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Curcumin Suppresses Human Papillomavirus Oncoproteins, Restores p53, Rb, and PTPN13 Proteins and Inhibits Benzo[a]pyrene-Induced Upregulation of HPV E7†

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Curcumin has great potential as a chemopreventive and chemotherapeutic agent; however, its effects on human papillomavirus (HPV)-associated molecular events are inadequately explored. This study examined the effects of curcumin on HPV-associated pathways involved in developing cervical cancer. We demonstrate for the first time that curcumin treatment suppresses cervical cancer cell growth in a three-dimensional raft culture system. Curcumin also inhibits tumorigenic characteristics as shown by decreases in both clonogenic potential and cell motility. Additionally, our findings show that curcumin treatment inhibits the transcription of HPV16 E6/E7 as early as 6 h posttreatment and restores the expression of tumor suppressor proteins p53, retinoblastoma protein, and PTPN13. While smoking is a recognized risk factor for cervical cancer, the molecular effects of smoke carcinogens on the expression of HPV E6/E7 oncogenes are not well known. We show for the first time that exposure to benzo[a]pyrene (BaP), a tobacco carcinogen, increases the expression of HPV E7 oncoprotein suggesting a molecular link between smoking and cervical cancer. Importantly, curcumin decreases the BaP induced increase in the expression of HPV E7 oncoprotein. The results of this study clearly demonstrate that curcumin alters HPV-associated molecular pathways in cervical cancer cells. These novel findings imply that curcumin may be an effective chemopreventive and therapeutic agent for cervical cancer prevention and treatment.

Key words: curcumin; cervical cancer; HPV E6/E7; p53; Rb; PTPN13; cell motility; benzo[a]pyrene (BaP)

INTRODUCTION

There are 470 000 new cases and approximately 233 000 deaths per year due to cervical cancer, making it one of the most common and deadly cancers in women worldwide [1,2]. Infection with a high-risk human papillomavirus (HPV) genotype is the most important risk factor for cervical cancer [3]. HPV is the most common sexually transmitted infection in the United States with approximately 80% of women having acquired HPV infection by age 50 [4]. The vast majority of HPV infections will clear naturally with no lasting physical manifestation. However, in some women persistent HPV infection may cause genital warts or lead to the development of cervical cancer, depending upon the genotype of HPV. While over 100 HPVs have been identified, about 40 infect mucosal surfaces and of these, HPV16 and HPV18 have been detected in approximately 70% of cervical cancers [5,6]. In addition to persistent HPV infection, cigarette smoking is a known risk factor for developing cervical cancer; however, the molecular interactions between tobacco smoke and HPV infection are not well known [7,8].

Currently, the standard of care in the United States suggests that sexually active women have regular Papanicolaou (Pap) smears to identify precancerous cellular changes at the uterine cervix and implementation of such cervical cancer screenings have dramatically reduced the incidence of invasive cervical cancer. However, the incidence of cervical

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Additional Supporting Information may be found in the online version of this article.

Abbreviations: HPV, human papillomavirus; BaP, benzo[a]pyrene; Rb, retinoblastoma protein; PARP, poly (ADP-ribose) polymerase; AP1, activator protein 1.

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cancer in developing countries and in low socioeconomic populations is considerably higher, in part due to the limited availability of screening methods [9]. For example, American Indian women of the Northern Plains have an increased incidence and mortality rate from cervical cancer compared to the general US population (mortality rates are 4.5 per 100 000 for American Indian and 2.7 per 100 000 for general population) [10]. Our own studies have identified a high prevalence of HPV infection (21.25% HPV positive) in American Indian women living on the Northern plains [11]. These women also have a higher incidence of smoking than Caucasian women [12]. Smoke carcinogens, such as benzo[a]-pyrene (BaP), have been detected in cervical mucus [13,14] and could directly affect the progression of HPV infection, but this has not been well studied. In addition to limited availability of medical care, a high prevalence of both HPV infection and smoking may contribute to increased incidence and mortality rate of cervical cancer.

The accepted form of treatment following a cytological diagnosis of high-grade squamous intraepithelial lesion (HSIL), histological diagnosis of cervical intraepithelial neoplasia (CIN) 2, 3 or carcinoma in situ is to excise the tissue encompassing the transformation zone (the junction of the stratified squamous epithelium of the ectocervix and the columnar epithelium of the endocervix) [15]. However, this strategy may allow for reoccurrence of cervical lesions and can negatively affect future reproductive outcomes, an important issue especially as the average maternal age increases [16,17]. The development of noninvasive strategies for treating healthy women at high risk for developing cervical cancer (i.e., women with a premalignant lesion, or known to be infected with high-risk HPV) would be extremely desirable. The relatively slow natural progression of HPV lesions and the accepted management practices of observing early lesions allows for the addition of chemopreventive treatments with compounds that are safe (no negative side-effects) to promote the clearance of HPV infection. Our goal is to identify a chemopreventive agent that would safely reduce the cervical cancer burden, especially in women living in under-served areas.

Curcumin, diferuloyl methane, a polyphenol derived from the rhizomes of turmeric, *Curcuma longa*, has received considerable attention due to its broad range of anti-cancer activities (as described in a recent review [18]). Curcumin acts through a variety of cellular pathways and induces a multitude of effects, including, but not limited to anti-inflammatory, anti-mutagenic, anti-metastatic, and anti-angiogenic properties [19–23]. Importantly, curcumin has demonstrated no toxicity to healthy organs at doses as high as 8 g/d [24]. Given curcumin's potential as a safe chemopreventive agent, we assessed the anti-cancer effects of curcumin on cervical cancer cell lines. Herein, we report that curcumin inhibits proliferation of a variety of cervical cancer cells, inhibits clonogenic potential, decreases cell motility, induces caspase-mediated apoptosis, decreases the expression of HPV oncoproteins and restores expression of tumor suppressor proteins, p53, retinoblastoma (Rb), and PTPN-13. Additionally, we found that a tobacco smoke carcinogen, BaP, causes an increase in the expression of HPV oncoproteins, which is effectively suppressed by curcumin treatment. The results of this study describe unique molecular mechanisms regulated by curcumin.

MATERIALS AND METHODS

Cell Culture

All cells were purchased from ATCC between 2001 and 2007. Upon receipt, cells were expanded and frozen aliquots (passage <4) were stored in liquid nitrogen. When needed, cells are thawed and grown for <6 mo. HeLa, SiHa, and C33A were maintained in DMEM containing 4.5 g/L of glucose, 10 nM of nonessential amino acids, 100 nM of sodium pyruvate, 1× antibiotic/antimycotic (Sigma, St. Louis MO), and 10% heat-inactivated FBS (Atlantic Biologicals, Lawrenceville, GA). Caski cells were grown in RPMI containing 10% heat-inactivated FBS and 1× antibiotic/antimycotic. SW756 were grown in Leibovitz's L-15 medium with 2 mM of L-glutamine, 10% heat-inactivated FBS, and 1× antibiotic/antimycotic. All cells were incubated at 37°C in a 5% CO_2 incubator.

Cell Proliferation Assays

Cervical cancer cells (5000 per well) were seeded in 96-well tissue culture plates and allowed to adhere for 18 h. Curcumin (Sigma #7727) was dissolved in DMSO, diluted in tissue culture media and added to the 96-well plate (each concentration was performed in triplicate). After 48 h incubation, the CellTiter96 Aqueous One Solution (Promega, Madison, WI) was added, incubated for 2 h at 37°C and the absorbency read at 490 nm. Each plate was read three times and the absorbency reading averaged. The percent proliferation was then determined by subtracting the background and dividing the curcumin-treated wells by the vehicle (DMSO) controls (thereby setting the vehicle (DMSO) control at 100%). The experiment was repeated three times and data shown are the average of the three experiments.

Colony Forming Assays

Colony forming assays were performed as described earlier [25]. Briefly, Caski, or SiHa cells were seeded at 1000 cells per dish (90 mm) and allowed to attach for 18 h. Curcumin (2, 5, or 10 μM) was added in fresh media the following day and the cells were cultured for an additional 14 d. The cells were washed, fixed in cold methanol and stained

with hematoxylin. Visible colonies (~50 cells) were counted manually and reported as the ratio of the number of colonies in treated cells divided by the number of colonies in the vehicle (DMSO) control.

Raft Cultures

Cervical cancer cells were grown on rafts according to the method published by Lambert et al. [26]. Briefly, Caski cells were grown on air-media interphase of a collagen/fibroblast gel (rafts). Curcumin was added 7 d after initial seeding of the cells and allowed to incubate for an additional 7 d. Rafts were fixed in formalin, paraffin embedded, and 4 μm sections were cut and stained with hematoxylin and eosin.

Cell Motility Assay

The cell motility assay was performed using a scratch assay format; however, to increase the reproducibility of the assay, we used the IBIDI cell culture inserts (Integrated BioDiagnositics, LLC, Verona, WI). Using a six-well plate, the cell culture inserts were aseptically placed into each well and Caski cells (4×10^4) were seeded into each chamber of the cell culture insert and incubated overnight. The following day, the media was removed and replaced with media containing curcumin (5 or 10 μM) or vehicle (DMSO) for 1 h. The cell culture insert was then carefully removed, leaving a 500 μm cell free gap. The media was replaced with fresh media containing curcumin or vehicle (DMSO). Immediately after creating the gap ($T = 0$ h) and 6 h later ($T = 6$ h), phase contrast photomicrographs were taken of two consecutive fields from the center of the gap (covering 4.5 mm) using a 4× objective. To ensure the same areas were captured, a mark was created on the bottom side of the plate. The area of the cell free gap was determined at $T = 0$ h and $T = 6$ h using the assisted polygon measuring tool in the Micron software (Westover Scientific, Advanced Microscopy Group, Mill Creek WA). The percent of the gap which was filled in at $T = 6$ h was determined and normalized to the vehicle control. For the time-lapse experiments cells were treated as described above. After removal of the insert, the cells were incubated in a heated chamber (37°C) with CO_2. Differential interference contrast (DIC) images were collected with an Olympus FluoView FV1000 Confocal Microscope every 2.5 min for 6 h (see video).

Flow Cytometry

To examine curcumin's effect on cell viability (as determined by the loss of plasma membrane integrity), Caski cells were treated with curcumin for 48 h, collected (both adherent and floating) and stained with 7-AAD (BD Biosciences, San Diego, CA) at 5 μL/mL of cell suspension. After a 20 min incubation, cells were analyzed with an Accuri C6 cytometer. Cells were gated based on the forward/side scatter profile and a minimum of 10 000 gated events were collected.

Immunoblotting

Cells (1×10^6) were allowed to attach for 18 h and then treated with curcumin for the indicated times. After curcumin treatment, both adherent and floating cells were collected in 2× SDS lysis buffer (Santa Cruz Biotechnologies, Santa Cruz, CA), sonicated and the protein concentration was normalized using SYPRO Orange (Invitrogen, Carlsbad, CA). SDS–PAGE electrophoresis was performed with a 4–20% gradient gel and resolved proteins were transferred onto a PVDF (BioRad, Hercules, CA) membrane. The following antibodies were used: poly (ADP-ribose) polymerase (PARP), Caspase 3, Caspase 9, Rb (Cell Signaling, Danvers, MA), HPV 16 E7 (Invitrogen), p53 (Santa Cruz Biotechnologies), and PTPN13 (Santa Cruz Biotechnologies). The primary antibody was detected by a species appropriate HRP-secondary antibody (Promega) followed by incubation with the Lumi-Light detection reagent (Roche, Nutley, NJ). Band intensity was normalized with the β-actin loading control and the expression level was compared to the vehicle control (DMSO).

Real-Time PCR Analysis

After curcumin treatment, adherent cells were collected and RNA was extracted with the Qiagen RNeasy kit (Qiagen Inc., Valencia, CA). Reverse transcription was performed with the high capacity RNA to cDNA kit and amplified with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The cDNA was amplified with primers specific for HPV16 E6, HPV16 E7, and β-2-microglobulin. E6-F: **caaaccgttgtgtgatttgttaatta**; E6-R: **gctttttgtccagatgtctttgc**; E7-F: **ccggacacagcccattacaa**; E7-R: **cgaatgtctacgtgtgtgctttg**; β2M-F: **tgagtatgcctgccgtgtga**; β2M-R: **tgatgctgcttacatgtctcgat** [27]. Cycling conditions: 95°C for 10 min, 30 cycles: 95°C for 30 s and 60°C for 30 s. A melting curve was generated to ensure specificity of the PCR product. Each reaction was performed in duplicate with a MyiQ single color real-time PCR thermocycler and analyzed with Genex (Microsoft Excel macro provided by BioRad). The results were first normalized to the endogenous control (β2-microglobulin) and then the expression level was scaled to the vehicle control (DMSO). The experiment was repeated three times and data shown is the average of the three experiments.

NFκB and AP1 Activation

Caski cells (1×10^6) were plated in 150 mm dishes and allowed to attach overnight. The following day, the media was changed and cells were exposed to freshly diluted BaP (stock in DMSO at 1000× for each

dose) at a final concentration of BaP (10 or 100 nM) or DMSO (vehicle control). After 24 h, the media was again changed. After 48 h of exposure to BaP nuclear lysates were collected (Active Motif). For experiments with both BaP and curcumin, cells were first treated with BaP (or DMSO, vehicle control) for 24 h and then treated with BaP and/or curcumin for an additional 24 h, as indicated. NFκB and activator protein 1 (AP1) activity was determined with the TransAM™ NFκB family kit and AP-1 family kit (Active Motif) following manufacturer's directions.

Statistical Methods

Statistical analysis was completed by using an unpaired, two tailed Student's *t*-test. The results were considered statistically significant if $P < 0.05$.

RESULTS

Curcumin Treatment Results in Decreased Cell Proliferation in a Variety of Cervical Cancer Cell Lines

Cell proliferation and enhanced clonogenic potential are two important measures of neoplastic behavior of cancer cells. To determine curcumin's ability to inhibit cervical cancer, we assessed curcumin's effect on cell growth in a variety of cervical cancer cells (SiHa and Caski: HPV16, HeLa and SW756: HPV18 and C33A: HPV negative). All cell lines tested showed an inhibitory effect of curcumin in a dose-dependent manner, with some differences in sensitivity, especially at the 20 μM concentration (Figure 1A). For further analysis of curcumin, we focused our efforts on the SiHa and Caski cell lines. SiHa cells have 1–2 copies of integrated HPV 16. Caski cells were chosen as a robust model of HPV because they contain about 600 copies of integrated HPV 16. To determine the long-term effect of curcumin on cervical cancer cell growth, we performed colony forming assays. Curcumin treatment significantly ($P < 0.05$) reduced the clonogenic potential of both Caski and SiHa cells (at 5 and 10 μM) (Figure 1B and C). Due to the prolonged incubation after curcumin treatment, it is not surprising that lower doses of curcumin had significant effects compared to the 48 h proliferation assay.

Curcumin Inhibits Cell Growth in Raft Culture Model of Cervical Cancer

It has been reported that raft culture models of cervical cancer have a gene expression profile that more closely relates to cervical cancer than a simple monolayer of epithelial cells [28]. The life cycle of HPV and the expression of HPV proteins are closely tied to the differentiation status of the epithelial cell. When cultured at the air/media interface on a collagen gel containing fibroblasts, epithelial cells will form a multilayered epithelium. Therefore, we also tested the effectiveness of curcumin in a raft culture system. Curcumin treatment (20 and 40 μM) dramatically reduced the thickness of epithelium

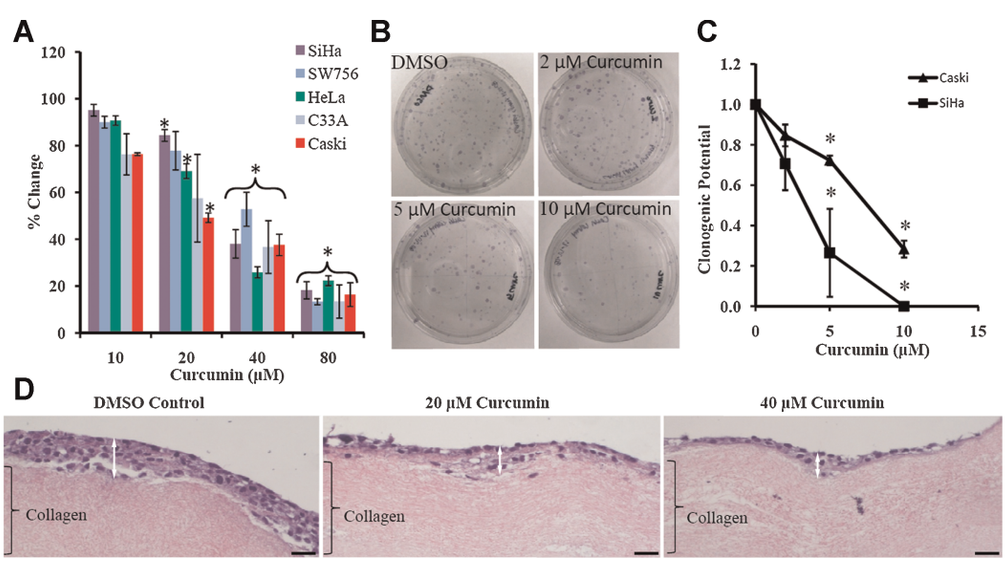

Figure 1. Curcumin treatment suppresses cervical cancer cell growth in monolayer and organotypic raft culture systems. (A) Curcumin treatment results in decreased proliferation in a variety of cervical cancer cell lines. Proliferation was determined with the CellTiter96 Aqueous One Solution (Promega) 48 h after drug addition and normalized to control wells treated with appropriate amounts of vehicle (DMSO set at 100%, not shown). Columns, mean; bars, SE. $n = 3$, $*P < 0.05$. (B and C) Curcumin reduces the clonogenic potential of Caski and SiHa cells. Cells (1000) were plated and subsequently treated with curcumin for 14 d (representative plates shown for Caski cells). Colonies were counted and expressed as a ratio compared to the vehicle control (DMSO). Columns, mean; bars, SE. $n = 3$, $*P < 0.05$. (D) Curcumin treatment inhibits cell growth in an organotypic raft culture system. In a raft culture model using HPV 16 positive Caski cells, curcumin dramatically inhibited cell growth and thickening of the epithelial layer. Caski cells were cultured on top of a collagen gel containing fibroblasts for 7 d and then treated with curcumin for 7 d. A representative photomicrograph of hematoxylin and eosin stained epithelium is shown for each treatment. Arrows indicate the different epithelial thickness of each treatment. Bar = 100 μm.

generated by Caski cells in a raft culture system (Figure 1D).

Curcumin Treatment Induces Cell Death and Apoptosis

Microscopic examination of curcumin-treated cells revealed classic signs of apoptosis, including cell shrinkage, rounding up of cells, and plasma membrane blebbing (Figure 2A). Within 48 h of curcumin treatment, the percent of dead cells (determined by loss of membrane integrity as shown by 7-AAD staining) had increased at each dose of curcumin (Figure 2B). To determine if curcumin was inducing cell death through apoptosis, we analyzed curcumin-treated cells for the expression of cleaved PARP, Caspase 3, and Caspase 9 through immunoblotting. Curcumin treatment (40 μM) clearly resulted in the cleavage of PARP, Caspase 3, and Caspase 9, indicating cell death by apoptosis (Figure 2C).

Curcumin Treatment Inhibits the Expression of HPV Oncoproteins

It is well established that the HPV oncoproteins E6 and E7 are capable of inducing continued cell division and in addition to other environmental/genetic factors, are responsible for the development of cervical cancer [29]. Therefore, the expression of HPV E6/E7 is an important consideration for chemoprevention/treatment of cervical cancer. We found that the level of HPV E7 protein was significantly decreased after 24 and 48 h of curcumin treatment (20 and 40 μM) (Figure 3A). For unexplained reasons, we were not able to consistently perform immunoblotting for HPV E6. However, quantitative RT-PCR clearly indicated a significant reduction of both HPV E6 and E7 mRNA (Figure 3B). Curcumin treatment also significantly reduced the expression of HPV E6 and E7 mRNA in SiHa cells, an additional HPV 16 positive cell line (Supplemental Figure 1A). Interestingly, in both Caski and SiHa cell lines, the level of HPV E6 and E7 mRNA was significantly decreased after only 6 h of curcumin treatment.

Curcumin Treatment Restores the Expression of p53, Rb, and PTPN13

HPV E6 and E7 are known to cause the down-regulation of tumor suppressor proteins p53, Rb, and PTPN13 [29,30]. The decrease in HPV E6/E7 protein following curcumin treatment prompted us to ask whether the expression of tumor suppressor proteins known to be suppressed by HPV E6/E7 could be restored by curcumin. Following curcumin treatment, we assessed the levels of p53, Rb, and PTPN13 by immunoblotting. We observed that these proteins were restored in response to curcumin treatment (Figure 4 and Supplemental Figure 1B). In a HPV negative cell line, C33A, curcumin treatment did not change the expression levels of p53 and Rb (Supplemental Figure 1C). In contrast to Caski cells, SiHa and C33A did not express consistent levels of PTPN13 (data not shown). These data indicate that the reduction of HPV E6/E7 by curcumin results in the increased expression of tumor suppressor proteins known to be inhibited by HPV oncoproteins.

Curcumin Treatment Inhibits Cell Motility

Increased cell motility is a characteristic displayed by many cancer cells; therefore, we utilized a scratch/wound closing assay to determine if curcumin treatment has a physiologic effect on cervical cancer cells. Rather than creating a scratch, we utilized the IBIDI cell culture inserts which leave a uniform gap of 500 μm. Compared to the vehicle control, treatment with 5 and 10 μM of curcumin significantly

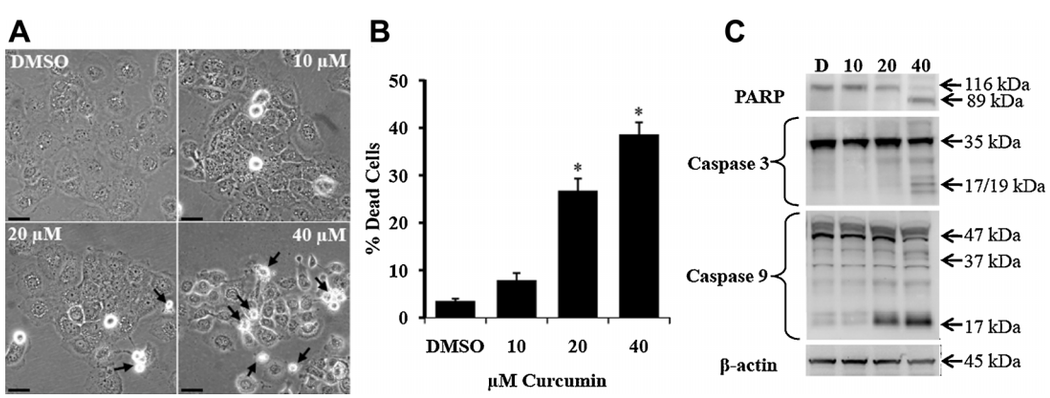

Figure 2. Curcumin treatment induces apoptosis in cervical cancer cells via caspase-mediated signaling. (A) Microscopic analysis of curcumin-treated cells indicates cell death via apoptosis. Phase contrast images of Caski cells treated with curcumin for 24 h (arrows indicate membrane blebbing). Bar = 10 μm. (B) Curcumin treatment increases the percent of dead cells. Curcumin-treated Caski cells were collected after 48 h, stained with 7-AAD and analyzed by FACS. Columns, mean; bars, SE. $n = 3$, $^*P < 0.05$. (C) Curcumin treatment induces apoptosis via caspase-mediated signaling. Caski cells were treated with curcumin (10, 20, or 40 μM) or DMSO (D, vehicle control). Both adherent and floating cells were collected in 2× SDS lysis buffer. Immunoblots were performed to detect the cleavage of PARP, Caspase 3, and Caspase 9. Cleaved products are denoted by an arrow. At least three independent experiments were performed and representative immunoblot is shown.

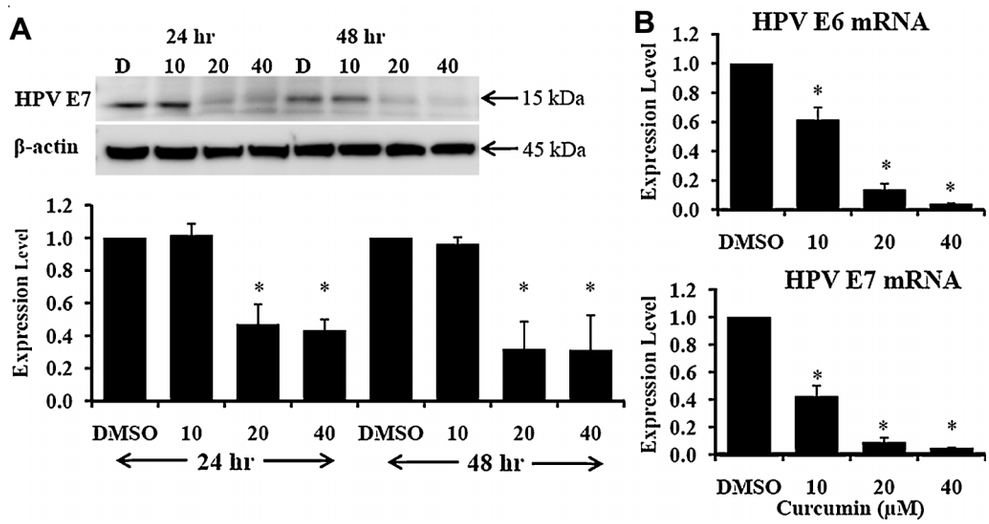

Figure 3. Curcumin treatment represses the expression of HPV oncogenes E6 and E7. (A) Curcumin treatment reduces the expression of HPV E7. Caski cells were treated with curcumin (10, 20, or 40 μM) or DMSO (D, vehicle control) and both the adherent and floating cells were collected in 2× SDS lysis buffer. Immunoblots were preformed to detect HPV E7 protein. Three independent experiments were performed and representative blot is shown. Band intensity was normalized to β-actin and scaled to the DMSO control. REL, relative expression level. Columns, mean; bars, SE. $n = 3$, $*P < 0.05$. (B) Curcumin treatment downregulates the expression of HPV E6 and E7 mRNA. Caski cells were treated with curcumin for 6 h. mRNA levels of HPV E6 and E7 were determined by quantitative PCR utilizing cyber green. Columns, mean; bars, SE. $n = 3$, $*P < 0.005$.

($P < 0.005$) decreased cell motility in a dose-dependent manner (Figure 5). It is important to note that we chose (1) a dose of curcumin that does not cause significant cell death within 48 h and (2) we stayed within a short period of time (6 h) to eliminate the concern of cell replication being responsible for filling in the gap. To better understand the dynamics regarding curcumin's affect on cell motility, we also performed time-lapse experiments on cells treated with 10 μM of curcumin (video). Compared to the vehicle control, curcumin-treated cells moved much more slowly throughout the 6-h observation period.

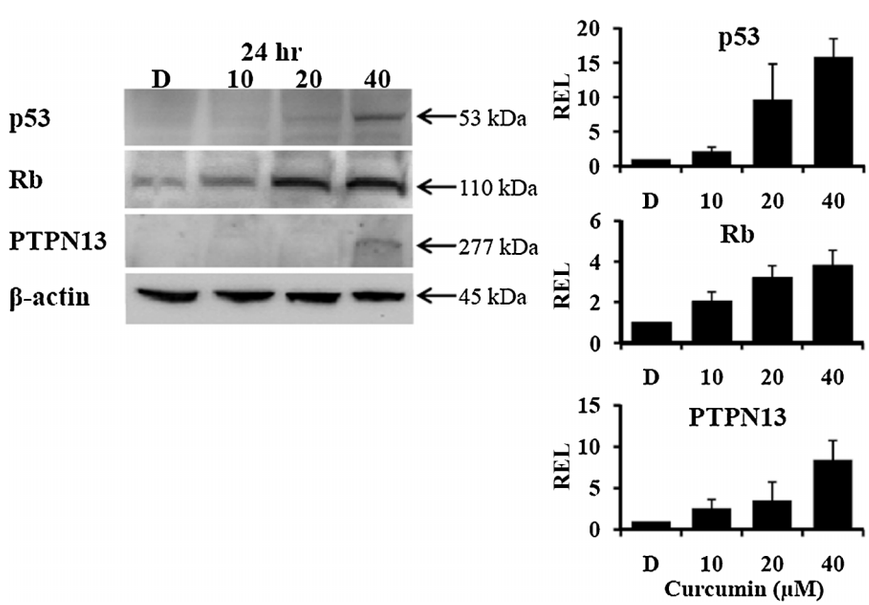

Figure 4. Curcumin treatment restores p53, Rb, and PTPN13. Caski cells were treated with curcumin (10, 20, or 40 μM) or DMSO (D, vehicle control) for 24 h and both the adherent and floating cells were collected in 2× SDS lysis buffer. Immunoblots were performed to detect p53, Rb, and PTPN13. Three independent experiments were performed and representative blot is shown. Band intensity was normalized to β-actin and scaled to the DMSO control. REL, relative expression level. Columns, mean; bars, SE. $n = 3$.

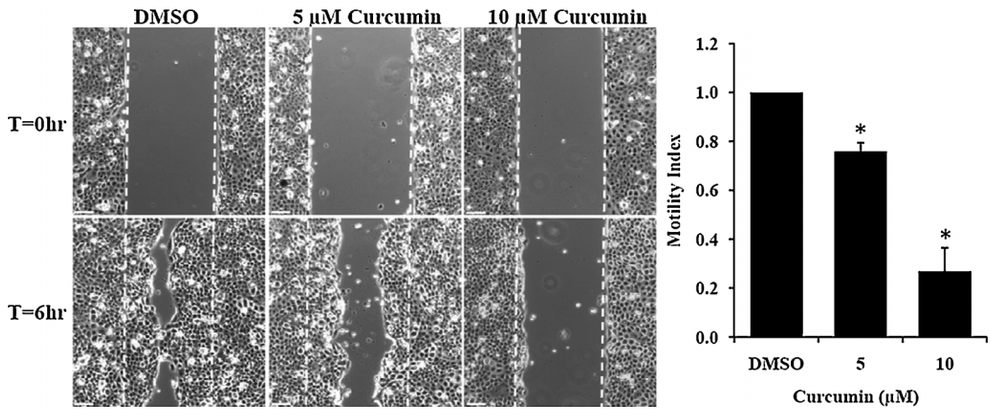

Figure 5. Curcumin treatment inhibits cell motility. Images were collected within 10 min of removing the IBIDI insert, creating a gap of ~500 μm (marked by the dashed lines) ($T = 0$ h). Images of the same areas were taken 6 h later ($T = 6$ h). Bar = 100 μm. The area of the gap was determined (at $T = 0$ h and $T = 6$ h) and used to calculate the motility index (normalized to the DMSO vehicle control). The experiment (performed in duplicate) was repeated three times. Columns, mean; bars, SE. $n = 3$, $*P < 0.005$.

Curcumin Suppresses BaP Induced Increase of HPV E7 Oncoprotein

While smoking has been recognized as a risk factor for developing cervical cancer, the molecular relationships between HPV infection and smoke carcinogens are not clearly understood. Surprisingly, we found that exposure to BaP (a component of tobacco smoke) causes a significant ($P < 0.05$) increase in HPV E7 protein expression (Figure 6A), suggesting a novel molecular mechanism by which BaP may affect HPV infection and cervical cancer development. Importantly, curcumin treatment suppressed BaP induced increase in HPV E7 expression (Figure 6A). BaP is known to induce NFκB activation in other cell types, which we also found to be true for cervical cancer cells (Figure 6B). To address the mechanism of increased HPV E7 protein expression, we assessed the ability of BaP to activate AP1 signaling. The AP1 family of transcription factors (including c-Fos) is known to induce transcription of HPV E6/E7 through binding to the upstream regulatory region (URR). We detected an increase in the amount of AP1 (c-Fos) in the nucleus of cervical cancer cells exposed to BaP, suggesting that BaP may increase HPV oncoprotein expression through modulation of AP1 (Figure 6B). Additionally, curcumin inhibited NFκB activation in the presence of BaP (Figure 6C). These data strongly suggest that curcumin may be an important chemopreventive and or therapeutic agent that could be effective against multiple carcinogenic challenges, such as HPV infection and tobacco carcinogens.

DISCUSSION

While the rate of invasive cervical cancer has decreased in areas with aggressive cervical cancer screening, the incidence of cervical cancer in developing nations and low socio-economic groups remains high. For example, in the United States, American Indian women have a higher rate of cervical cancer than the Caucasian population. Our laboratory has recently shown that American Indian women of the northern plains have a high rate of HPV infection [11]. Additionally, these women also have a high prevalence (29%) of cigarette smoking [12]. In combination with the lack of readily available health care, the high rates of HPV infection and smoking likely contribute to the higher incidence of cervical cancer. Curcumin has potential to be both a chemopreventive and therapeutic agent for cervical cancer; however, its effect on HPV-associated cellular signaling in cervical cancer has not been fully explored.

While many factors contribute to the development of cancer, one hallmark is the loss of balance between cell proliferation and cell death, resulting in increased cell replication and the failure of damaged cells to be removed through apoptosis [31]. We show that curcumin inhibits cell viability in a variety of cervical cancer cell lines. Curcumin also inhibited the clonogenic potential of cervical cancer cells. These results support other reports indicating the anti-proliferative effects of curcumin in a variety of different cancer cells [19,32–34]. While a common research tool, cells grown in a monolayer obviously do not mimic a stratified epithelial surface. Research on HPV has been significantly hampered due to the requirement of a stratified epithelium for HPV replication, although the development of raft culture techniques supporting the growth of multilayered and differentiated epithelial cells has provided a physiologically relevant model to study HPV in vitro. Therefore, we assessed the effect of curcumin on HPV16 infected Caski cells grown in a raft culture system and indeed, curcumin dramatically reduced cell growth and thickening of the raft. Curcumin also induces significant cell death and

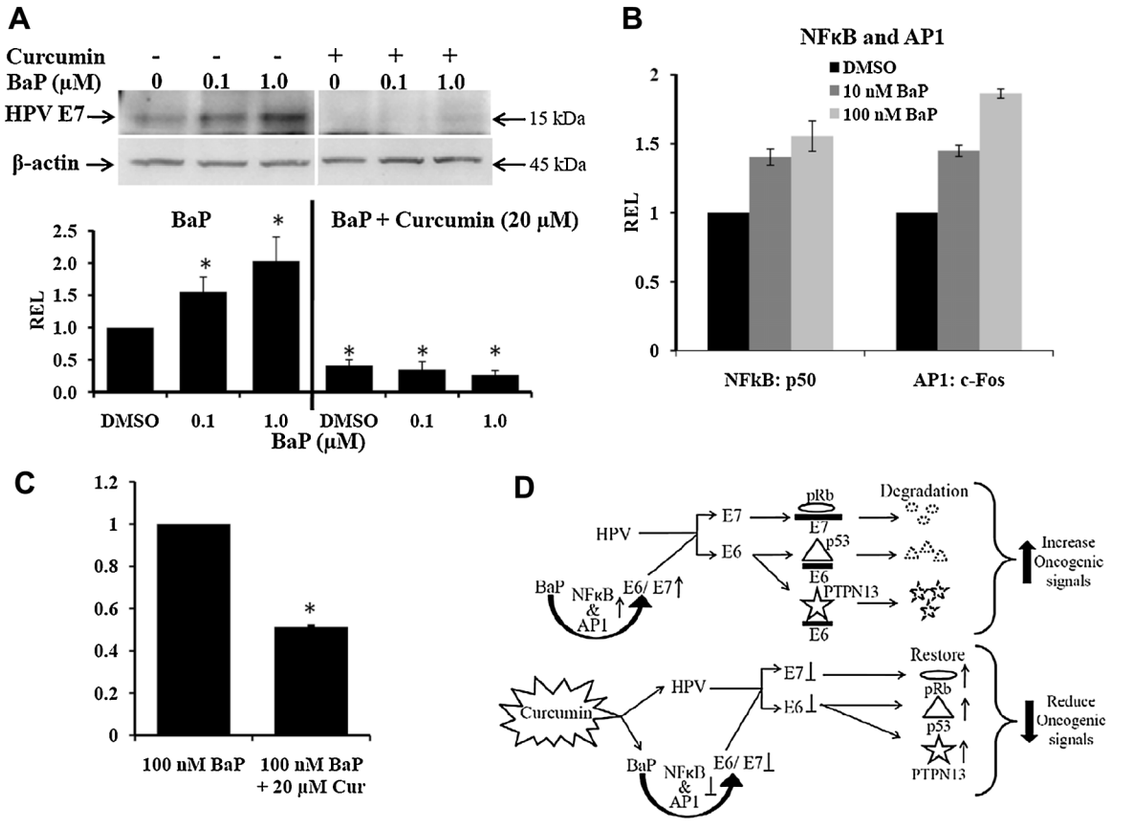

Figure 6. Tobacco smoke compound BaP upregulates the expression of HPV oncogenes, NFκB, and AP1 but is suppressed by curcumin treatment, and schematic model for mechanism of action of curcumin. (A) Curcumin inhibits BaP induced increase of HPV E7. Caski cells were exposed to BaP for 24 h followed by 24 h of curcumin or vehicle control (DMSO) treatment (as indicated). Immunoblots were performed to detect HPV E7 protein. Band intensity was normalized to β-actin and scaled to the DMSO control. REL, relative expression level. Columns, mean; bars, SE. $n=3$, $*P<0.05$. (B) Exposure to BaP activates the NFκB and AP1 signaling pathways. Caski cells were exposed to BaP (as indicated) for a total of 48 h and nuclear lysates were collected. NFκB and AP1 activity was determined and normalized to the DMSO vehicle control. REL, relative expression level. Columns, mean; bars, SE. $n=2$. (C) Curcumin inhibits NFκB activation, even in the presence of BaP. Caski cells were exposed to BaP for 24 h followed by 24 h of BaP and curcumin treatment (as indicated). Nuclear lysates were collected and NFκB activity was determined and normalized to the BaP-exposed sample. REL, relative expression level. Columns, mean; bars, SE. $n=2$. (D) Schematic model of increased oncogenic signals via HPV E6/E7 oncoproteins and molecular effect of curcumin on HPV-associated cellular events.

apoptosis (as indicated by loss of membrane integrity and cleavage of PARP, Caspase 3, and Caspase 9) at micromolar concentrations. It is interesting to note that curcumin possibly induces apoptosis through non-PARP-mediated pathways, as indicated by the high percent of dead cells present at the 20 μM concentration, but only minimal PARP cleavage. Recently, the role of apoptosis inducing factor (AIF) in curcumin-mediated apoptosis has been reported [34]. Taken together, these results suggest that curcumin-mediated cell death may involve multiple apoptotic pathways.

Curcumin affects a wide variety of cellular pathways and can have multiple functions; therefore, we were interested in assessing curcumin's effect specifically on the expression of HPV oncoproteins (HPV E6 and E7), as the development of cervical cancer is considered to require the expression of both HPV E6 and E7 (depicted in Figure 6C and D). We found that within 24 h of curcumin treatment, the protein level of HPV E7 was dramatically reduced. To determine if the reduction in protein was due to decreased transcriptional activity, we assessed the level of HPV E6/E7 mRNA by real-time PCR analysis. Impressively, the amount of mRNA for both E6 and E7 was significantly reduced within 6 h of curcumin treatment. Regulation of HVP E6/E7 transcription is complicated and controlled by a variety of transcription factors, including the AP-1 family of transcription factors which increases transcription of HPV E6/E7. AP-1 activity is known to be reduced by curcumin, which likely explains the down regulation of HPV E6/E7 [32,33]. HPV E6 and E7 have a multitude of effects on epithelial cells. HPV E6 causes the decrease of p53 through interaction with the E6-associated protein (E6-AP), an E3 ubiquitin ligase, which causes proteosomal degradation of p53 (and E6-AP), allowing for increased proliferation [35].

Additionally, HPV E6 is also known to cause the loss of tumor suppressor PTPN13, a nonreceptor phosphatase important for preventing anchorage-independent growth [30]. HPV E7 is well known for its interaction with Rb and family members p107 and p130, causing the release of transcription factor E2F from Rb inhibition and allowing cells to enter into the S phase in an unregulated manner [29,35]. The result that curcumin reduced HPV E6/E7 expression prompted us to determine if protein levels of tumor suppressor proteins known to be targeted by the HPV oncoproteins would be restored by curcumin. Indeed, curcumin treatment resulted in the increased expression of p53, Rb, and PTPN13.

Cancer cells often exhibit an increased ability to migrate and increased cell motility is a characteristic used to measure the oncogenic potential of cancer cells. Curcumin's reduction of HPV E6/E7 and the increase in expression of tumor suppressor proteins, lead us to assess curcumin's ability to inhibit cell motility using the well-established scratch/wounding assay. We observed a dramatic decrease in cell motility upon curcumin treatment. To provide additional insight into the kinetics regarding curcumin treatment and cell motility, we performed time-lapse microscopy. The videos clearly show that curcumin slows the movement of cervical epithelial cells. Further experiments are necessary to determine the molecular mechanisms involved in curcumin's inhibition of motility. With curcumin's ability to affect a variety of cell signaling pathways, we anticipate multiple alterations are responsible for inhibiting motility. One interesting possibility is that curcumin's rescue of PTPN13 could result in decreased cell motility as the expression of PTNP13 is associated with a decrease in anchorage-independent growth [30].

Given the high rate of smoking in American Indian women, we wanted to assess curcumin's functions in the presence of a tobacco carcinogen. While cigarette smoking is a known risk factor for the development of cervical cancer, the molecular interactions between smoke carcinogens and HPV are not well understood. It was recently reported that exposure to nicotine resulted in an increase in cell proliferation [36]. Additionally, BaP, a smoke carcinogen, has been detected in cervical mucus of smokers and was recently reported to increase HPV virion production [13,14,37]. We determined that BaP exposure increased the expression of HVP E7 oncoprotein, a novel finding suggesting a molecular mechanism by which BaP may influence HPV infection and the development of cervical cancer (Figure 6C and D). Although the mechanisms have not been fully explored, cigarette smoke and/or BaP have been shown to activate both NFκB and AP-1 pathways in a variety of cell lines, either through independent mechanisms or through interactions between the two pathways [38–42]. Our data indicates that BaP increases both NFκB and AP1 signaling pathways in cervical cancer cells. Additional data from our laboratory also indicates that BaP exposure increases the level of reactive oxygen species, which can act as a stimulus for NFκB activation (unpublished data). AP-1 is a well-known important regulator of HPV oncoprotein expression; therefore, it is possible that the increase in HPV E7 is via the increase in nuclear AP-1 family member, c-Fos (Figure 6B). Importantly, BaP's induction of HPV E7 was inhibited by curcumin, which is known to inhibit both NFκB and AP-1 activity. These findings suggest a novel molecular link between smoking and cervical cancer and our future studies will examine the molecular pathways altered in cervical cells following BaP exposure.

One concern regarding curcumin, is its low bioavailability and poor pharmacokinetics. Clearly, curcumin has anti-cancer effects in animal models and is currently in Phases I and II clinical trials for a variety of cancers and other health conditions [43]. The doses used in our report achieved significant results within 24–48 h; however, a lower dose sustained over 7–10 d may also result in similar findings. In fact, we are currently exploring curcumin's effect on cervical cancer cells in an extended, low-dose model. Also, our laboratory is involved in formulating antibody targeted nanoparticles loaded with curcumin, as one method to increase the bioavailability of curcumin into desired locations, that is, tumor tissue [44]. Additionally, in respect to cervical cancer, it would be possible to use curcumin as a topically applied treatment, where the local dose would be controlled more easily.

Curcumin affects a multitude of cellular pathways, each working together to ''quiet'' a cell's behavior. Especially considering the capacity of cancer cells to evade single target therapies, the pleiotropic effects of curcumin are likely to positively enhance its chemopreventive and therapeutic activities. The data presented here provides evidence that curcumin may be an effective chemopreventive agent through its ability to target multiple pathways, including induction of apoptosis, inhibition of cell motility, decrease in expression of HPV oncoproteins, and restoration of expression of tumor suppressor proteins. Importantly, curcumin is also able to inhibit BaP's induced increase in HPV oncoproteins. Taken together, our novel findings suggest that curcumin treatment is as an effective chemopreventive and therapeutic approach for cervical cancer.

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