxin, 5-µg/mL transferrin, 5-µg/mL insulin, 1.36-ng/mL triiodothyronine, and 5-ng/mL epithelial growth factor. Cells in serum-containing media required the addition of irradiated murine J2-3T3 feeder cells for maximal growth and viability.

Replication incompetent retrovirus was produced by plasmid transfection in packaging cell lines using previously reported techniques. Early-passage mouse TECs (MTECs) were transduced at early passage. Retroviruses expressing E6, E7, H-ras, and a control empty vector (LXSN) were used to transduce approximately 40% confluent normal MTECs for 12 hours at 37°C in 5% carbon dioxide. After the media change, the cells were cultured for 16 hours, split at a ratio of 1:4, and subjected to initial antibiotic selection. Transduced cells were selected by the addition of 150-µg/mL G418 (Invitrogen Corp) for E6 and LXSN, 15-µg/mL hygromycin B (Invitrogen Corp) for E7, and 1-µg/mL puromycin for H-ras beginning 24 hours after retroviral transduction. The cells were maintained at this high antibiotic concentration until 100% untransduced control cells had died (approximately 5-10 days). Stable expressing cell lines were maintained in 30-µg/mL G418, 1-µg/mL hygromycin, and 0.2-µg/mL puromycin, respectively.

**WESTERN BLOT ANALYSIS**

Protein was isolated with 150mM sodium chloride, 5mM EDTA, 2mM sodium orthovanadate, 10mM tetrasodium diphosphate decamolybdate, 100mM sodium fluoride, and 10% glycerol, in 50mM tris hydrochloride (pH, 7.5), plus 1% detergent (Triton-X 100) and phosphatase inhibitors leupeptin (1 µg/mL), aprotinin (1 µg/mL), and pepstatin (1 µg/mL), which were added at the time of use. Total protein levels were determined using the Bradford method, and 75 µg of protein was loaded into each lane. Standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis was then carried out using a 10% polyacrylamide resolving gel and a 5% stacking gel. Proteins were transferred to a polyvinyl difluoride membrane (Immobilon-P; Millipore, Billerica, Massachusetts) overnight. Membranes were blocked at room temperature in 10% nonfat dry milk in tris-buffered saline for 1 hour, followed by immunoblotting with an anti–mouse p53 monoclonal antibody (p53-1C12; Cell Signaling Technology Inc, Danvers, Massachusetts) or anti–human p53 monoclonal antibody (Clone DO-1; Calbiochem, San Diego, California). The secondary antibody was goat anti–mouse conjugated to horseradish peroxidase. Then, enhanced chemiluminescence was performed according to the manufacturer’s instructions (SuperSignal West Pico Kit; Pierce, Rockford, Illinois).

**CELL COUNTING**

The cells were briefly trypsinized with dilute trypsin to remove feeder fibroblasts, washed once with Dulbecco phosphate-buffered saline with calcium and magnesium, and trypsinized with 0.05% trypsin (Gibco) at 37°C to remove all epithelial cells. They were centrifuged at 1000g for 5 minutes, resuspended in 1 mL of complete media, and then counted in quadruplicate using a Coulter counter (Z1 Series; Beckman Coulter Inc, Fullerton, California).

**THE CASPASE ENZYME ASSAY**

The caspase 3 enzyme assay, an early marker of p53-related apoptosis, was performed using a commercially available caspase 3 assay kit (Enzo Lyte Rh110 Caspase-3 Assay Kit; AnaSpec, San Jose, California). The cells (5 X 10⁶) were plated in black-walled 96-well plates and allowed to attach. Substrate was applied according to the manufacturer’s instructions. Detection of caspase 3 was through specific cleavage of a fluorophore, with green fluorescence and detection at an excitation/emission of 496 nm/520 nm.

**IN VIVO STUDIES**

In vivo studies in mice were performed to evaluate the thera

mune-competent, age- and sex-matched, 6-month-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine). Institutional guidelines regarding animal experimentation were followed. The tumors grew to 5 mm in greatest dimension at 10 days, and semi-weekly treatment was initiated using 10-mg/kg MG132 by intraperitoneal injection or carrier alone (dimethyl sulfoxide). To image, the animals were shaved and injected intraperitoneally with 100-µL/10 g body weight of D-luciferin (15 mg/mL in phosphate-buffered saline) (Xenogen, Hopkinton, Massachusetts) using a 25-gauge needle. Approximately 10 minutes after injection with luciferin, the animals were anesthetized by 1% to 3% isoflurane delivered through nose cones on a manifold. The optimal exposure time for the camera (IVIS Imaging System; Xenogen) was determined empirically. Care was taken to ensure uniformity in imaging between time points. Imaging data were analyzed and signal intensity was quantitated with commercially available software (Living Image; Xenogen).

RESULTS

PROTEASOME INHIBITION REVERSES E6-MEDIATED DEGRADATION OF p53

The proteasome-mediated degradation of cellular p53 is the most clearly understood function of HPV-16 E6. We hypothesized that inhibition of the proteasome with MG132 would reverse the degradation. Both MTECs and human TECs (HTECs) were transduced with retroviral E6 (or E6 and E7 in the case of the HTECs, because cells with only E6 do not survive selection). At baseline, control TECs had significant levels of p53, while in E6+ cells p53 was nearly undetectable (Figure 1A-D, lane 1). Loss of cellular p53 correlates with E6 expression for both HTECs and MTECs. Following treatment with MG132, p53 levels increased dramatically in a concentration-dependent manner in cells expressing E6, while only a small increase of p53 was observed in control cells not expressing E6 (Figure 1A-D, lanes 2-6). Transduction with an empty vector control (LXSN) showed that p53 was present at baseline and minimally increased after the addition of MG132 (Figure 1E). These results indicate that p53 is present in primary MTECs and HTECs. E6 results in degradation of p53. Proteasome inhibition with MG132 restores p53 levels in cells expressing E6.

PROTEASOME INHIBITION SELECTIVELY DECREASES PROLIFERATION IN CELLS EXPRESSING E6

We next assessed the physiologic significance of MG132 rescue of p53 in cells expressing E6 compared with normal controls. Whether the rescue of E6-mediated loss of p53 would change proliferation rate in transformed TECs was unknown. Cell cycle arrest is known to be induced by p53. To determine whether the substantial and MG132-associated increase in p53 in E6+ cells (compared with the small increase of p53 in control cells) would then preferentially block cell proliferation, we compared growth in the presence of MG132. The TECs that were transduced with an empty viral vector, a vector expressing E6, or primary TECs alone were treated with increasing concentrations of MG132 until the untreated cells reached confluence. E6+ cells proliferate at a faster rate (1.3 days per doubling) than primary epithelial cells (1.7 days per doubling) or vector control (1.7 days per doubling) (J.H.L., written communication, June 2005); therefore, a curve was generated that plotted the percentage of cells present relative to the untreated group for each cell line. These data, which are shown in Figure 2, revealed a dose-dependent decrease in proliferation that was more pronounced in cells with E6 than in those without E6. The difference was significant for doses greater than 0.25 µmol/L (P < .05). The preferen-

Figure 1. MG132 reverses p53 degradation in E6+ cells. Western blot of primary culture mouse tonsil epithelial cells (MTECs) (A), human tonsil epithelial cells (HTECs) (B), MTECs plus E6 (C), and HTECs plus E6/E7 (D) at 8 hours with or without MG132; p53 for empty vector (LXSN) control is shown at baseline and after treatment with 10-µmol/L MG132 (E) (25-µg protein per lane). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control.
tial inhibition in growth was also observed in human cells that express E6 and E7 compared with control primary HTECs. Therefore, TECs that contain E6 and degrade p53 have a reduced capacity to proliferate in the presence of MG132.

CASPASE 3 ACTIVITY, AN EARLY MARKER OF P53-MEDIATED APOPTOSIS, IS INDUCED BY MG132 IN E6-EXPRESSING MTECS

A known function of p53 is activation of the caspase pathway, resulting in apoptosis. Decreased proliferation as a result of MG132 treatment was more profound in E6 cells than in control cells, and because of the evidence of the restoration of p53, we hypothesized that cells expressing E6 were undergoing apoptosis to a greater extent. As an early marker for apoptosis, we assayed for caspase 3 at doses below 4 µmol/L, there was an increase in the amount of caspase 3 activity in cells containing E6 compared with LXSN control cells (Figure 3A). The trend continued through doses in excess of 40 µmol/L (Figure 3B). These results show that proteasome inhibition with MG132 increases via caspase 3 activity, an early marker of apoptosis, much more in cells containing E6 than in those without E6.

GROWTH INHIBITION IS p53 INDEPENDENT

Surprisingly, we noted that the induction of caspase 3 and maximum growth inhibition in cells expressing E6

Figure 2. Proteasome inhibition preferentially decreases proliferation in E6+ cells. Primary mouse tonsil epithelial cells (MTECs) (A) and human tonsil epithelial cells (HTECs) (B) were transduced with either LXSN or E6 and grown with or without MG132 until the fastest-growing (untreated) group reached confluence. The cell number was plotted as the percentage of untreated cells at confluence; P<.05 for all doses greater than 25 µmol/L by analysis of variance and t test, respectively. Error bars represent standard deviations. LXSN data were not available for HTECs because of the inability of the cells to survive selection.

Figure 3. Caspase 3 activity is detected in treated E6+ cells. Mouse tonsil epithelial cells with or without E6 and with or without MG132 at low (A) and high (B) concentrations for 8 hours and assayed for caspase 3 by detection of a cleaved fluorogenic substrate, Ex/Em 496 nm/520 nm. It is reported in relative fluorescent units. P<.05 for all conditions. Error bars represent standard deviations.
occurred at a substantially lower concentration than detection of p53. To investigate whether decreased proliferation was attributable primarily to restoration of p53, we transfected MTECs from p53 knockout mice with E6, knowing that in these cells p53 levels could not return, even when E6-mediated proteasomal degradation of p53 was inhibited. P53 knockout MTECs expressing E6 were then treated and growth assays were performed using the same method as described above. As shown in Figure 4, p53 knockout cells with E6 were as sensitive to MG132 as wild-type E6 cells that could restore p53. These data strongly suggest that the observed growth inhibition is not related to proteasomal rescue of p53.

**MG132 DOES NOT INHIBIT HPV-16 E6 INVASIVE GROWTH IN VIVO**

Although the preferential growth inhibition was found not to require p53, it is still possible that proteasome inhibition would have a potential therapeutic role for HPV-related malignancy, as apoptosis is preferentially induced in E6 cells. We previously described a mouse model that uses our E6 MTECs to reproduce invasive growth in vivo. In this model, the cells require the functions of E6 to grow invasively. We established small (5-8 mm) tumors in immune-competent C57BL/6 mice by injecting 1×10^5 MTECs expressing E6, ras, and luciferases. Previous dosage studies had determined an optimal MG132 dose for mice to observe maximal proteasome inhibition. Mice were then either treated with MG132 or a control Dulbecco phosphate-buffered saline injection for 4 weeks. To quantitate in vivo growth, the mice were then imaged using a bioluminescence imaging system (IVIS; Xenogen) and the light flux was compared. After 4 weeks, 80% of the mice that received control injections had stable tumors, while 20% had spontaneous clearance of their tumor cells, yet 100% of the MG132 treatment group contained tumors (Figure 5A). In addition to reduced spontaneous clearance of the tumor cells, the tumors in the MG132-treated mice also grew at a statistically significant greater rate (Figure 5B). Tumor size directly correlated with average flux. Although further studies need to be completed to explain these observations, these findings suggest that proteasome inhibitors may not be a valuable therapy for HPV-related malignant neoplasms of the head and neck.

**COMMENT**

Human papillomavirus–related HNSCC offers a unique opportunity to design therapies based on known molecular targets of HPV oncogenes. Studies of the role of HPV E6 in HNSCC are hampered because current HPV+ HNSCC cell lines produce discordant results, for reasons that are not clear (J.H.L., written communication, June 2005). Therefore, we turned to a model of E6 expression in TECs at early passage. Although this model may not fully recapitulate all cellular changes during HPV transformation, it allows direct examination of the changes related to specific viral oncogenes. This approach in other cell types has shown that HPV-16 E6 partners with E6-AP (a ubiquitin ligase) to rapidly target p53 for proteasome-mediated degradation at a rate that is at least 2.5 times greater than this cellular mechanism would normally cause. Loss of functional p53 is likely a common cellular change that occurs in most cases of HNSCC. In most HPV-negative tumors, mutations in the p53 gene either prevent expression or make the gene nonfunctional. However, HPV-positive cancers do not show increased mutations in p53 likely because the viral oncoprotein E6 effectively blocks its function. Therefore, if the protein could be restored, it should function. We have demonstrated that MG132 substantially reverses p53 degradation in HTECs and MTECs that express E6 in a model keratinocyte cell line. The slight increase in p53 levels in control cells is also consistent with previous reports that show that the degradation pathway for p53 occurs through the proteasome. Because p53 return is more substantial in cells expressing E6, the reversal of E6-mediated p53 degradation is a potential therapeutic effect of proteasome inhibition.

Although our data demonstrate a preferential return of p53 with MG132, the inhibition of growth in an E6 model with this compound does not appear to depend on functional p53. There are several lines of evidence to suggest that p53 function was not responsible for the preferential inhibition of growth: (1) caspase assays show an induction of caspase 3 activity before p53 return is detectable by Western blot; (2) growth inhibition is most substantially different between E6 and control cells at doses when p53 is not detected; and (3) we noted the same inhibition of growth in wild-type and p53 knockout cells expressing E6. Therefore, although we saw preferential growth inhibition in E6-containing cells, the mechanism responsible for this inhibition is not induction of p53. This finding may suggest that the observed differences in proliferation with treatment are related to the intrinsic growth rate differences.
Intraperitoneal MG132 (10 mg/kg) therapy was initiated and administered semiweekly for 4 weeks. A, Sequential imaging is shown over a period of 55 days. B, Average flux was recorded at several points. Error bars represent standard deviations.

Why cells expressing E6 are preferentially growth inhibited by MG132 is not clear. Our data would support at least 2 possibilities. First, E6 has multiple functions other than p53 degradation. Some of these functions also result in proteasomal-mediated degradation of their target protein. To our knowledge, the functional significance of these non–p53-related mechanisms in regard to growth has not been examined. It is possible the inhibition in growth by MG132 is related to a rescue of one of these other E6 functions. A second explanation of our results could be based on the fact that MG132 more effectively induces apoptosis and inhibits growth in more rapidly proliferating cells. Cells expressing E6 do proliferate at a faster rate than control cells. Other investigators have observed that broad proteasome inhibition selectively reduces proliferation of rapidly dividing cells in a p53-independent manner.

In future studies, we will design experiments to identify the mechanism of this inhibition.

Although the in vitro data did not clearly define a mechanism for the increased apoptosis and decreased proliferation in MG132-treated cells expressing E6, it is still possible that such a treatment could have therapeutic potential for cancer in humans. However, our in vivo data suggest that MG132 may not be beneficial. We used intraperitoneal injection of MG132 because previous authors had documented rapid and complete proteasome inhibition using a very sensitive luminescence method. Other methods, such as intratumoral injection, were not considered because we did not have a method for ensuring even distribution or for estimating an appropriate dose. Multiple possibilities could explain the observation that tumors grew more quickly and in more animals with treatment. From previous experiments, we know that in some animals an immune response is activated after the animals receive the tumor cells, resulting in clearance of the tumor (J.H.L., written communication, January 2007). One possible explanation for our findings is that MG132 alters the immune response in a manner that preferentially improves tumor growth. It is known that other proteasome inhibitors kill rapidly dividing immune cells. Further studies are needed to prove this hypothesis.

In conclusion, the present study attempts to develop a therapeutic target for HPV-related HNSCC based on viral oncogene function. Using a model of HPV oncogene function, we have shown that p53 can be restored preferentially in cells expressing E6 by MG132, which broadly inhibits proteasomal degradation. Although we show that inhibition of growth of E6-expressing cells is independent of the return of p53, it is still possible that this known function of E6 may be a therapeutic target by other agents that selectively target only this interaction or that established cancers will behave differently than those in our model.

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Correspondence: John H. Lee, MD, Department of Otolaryngology–Head and Neck Surgery, The University of Iowa, 200 Hawkins Dr, Iowa City, IA 52242-1078 (john-h-lee@uiowa.edu).

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