Dihydroartemisinin Is Cytotoxic to Papillomavirus-Expressing Epithelial Cells *In vitro* and *In vivo*

Gary L. Disbrow,[1] Astrid C. Baege,[1] Katie A. Kierpiec,[1] Hang Yuan,[1] Jose A. Centeno,[2] Clare A. Thibodeaux,[1] Dan Hartmann,[1] and Richard Schlegel[1]

[1]Department of Pathology, Georgetown University Medical Center and [2]Department of Environmental and Infectious Disease Sciences, Armed Forces Institute of Pathology, Washington, District of Columbia

Abstract

Nearly all cervical cancers are etiologically attributable to human papillomavirus (HPV) infection and pharmaceutical treatments targeting HPV-infected cells would be of great medical benefit. Because many neoplastic cells (including cervical cancer cells) overexpress the transferrin receptor to increase their iron uptake, we hypothesized that iron-dependent, antimalarial drugs such as artemisinin might prove useful in treating HPV-infected or transformed cells. We tested three different artemisinin compounds and found that dihydroartemisinin (DHA) and artesunate displayed strong cytotoxic effects on HPV-immortalized and transformed cervical cells *in vitro* with little effect on normal cervical epithelial cells. DHA-induced cell death involved activation of the mitochondrial caspase pathway with resultant apoptosis. Apoptosis was p53 independent and was not the consequence of drug-induced reductions in viral oncogene expression. Due to its selective cytotoxicity, hydrophobicity, and known ability to penetrate epithelial surfaces, we postulated that DHA might be useful for the topical treatment of mucosal papillomavirus lesions. To test this hypothesis, we applied DHA to the oral mucosa of dogs that had been challenged with the canine oral papillomavirus. Although applied only intermittently, DHA strongly inhibited viral-induced tumor formation. Interestingly, the DHA-treated, tumor-negative dogs developed antibodies against the viral L1 capsid protein, suggesting that DHA had inhibited tumor growth but not early rounds of papillomavirus replication. These findings indicate that DHA and other artemisinin derivatives may be useful for the topical treatment of epithelial papillomavirus lesions, including those that have progressed to the neoplastic state. (Cancer Res 2005; 65(23): 10854-61)

Introduction

Cervical cancer is a global health problem, representing the primary cause of cancer-related female deaths in developing countries (1). Infection with high-risk human papillomaviruses (HPV) has been established as a precursor of cervical cancer and ~99% of cervical cancers contain a "high-risk" papillomavirus genome, with 50% to 60% of these cases due to a single HPV genotype, HPV-16 (2). Given the significant economic burden to health care systems for detecting and treating HPV-induced lesions and the need for inexpensive therapeutic approaches to treat such lesions, we initiated a study to investigate the efficacy of artemisinin compounds to kill HPV-16 E6 and E7 immortalized ectocervical cells and several established cervical cancer cell lines.

Artemisinin, the active principle of the Chinese medicinal herb *Artemisia annua* (3, 4), and its derivatives are very effective blood schistocidal antimalarials with fewer adverse side effects than any other antimalarial drug. Recommended by the WHO, the drug has been used to treat more than 2 million people, mainly in Africa and Asia (5). Artemisinin contains an endoperoxide bridge that reacts with ferrous iron to generate free radicals, leading to macromolecular damage and cell death (4–6). Recently, certain artemisinin derivatives were shown to inhibit the growth of a limited set of human cancer cell lines (7). In a subsequent study, the cervical cancer cell line HeLa was also shown to be sensitive to DHA although a mechanism for cell death was not elucidated (8). We speculated that both cervical cancer and immortalized cell lines, which are sensitized to apoptotic cell death by the expression of the *E6* and *E7* oncogenes (9–13) and overexpress the transferrin receptor (14–16), might be killed by these antimalarial compounds.

In this study, we investigated the specificity of artemisinin and two of its derivatives on a panel of cell lines that represent various stages of cancer progression. These cells included primary, HPV-immortalized, and tumorigenic cervical cells. We show that the HPV-immortalized and tumorigenic cervical cells are sensitive to DHA and artesunate and that these cells express higher levels of transferrin receptor and intracellular iron (compared with normal ectocervical cells). Indeed, iron is required for the drug-induced formation of reactive oxygen species (ROS), the activation of caspases, and consequent apoptosis. Most importantly, we show that DHA can inhibit mucosal tumors induced in animals by papillomavirus, suggesting that this class of drugs may have clinical applications additional to the well-studied antimalarial activities. For example, the topical application of artemisinin derivatives to early cervical dysplasia could greatly simplify the treatment of such papillomavirus-related lesions, including those in immunocompromised patients.

Materials and Methods

Cells and cell culture. Control ectocervical cells, transduced with empty retrovirus, and immortalized ectocervical cells representing preimmortalization (five passages after transduction with the *E6* and *E7* oncogenes) and postimmortalization (50 passages after transduction) have previously been described (17, 18). Ectocervical cells expressing E6 or E7 independently were transduced with retroviruses encoding each gene and selected as previously described (17). All ectocervical cells were maintained in modified Keratinocyte Growth Medium (Invitrogen, Carlsbad, CA) as described (17, 18). HeLa, SiHa, and Caski cells were obtained from the American Type Culture

Note: G.L. Disbrow, A.C. Baege, and K.A. Kierpiec contributed equally to this work.
Requests for reprints: Richard Schlegel, Department of Pathology, Georgetown University Medical Center, 3900 Reservoir Road Northwest, Washington, DC 20057. Phone: 202-687-7733; Fax: 202-687-8934; E-mail: schleger@georgetown.edu.

doi:10.1158/0008-5472.CAN-05-1216

Collection (Manassas, VA) and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Quality Biological, Inc., Gaithersburg, MD) and penicillin/streptomycin (Invitrogen).

Neutral red assay for cell survival. Cells, 5×10^4, were plated in duplicate in 12-well plates (Falcon). Twenty-four hours after plating, cells were treated for 3 days with the indicated concentrations of artemisinin (Sigma Chemical, St. Louis, MO), dihydroartemisinin [DHA; Calbiochem, San Diego, CA (discontinued) or a gift from Holley Pharmaceuticals, Inc., Fullerton, CA], or artesunate (gift from Holley Pharmaceuticals). All three compounds were dissolved in DMSO (Sigma Chemical). The wells were washed with PBS and appropriate medium containing neutral red (Invitrogen) was added. The cells were incubated at 37°C for 2 hours in 5% CO_2. Cells were washed thrice with PBS and then lysed in a solution of glacial acetic acid (1%)/ethanol (100%) (50:50; both from Baker Scientific, Phillipsburg, NJ) with rocking for 2 to 5 minutes at room temperature. Lysates were collected and 100 μL were analyzed in an Opsys MR plate reader (Dynex, Chantilly, VA) at a wavelength of 540 nm. The resulting cell viability number is the average of two wells and the experiment was repeated thrice with identical results. The assay was standardized and shown to be linear over the values obtained in these experiments (data not shown).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell viability. HeLa cells were plated in triplicate in 96-well microtiter plates (Falcon) at 2×10^4 cells per well. Twenty-four hours later, the cells were treated with either vehicle (H_2O) or desferrioxamine dissolved in H_2O at the concentrations indicated for 6 hours. After the pretreatment, the media was replaced with media containing various concentrations of DHA with additional desferrioxamine added to maintain the concentration of desferrioxamine in the wells. After 24 hours of DHA treatment, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Chemicon International, Temecula, CA) as per protocol of the manufacturer. The resulting cell viability number is the average of three wells and the experiment was repeated thrice with identical results.

Annexin V and propidium iodide staining. Control ectocervical cells (HCX) were plated at 1×10^5 and HeLa cells at 2×10^5 both in 60-mm tissue culture dishes (Falcon). Twenty-four hours later, the cells were treated with various concentrations of DHA for 3 days. At the end of 3 days, the cells were trypsinized and stained with the Annexin V propidium iodide kit (BD PharMingen, San Diego, CA) as per protocol of the manufacturer. Cells were subjected to fluorescence-activated cell sorting (FACS) analysis. The data presented are representative of results from at least two independent experiments.

Western blot. Cells, 1×10^6, were plated in 100-mm tissue culture dishes (Falcon). After 24 hours, the cells were pretreated with desferrioxamine or left untreated for 6 hours, then treated overnight with various concentrations of DHA or DHA + desferrioxamine. The cells were lysed in 2× Laemmli's buffer and boiled for 10 minutes. Protein concentrations were determined using a detergent-compatible protein assay kit from Bio-Rad (Hercules, CA). β-Mercaptoethanol (Sigma Chemical) was added to 10% of the final volume and equal amounts of protein were loaded on 4% to 20% gradient polyacrylamide gels (Invitrogen) and separated by eloctrophoresis. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and probed with antibodies against total caspase-9, caspase-3, caspase-7, and poly(ADP-ribose) polymerase or cleaved caspase-9, caspase-3, caspase-7, and poly(ADP-ribose) polymerase obtained from the apoptosis kit (Cell Signal, Beverly, MA) at dilutions of 1:2,000. To determine activation of other caspases, antibodies against caspase-12 (BD PharMingen) at a concentration of 1:2,000, caspase-8 (CN Biosciences, San Diego, CA) at 1:100, and caspase-10 (Trevigen, Gaithersburg, MD) at 1:1,000 were used. Additionally, p53 was detected using an anti-p53 antibody (Cell Signal) at 1:1,000 and E7 was detected using an anti-E7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500. To analyze for the level of transferrin receptor, 2×10^6 cells were plated (all cell lines) and lysed 24 hours later in SDS lysis buffer as previously described (18). Samples were separated on a 10% Tris-glycine gel (Invitrogen) and the membrane was probed with antitransferrin receptor antibody (BD PharMingen) at 1:1,000. To ensure equal loading, all membranes were reprobed using an anti-β-actin antibody (Sigma Chemical) at 1:10,000.

Measurement of reactive oxygen species. Production of ROS was determined using 6-carboxy-2′,7′-dihydrofluorescein-diacetate (Molecular Probes, Eugene, OR) and FACS analysis as described (19, 20). HeLa cells, 1×10^6, were seeded into 100-mm plates. Twenty-four hours later, the cells were incubated for 1 hour in 6-carboxy-2′,7′-dihydrofluorescein-diacetate at a concentration of 5 μmol/L in DMEM and simultaneously treated with 25 or 100 μmol/L DHA. Some cells also were pretreated with 150 μmol/L

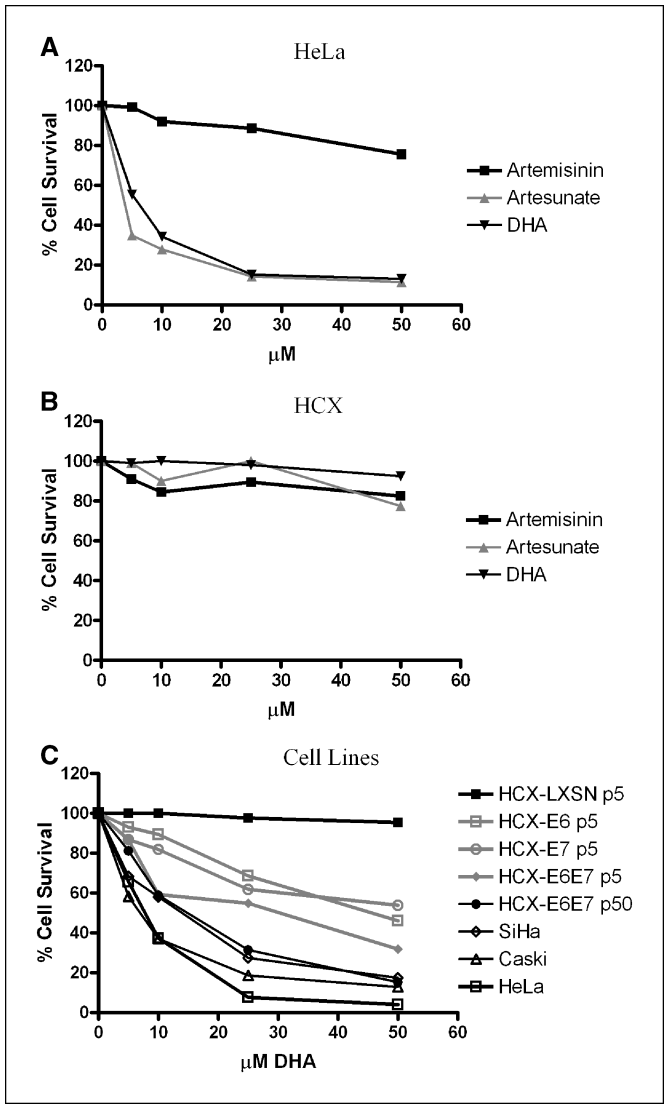

Figure 1. Effects of artemisinin, artesunate, and dihydroartemisinin on normal, immortalized, and tumorigenic cervical cells. *A,* HeLa cells were treated with various concentrations of artemisinin, artesunate, or DHA for 3 days. Cell survival was measured using the neutral red assay. The IC_{50} for artesunate and DHA was determined to be 5 and 7.5 μmol/L, respectively. *B,* normal cervical epithelial cells (HCX) were treated as in (*A*). At the end of the 3-day treatment, cell survival was measured using the neutral red assay. Normal cervical cells were very resistant to all three compounds tested. *C,* ectocervical epithelial cells transduced with empty retrovirus (HCX), ectocervical cells transduced with HPV-16 E6 or E7 independently, cervical cells transduced with HPV-16 E6/E7, preimmortalization (early p5) and postimmortalization (late p50), and cervical cancer cells (HeLa, SiHa, and Caski) were treated with DHA for 3 days. At the end of the treatment, cell viability was measured with a neutral red assay. All tumorigenic and HPV-immortalized cell lines were sensitive to DHA with HeLa showing the greatest sensitivity. After treatment with 50 μmol/L DHA for 3 days, 98% of HeLa cells were killed. In contrast, normal cervical cells were minimally affected. *Points,* mean of two wells from experiments carried out three separate times.

desferrioxamine as mentioned above. The probe was removed and cells were rinsed with PBS. Cells were incubated in fresh DMEM, DMEM + DHA, or DMEM containing desferrioxamine and DHA for 3 hours. Cells were then trypsinized, resuspended in PBS, and subjected to FACS analysis (Coulter EPICS equipped with an argon laser lamp; emission 480 nm, band pass filter 530 nm). Repeat experiments gave similar results.

Chemical analysis for total iron. Chemical analysis for total iron was conducted on several preparations of cervical cancer cell lines employing inductively coupled plasma-optical emission spectrometry (Optima 3000, Perkin-Elmer, Norwalk, CT). Elemental determinations for iron in each cell line preparation were established employing the internal standard method and a five-point calibration curve using 0.1, 0.5, 1.0, 2.5, and 5 μg/mL standard solutions prepared by serial dilution from a 1,000-μg Fe/L stock solution (Spex Industries, Edison, NJ). We used the method of internal standard to accurately measure iron by inductively coupled plasma-optical emission spectrometry. Using this method, a known amount of internal standard (0.1 mL of 70 μg/mL gallium standard) is added to both the standards and the samples. Known mixtures of standard and analyte are used to construct a calibration curve and the ratio of varied Fe signal to the constant Ga concentration in the sample is used to calculate the sample concentration. An internal standard is useful to correct for any interference due to sample matrix, particularly with biological samples, and to account for any sample loss. Distilled deionized water from the Millipore Ultrapure Water System was used for the preparation and dilution of all standards and cell cultures. Before the analysis, samples were digested in 1% nitric acid (Ultra-Pure Optima Grade, Sigma Chemical) employing a microwave digestor (CEM Industries, Salt Lake City, UT) at a constant temperature and pressure. The samples and standards were introduced into the inductively coupled plasma-optical emission spectrometry at a flow of 1 mL/min and were analyzed at 1,200-W radio frequency power, 0.8 L/min nebulizer gas flow, 15 L/min plasma gas glow, and 1.2 L/min auxiliary gas flow. Two emission wavelengths were used for the determination of iron by inductively coupled plasma-optical emission spectrometry at 239.562 and 259.940 nm. The gallium internal standard was monitored at an emission wavelength of 294.364 nm. Samples containing the cell lines were prepared in duplicate and each sample was measured in triplicate (accumulation time of 20 seconds per replicate) with a relative SD of <2 relative units.

***In vivo* canine papillomavirus assay.** Although under anesthesia, both sides of the oral mucosa of six 10-week-old, female beagle dogs was abraded and infected with canine oral papillomavirus (500 ng based on L1 concentration) as previously described (21). Twenty-four hours later, the infected areas of three dogs were treated with 100 μL of DHA dissolved in DMSO (78 mmol/L). Control dogs were treated with 100 μL of DMSO. The dogs were treated once daily for 1 minute, five times a week, for the duration of the experiment. Every 3rd day, the dogs were anesthetized for a more thorough treatment and photography. Blood was drawn on three separate occasions at 1, 3, and 5 weeks after tumor formation started.

ELISA analysis of dog serum for L1 antibody production. Blood was collected by phlebotomy and serum fractions were prepared. Sodium azide (Sigma Chemical, 20% stock) was added to the serum samples (final concentration of 0.02%) to prevent microbial growth. Wells from a 96-well ELISA plate (Dynex Immulon 2 HB) were coated with the canine oral papillomavirus at a dilution of 1:1,000 (0.1 ng per well) in 5% skim milk for 1 hour at 37°C. Wells were rinsed with PBS and serum was added (diluted 1:50 in 5% skim milk) to the plate and incubated for 1 hour at 37°C. Secondary sheep anti-dog immunoglobulin G conjugated to horseradish peroxidase (HRP) was added at a dilution of 1:5,000 for 1 hour at 37°C. HRP was activated with the TMB Microwell Peroxidase substrate and the reaction was terminated with stop solutions (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Samples were then read in an ELISA plate reader at 450 nm. The results shown are representative of three individual experiments with similar results.

Results and Discussion

Artemisinin and derivative compounds exhibit a differential cytotoxicity to human papillomavirus–expressing cell lines. Artemisinin and several of its natural and synthetic derivatives have been shown to exhibit varying antimalarial activities (3, 22, 23). To determine if artemisinin and its derivatives also might differ in their antitumor cell activity, we compared their abilities to inhibit

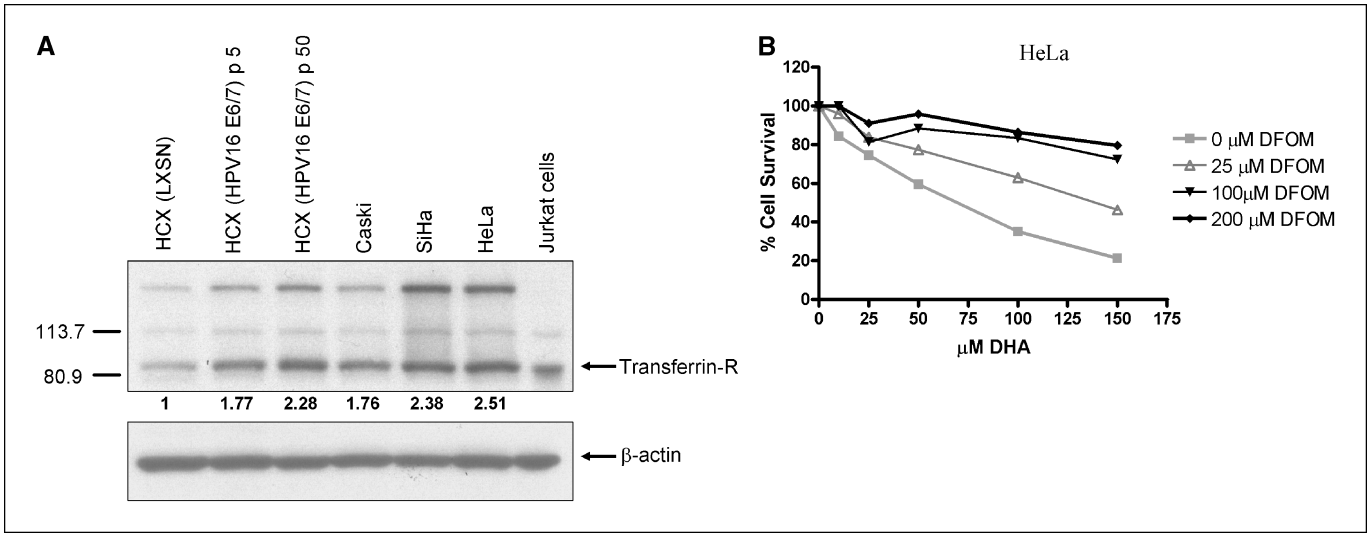

Figure 2. Transferrin receptor levels correlate with sensitivity to DHA and with iron-dependent toxicity in HeLa cells. *A,* the same panel of cells as in Fig. 1*C* was examined for transferrin receptor level. Equal numbers of cells were plated and 24 hours later were harvested for Western blot analysis. The protein was transferred to a PVDF membrane and probed with antitransferrin receptor antibody (1:1,000). The blot was reprobed with anti-β-actin antibody (1:10,000) to ensure equal loading. The blot shows a clear increase in the amount of transferrin receptor in immortalized and tumorigenic cell lines as compared with normal cells and this correlates with their increased sensitivity to DHA treatment. Densitometry was done on the scanned image; *numbers below the lanes,* increase in transferrin receptor levels when HCX was set at 1. Jurkat cell extract was included as a positive control. *B,* HeLa cells were plated in 96-well microtiter plates at 2×10^4 cells per well. Twenty-four hours later, the cells were either treated with desferrioxamine at the concentrations indicated for 6 hours or left untreated. Medium containing various concentrations of DHA and desferrioxamine at the original concentration was then added. After 24 hours of DHA treatment, cell viability was measured using MTT assay. Desferrioxamine antagonized the cytotoxic effects of DHA and increased cell survival in a dose-dependent manner with >85% cell survival when the cells were pretreated with 200 μmol/L desferrioxamine. *Points,* mean cell viability of three wells; experiment was repeated thrice with identical results.

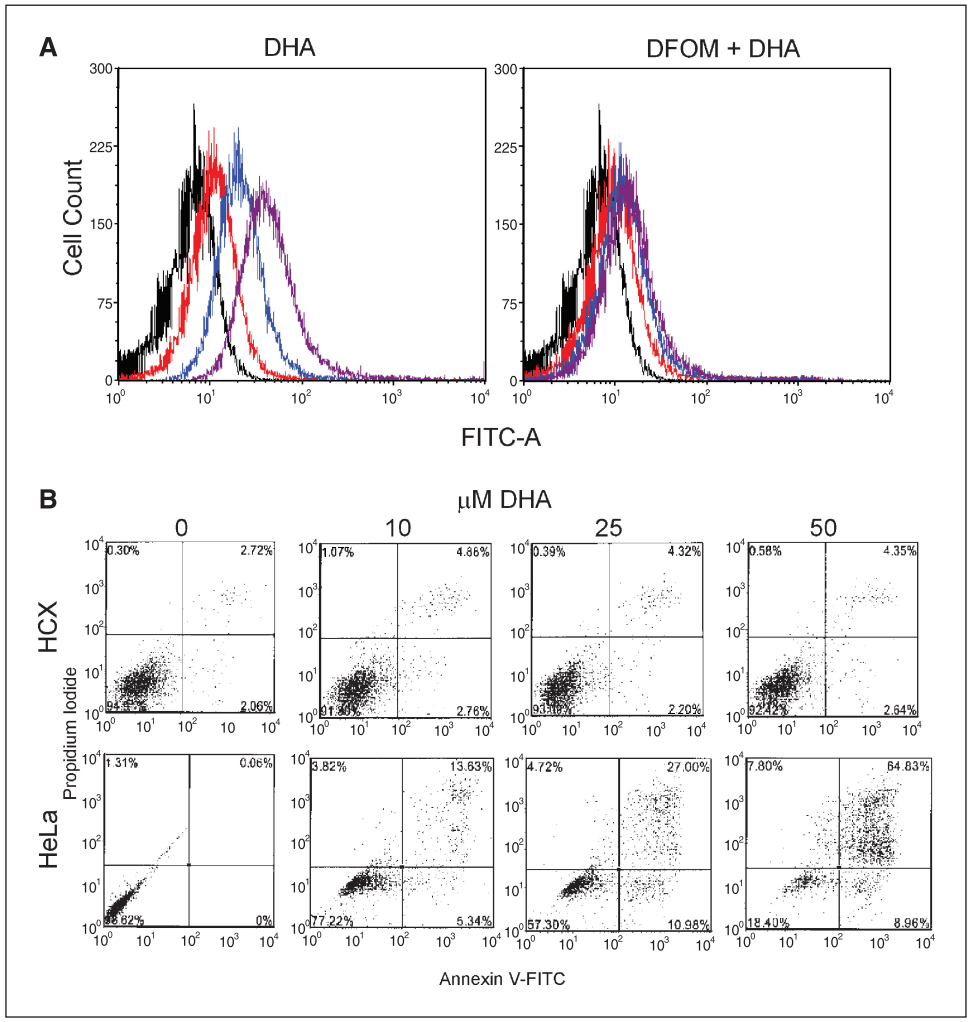

Figure 3. DHA-induced apoptosis is caused by iron-dependent formation of ROS. *A,* HeLa cells were plated in 100-mm tissue culture dishes and treated with 5 μmol/L 6-carboxy-2′,7′-dihydrofluorescein-diacetate (fluorescent probe for ROS) and simultaneously treated with 0 μmol/L (*red line*), 25 μmol/L (*blue line*), or 100 μmol/L (*purple line*) DHA for 3 hours (*left*). *Black lines,* untreated cells. Cells were collected and analyzed by flow cytometry. A 2-fold and 4.2-fold increase in fluorescence was observed in cells treated with 25 and 100 μmol/L DHA, respectively, indicating a dose-dependent increase in production of ROS in response to DHA treatment (*left*). The production of ROS was markedly inhibited by pretreating the cells with desferrioxamine (*right*), indicating that the formation of ROS in response to DHA treatment is iron dependent. *B,* after a 3-day treatment with various concentrations of DHA, control cervical cells (HCX) and HeLa cells were collected and stained with FITC-conjugated Annexin V and propidium iodide for 10 minutes before being subjected to flow cytometry. Control cervical cells (HCX) showed only a minor increase (from 2.7% to 4.4%) in the number of Annexin V– and propidium iodide–positive cells following treatment with DHA (50 μmol/L; *top*). In contrast, the HeLa cervical cancer cells showed an increase in Annexin V and propidium iodide positivity from 0.1% to 64.8%. In addition, there were 7.8% and 9% propidium iodide and Annexin V singly positive cells, respectively. It seems that there is a rapid progression of Annexin V–positive cells to propidium iodide–positive cells (*bottom*).

the growth of the HeLa cervical cancer cell line (Fig. 1*A*). HeLa cells were treated for 3 days with the compounds at 5 to 50 μmol/L concentrations and viability was measured with neutral red staining as described. Artemisinin had little biological effect on these cells although it is known to exhibit strong antimalarial activity *in vivo*. We presume that the *in vivo* activity of artemisinin may rely on its rapid conversion in the liver (and other potential sites) into dihydroartemisinin. Consistent with this hypothesis, we found that DHA was highly effective in killing HeLa cells. Artesunate also displayed a cytotoxic activity equivalent to that observed with DHA. Most importantly, DHA and artesunate had little or no cytotoxic effect on normal cervical epithelial cells (Fig. 1*B*) even at 50 μmol/L concentrations. As anticipated from its lack of effect on HeLa cells, artemisinin also had no effect on normal cervical cells.

Because DHA is the predominant metabolite of artemisinin and displayed a highly selective cytotoxic activity for the HeLa cell line, we selected this compound for further study and screened its activity against two additional tumorigenic cervical cancer cell lines, SiHa and Caski, two HPV-immortalized nontumorigenic cervical cell lines expressing E6 and E7 together [preimmortalization (p5) and postimmortalization (p50)], and two cell lines expressing E6 and E7 independently. Again, cervical cells transduced with empty retrovirus were included as a control. Figure 1*C* shows that DHA is cytotoxic to all HPV-expressing cells regardless of tumorigenicity. Because the HPV-immortalized cells express only the HPV-16 *E6* and *E7* genes (17, 18), we conclude that the expression of these two viral genes is sufficient for inducing DHA sensitivity. In addition, the sensitivity to DHA seemed to be early after transduction of the *E6/E7* genes, suggesting that DHA sensitivity precedes cellular immortalization. In an attempt to determine the potential role of E6 and E7 in the induction of sensitivity to DHA, we examined cells expressing each gene independently. Although both cell lines were slightly less sensitive to DHA when compared with the cell line expressing both genes, we were unable to attribute DHA sensitivity to one gene nor observed an additive effect. Neither gene alone is sufficient to immortalize ectocervical cells whereas each gene is capable of extending the life span of ectocervical cells (data not shown). Thus, whereas all the early passage cells grew at approximately the same rate, the increased growth potential seems to be sufficient to sensitize the cells to DHA. Similar to the results in Fig. 1*A*, DHA had little effect on the normal cervical epithelial cells.

Our results refine and extend published data showing that DHA is twice more effective than artemisinin at inhibiting the growth of four cancer cell lines, one of which was HeLa (8). The previous study determined the IC_{50} of DHA and artemisinin to be ~16 and 39 μmol/L, respectively, whereas our data suggest that HeLa cells, although sensitive to DHA (IC_{50} of ~5 μmol/L), are very resistant

to artemisinin. It is possible that differences in artemisinin preparations, the specific clone of HeLa cells, or the duration of treatment might explain the discrepant findings. Our inclusion of the relevant normal cervical cells allows us to conclude that drug-induced cytotoxicity is selective for HPV-expressing cells and permits the estimation of a therapeutic index. Finally, our finding that HPV-immortalized, nontumorigenic cervical cells are sensitive to DHA suggests that this compound may be useful for treating premalignant, dysplastic cervical lesions.

Dihydroartemisinin cytotoxicity is iron dependent. Because the antimalarial activity of DHA and artesunate is dependent on high intraparasite iron content (3), we examined whether the DHA sensitivity of HPV-expressing cells was related to cellular iron content. First, we found that all of the sensitive cell lines expressed higher levels of the transferrin receptor (1.7- to 2.5-fold) than corresponding normal cells (Fig. 2*A*). The increased level of transferrin receptor *in vitro* correlates with *in vivo* data showing increased transferrin receptors in high-grade cervical lesions and cancers as measured by immunohistochemistry (14–16). Thus, the increase in transferrin receptors is not an artifact of *in vitro* culturing of the cells. This receptor overexpression also was accompanied by higher levels of total intracellular iron. When the intracellular iron content of HeLa cells and normal ectocervical cells was measured by inductively coupled plasma optical emission spectrometry, we found that HeLa cells contained 50% more iron (1.3 versus 0.88 pg per cell, respectively). Whereas the absolute level of transferrin receptor and total cellular iron in HeLa cells was elevated, it did not seem to correlate strictly with the dramatic increase in DHA sensitivity. This could have several etiologies. For example, although we have measured total cellular iron, we have not been able to quantify the level of ferrous iron, the form that reacts with the DHA endoperoxide bond to generate ROS. It is possible that there is a greater difference in the level of ferrous iron in the normal and HPV-expressing cells. In addition, because HPV-expressing cells are sensitized to apoptosis by the E6 and E7 proteins, it is possible that it only takes slight increases in iron content to amplify DHA cell toxicity.

To provide a more direct evidence for the role of iron in DHA-induced cytotoxicity, we specifically chelated the iron in the cell cultures with desferrioxamine. As shown in Fig. 2*B*, DHA cytotoxicity was reversed in a dose-dependent manner, indicating an essential role for iron. To circumvent potential toxicity associated with prolonged exposure of cells to desferrioxamine (24), we did these experiments with increased amounts of DHA, allowing the exposure time to be decreased from 3 days to overnight. Under these conditions, there was no detectable effect of desferrioxamine on cell viability and cells were strongly protected against DHA effects.

Dihydroartemisinin induces reactive oxygen species in cervical cancer cells via an iron-dependent mechanism. To determine if the DHA cytotoxicity observed in HeLa cells truly reflected the hypothesized generation of iron-dependent ROS, we measured the induction of ROS in HeLa cells using 6-carboxy-2′,7′-dihydrofluorescein-diacetate as shown in Fig. 3*A*. This cell-permeating, nonfluorescent probe is oxidized by ROS and converted into a fluorescent product, 2′,7′-dichlorofluorescein, which can be measured using FACS. Thus, an increase in fluorescence indicates an increase in the level of ROS. HeLa cells show a significant, dose-dependent increase in ROS (Fig. 3*A, left*). Importantly, this increase in ROS was abrogated when the cells were pretreated with desferrioxamine (Fig. 3*A, right*). Together, these data show the essential role for intracellular iron in mediating the cytotoxic effects of DHA and that ROS, in fact, are generated in HeLa cells treated with DHA.

We hypothesized that the cytotoxicity secondary to ROS was due to the induction of apoptosis. This first was suggested by the morphologic changes that occur in DHA-treated cervical cancer cells. Treated cells ceased proliferating, rounded up, and detached

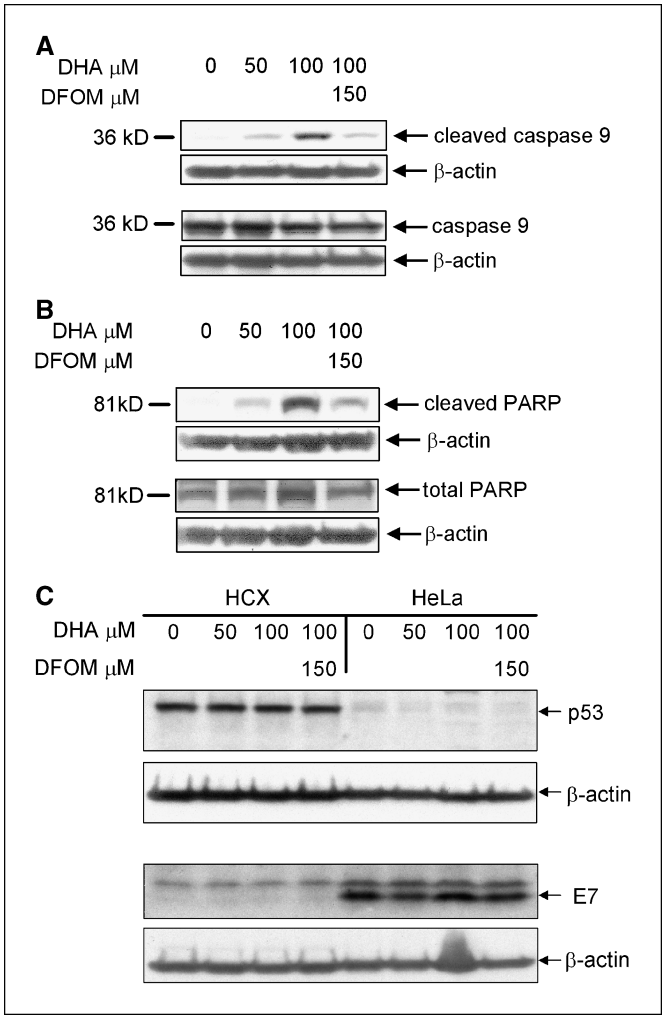

Figure 4. p53-independent induction of the mitochondrial apoptotic pathway in cervical cancer cells. *A,* HeLa cells were plated in 100-mm tissue culture dishes and 24 hours later were pretreated with desferrioxamine or left untreated as previously described. The cells then were treated overnight with various concentrations of DHA and harvested for Western blot analysis. Total caspase-9 and cleaved caspase-9 (a marker of mitochondrial-generated apoptosis) were detected using the apoptosis kit from Cell Signal. To ensure equal loading, the membranes were reprobed using an antibody against β-actin at 1:10,000. DHA induces detectable cleavage of caspase-9 and this cleavage is prevented by pretreating the cells with the iron chelator desferrioxamine. *B,* poly(ADP-ribose) polymerase (*PARP*) cleavage is one of the terminal alterations in cell apoptosis and is induced by many apoptotic pathways, including the mitochondrial pathway. Similar to the results with caspase-9, HeLa cells treated with DHA showed a dose response increase in the level of cleaved poly(ADP-ribose) polymerase. This cleavage event was reversed when the cells were pretreated with the iron chelator desferrioxamine. *C,* p53 is not increased in DHA-treated HCX or HeLa cells. Cells were treated as in (*A* and *B*). The p53 protein was detected using monoclonal p53 antibody at 1:2,000. The lack of p53 induction suggests that the E6 protein is functioning and not altered by DHA treatment. The E7 protein was detected using a monoclonal antibody at 1:500. Both blots were reprobed with anti-β-actin antibody (1:10,000) to ensure equal loading.

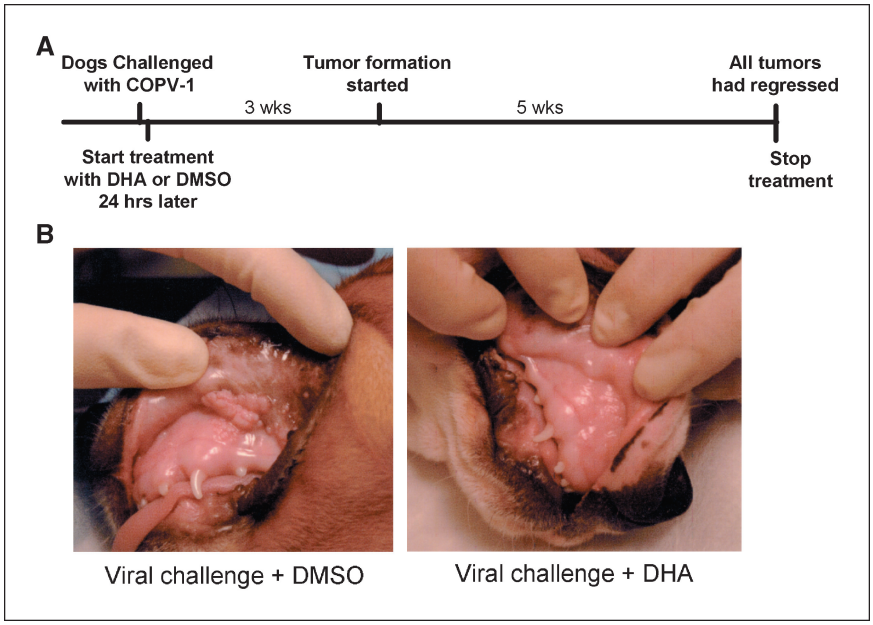

Figure 5. *In vivo* testing of DHA as a topical therapeutic agent. *A,* a schematic representation of the experimental protocol. Dogs were challenged with canine oral papillomavirus on day 1 with treatments starting 24 hours later. Tumors appeared at ~3 weeks and lasted ~4 to 5 weeks. The experiment was stopped 1 week later, ensuring that no additional tumors were formed. *B,* representative pictures of dogs with and without tumor formation (*left* and *right*, respectively).

from the plate, suggesting an apoptotic process (data not shown). To determine the mode of DHA-induced cell death, we stained normal and cancer cells with fluorescent Annexin V and propidium iodide (which measure early and late events in apoptosis, respectively) and quantified their fluorescence by flow cytometry. Untreated normal cervical cells showed a low background of staining with either Annexin V or propidium iodide (2-3%) and this staining was only marginally increased (2-4%) when treated with increasing concentrations of DHA for 3 days (Fig. 3*B, top*). In contrast, DHA-treated cervical cancer cells stained with Annexin V and rapidly became propidium iodide positive (Fig. 3*B, bottom*), indicating irreversible loss of membrane integrity. After 3 days in 50 μmol/L DHA, ~65% of the cervical cancer cells were stained positively with propidium iodide with an additional 9% staining positive for Annexin V.

Dihydroartemisinin-induced apoptosis proceeds via the mitochondrial pathway and is p53 and viral oncogene independent. To define the apoptotic pathway(s) being activated by DHA, we did Western blot analysis of DHA-treated cells using a panel of caspase antibodies. Figure 4*A* shows that caspase-9 was activated in response to DHA, indicating the involvement of the mitochondrial apoptotic pathway (reviewed in ref. 25). However, there was no activation of caspase-12 (an indicator of endoplasmic reticulum stress; reviewed in ref. 26) or caspase-8 and caspase-10 (indicative of receptor-mediated pathways; ref. 27 and data not shown). Apparently, the mitochondrial apoptotic pathway is activated preferentially when cells are treated with DHA. The induction of caspase-9 activates downstream effector caspases, which ultimately leads to the cleavage of cytoskeletal and nuclear proteins such as poly(ADP-ribose) polymerase (28). As shown in Fig. 4*B*, cleaved poly(ADP-ribose) polymerase was detected easily in cells treated with DHA. The activation of caspase-9 and cleavage of poly(ADP-ribose) polymerase occurred in a dose-dependent manner and was significantly inhibited by preincubating the cells with desferrioxamine (Fig. 4*A* and *B*), showing the iron dependence of DHA.

One of the key mediators of the mitochondrial apoptotic pathway is the p53 protein (reviewed in ref. 25), and the treatment of cervical cancer cell lines with chemotherapeutic compounds such as cisplatin often induces elevated levels of p53 even in the presence of the HPV E6 protein (11, 29). It was therefore possible that DHA might be inducing apoptosis by increasing cellular p53 levels. However, as shown in Fig. 4*C*, we found no increase in the amount of p53 in DHA-treated HeLa cells even at the highest dose of DHA. In addition, there was no increase in the level of p53 in the normal ectocervical cells, which may not be surprising due to the lack of toxicity in these cells. The finding that p53 was not induced in HeLa cells on treatment with DHA suggests a p53-independent mechanism for the activation of caspase-9 and the mitochondrial apoptotic pathway.

The lack of p53 increase in DHA-treated cells also suggests that the expression of the viral E6 protein, in HeLa cells, was not inhibited. Normally, E6 mediates the ubiquitin-dependent degradation of p53 and the continued lack of detectable p53 suggests that E6 protein expression continued in the presence of DHA. However, to independently verify that viral oncoproteins were not altered by DHA, we also quantified the expression of the E7 viral protein by Western blotting. As shown in Fig. 4*C* (*bottom*), the level of E7 protein was unaltered by DHA exposure. Thus, apoptosis occurs in HeLa cells even with continued expression of the viral oncoproteins and lack of cellular p53 protein.

***In vivo* activity of dihydroartemisinin: the canine papillomavirus model.** Thus far, our data show the ability of DHA to selectively kill cervical cancer cell lines and immortalized cervical cells *in vitro*. To determine whether these *in vitro* activities were applicable *in vivo*, we employed the canine oral papillomavirus model. This animal model has been used for evaluating the efficacy of papillomavirus vaccines and has several features which make it an ideal model for mimicking human mucosal papillomavirus infections, both oral and genital (30). One important advantage of this model is that 100% of challenged animals become infected and develop tumors. A second advantage is that tumor formation can be monitored easily and correlated with immunologic responses. Lastly, the easy accessibility of the tumors allows for topical application of potential antiviral or antitumor compounds.

A Tumor Formation in Dogs

Dog ID	DMSO or DHA	Right Side	Left Side
1	DMSO	+	+
2	DMSO	+	+
3	DMSO	+	+
4	DHA	-	-
5*	DHA	+	+
6	DHA	-	-

*Tumors regressed two weeks earlier than the DMSO-treated animals

B Anti-L1 capsid protein antibody titers

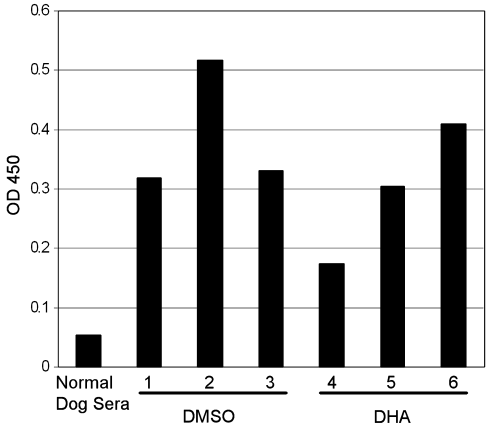

Figure 6. DHA significantly inhibits tumor formation while apparently not being able to prevent infection and replication of canine oral papillomavirus. *A,* tabulation of tumor formation in the dogs. +, presence of a tumor (similar to that shown in Fig. 5*B, left*). All tumors that formed reached approximately the same size. The tumors in DHA-treated dog regressed slightly faster than the tumors in DMSO-treated dogs. *B,* ELISA results from dog serum collected during the last week of the study. All dogs were positive for antibodies against L1, indicating an initial infection in all dogs. Dogs 4 and 6 failed to form tumors although they had antibodies against L1, suggesting DHA can inhibit tumor formation without inhibiting the initial infection or replication of the virus.

Ten-week-old beagles were challenged with purified canine oral papillomavirus on the right and left sides of the gum and buccal mucosa as previously described (21). Twenty-four hours later, the dogs were treated with either DHA or vehicle (DMSO). In experimental group 1, three dogs were treated topically with 2.22 mg DHA dissolved in 100 μL DMSO. Treatment typically lasted from 30 seconds to 1 minute each day. In experimental group 2, challenged dogs were treated with DMSO using an identical application protocol. Figure 5*A* illustrates the time line of viral challenge, drug treatment, tumor appearance, and tumor regression.

Figure 5*B* is a photograph of virus-infected dogs treated with DMSO (*left*) or DHA (*right*) at ~6 weeks post challenge. The tumor shown in the DMSO-treated dog is representative of the size of tumors observed in all tumor-positive dogs and was taken at the pinnacle of tumor growth. The DHA-treated dog is free of tumor. In addition, the oral mucosa treated with DHA shows no evidence of ulceration or inflammation, consistent with the lack of DHA cytotoxicity for normal epithelial cells *in vitro*.

Topical application of dihydroartemisinin inhibits the formation of tumors but does not seem to prevent canine oral papillomavirus infection or replication in oral mucosa. Figure 6*A* summarizes the results of tumor formation in the six dogs. All virus-infected dogs treated with DMSO developed tumors at 3 to 4 weeks post challenge. In contrast, DHA treatment abolished tumor formation in two of three infected animals. In the DHA-treated dog that did develop a tumor (dog 5), the tumor regressed 2 weeks earlier than those in DMSO-treated dogs although the tumor mass was of comparable size to those in control animals. Thus, the absence of tumors in two of the three DHA-treated dogs suggests that DHA is effective in the prevention of tumor formation. Interestingly, visual observations of the oral mucosa at ~3 weeks postinfection showed a similar mucosal "roughness" in all of the challenged dogs (including the DHA-treated animals), which is indicative of early papilloma formation. We postulated, therefore, that DHA might be inhibiting tumor formation rather than virus infection. In an effort to determine if the DHA-treated dogs really had a subclinical viral infection, we did ELISA (in triplicate) on serum samples (taken at the end of the trial) to detect and quantify antibody responses to the L1 viral capsid protein (Fig. 6*B*). Despite the finding that only one DHA-treated dog developed a tumor, we observed that all DHA-treated dogs developed antibody titers against the L1 protein. This suggests that the tumor-free animals indeed had been infected with virus and that the virus had replicated and synthesized significant amounts of L1 protein. Thus, it seems that DHA inhibits tumor formation rather than viral replication. As anticipated, all of the control animals also developed antibodies to L1, which is normally observed during the natural regression of these tumors.

In summary, our results indicate that DHA can effectively induce apoptosis in HPV-expressing cervical cells and inhibit viral-induced tumor formation in animals. Although our study groups were small, preventing us from performing statistical analysis, it should be emphasized that the canine oral papillomavirus model results in a 100% tumor formation rate and in the past 12 years of working with this model, we have not had a challenged dog that failed to develop tumors. Thus, the absence of tumors in 66% of the treated animals is biologically significant. We are continuing these studies to examine both the inhibition of tumor formation as well as the increased rate of tumor regression. Coupled with the observation that DHA can penetrate mucosal surfaces without inducing cellular damage, it seems that DHA may have clinical applications for the topical treatment of cervical dysplasia and possibly of other mucosal or epidermal neoplasias.

Acknowledgments

Received 4/7/2005; revised 8/19/2005; accepted 9/15/2005.

Grant support: Mary Kay Ashe Foundation and NIH National Center for Alternative Medicine grant R21AT002687-01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Vjekoslav Tomaić and members of the Department of Comparative Medicine, Dr. Mary Martin, Jennifer Pass, and Mark Williams, for assistance with the dogs and Michael Liu of Holley Pharmaceuticals for providing us with the dihydroartemisinin and artesunate used in this study.

References

1. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
2. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12–9.
3. Meshnick SR. Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol 2002;32:1655–60.
4. O'Neill PM, Posner GH. A medicinal chemistry perspective on artemisinin and related endoperoxides. J Med Chem 2004;47:2945–64.
5. Hien TT, White NJ. Qinghaosu. Lancet 1993;341:603–8.
6. Posner GH, O'Neill PM. Knowledge of the proposed chemical mechanism of action and cytochrome P450 metabolism of antimalarial trioxanes like artemisinin allows rational design of new antimalarial peroxides. Acc Chem Res 2004;37:397–404.
7. Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR. The anti-malarial artesunate is also active against cancer. Int J Oncol 2001;18:767–73.
8. Chen HH, Zhou HJ, Fang X. Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives *in vitro*. Pharm Res 2003;48:231–6.
9. DeFilippis RA, Goodwin EC, Wu L, DiMaio D. Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. J Virol 2003;77:1551–63.
10. Jones DL, Thompson DA, Munger K. Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to HPV type 16 E7-induced apoptosis. Virology 1997;239:97–107.
11. Liu Y, McKalip A, Herman B. Human papillomavirus type 16 E6 and HPV-16 E6/E7 sensitize human keratinocytes to apoptosis induced by chemotherapeutic agents: roles of p53 and caspase activation. J Cell Biochem 2000;78:334–49.
12. Simbulan-Rosenthal CM, Velena A, Veldman T, Schlegel R, Rosenthal DS. HPV-16 E6/7 immortalization sensitizes human keratinocytes to ultraviolet B by altering the pathway from caspase-8 to caspase-9-dependent apoptosis. J Biol Chem 2002;277:24709–16.
13. Stoppler H, Stoppler MC, Johnson E, et al. The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. Oncogene 1998;17:1207–14.
14. Keesee SK, Domanik R, Patterson B. Fully automated proteomic detection of cervical dysplasia. Anal Quant Cytol Histol 2002;24:137–46.
15. Lloyd JM, O'Dowd T, Driver M, Tee DE. Demonstration of an epitope of the transferrin receptor in human cervical epithelium—a potentially useful cell marker. J Clin Pathol 1984;37:131–5.
16. Mizuuchi H, Kudo R, Tamura H, et al. Identification of transferrin receptor in cervical and endometrial tissues. Gynecol Oncol 1988;31:292–300.
17. Baege AC, Berger A, Schlegel R, Veldman T, Schlegel R. Cervical epithelial cells transduced with the papillomavirus E6/E7 oncogenes maintain stable levels of oncoprotein expression but exhibit progressive, major increases in hTERT gene expression and telomerase activity. Am J Pathol 2002;160:1251–7.
18. Baege AC, Disbrow GL, Schlegel R. IGFBP-3, a marker of cellular senescence, is overexpressed in human papillomavirus-immortalized cervical cells and enhances IGF-1-induced mitogenesis. J Virol 2004;78:5720–7.
19. Gao F, Yi J, Yuan JQ, Shi GY, Tang XM. The cell cycle related apoptotic susceptibility to arsenic trioxide is associated with the level of reactive oxygen species. Cell Res 2004;14:81–5.
20. Vowells SJ, Sekhsaria S, Malech HL, Shalit M, Fleisher TA. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. J Immunol Methods 1995;178:89–97.
21. Yuan H, Estes PA, Chen Y, et al. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. J Virol 2001;75:7848–53.
22. Deponte M, Becker K. Plasmodium falciparum—do killers commit suicide? Trends Parasitol 2004;20:165–9.
23. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, et al. Artemisinins target the SERCA of Plasmodium falciparum. Nature 2003;424:957–61.
24. Simonart T, Boelaert JR, Mosselmans R, et al. Antiproliferative and apoptotic effects of iron chelators on human cervical carcinoma cells. Gynecol Oncol 2002;85:95–102.
25. Orrenius S. Mitochondrial regulation of apoptotic cell death. Toxicol Lett 2004;149:19–23.
26. Szegezdi E, Fitzgerald U, Samali A. Caspase-12 and ER-stress-mediated apoptosis: the story so far. Ann N Y Acad Sci 2003;1010:186–94.
27. Lee MW, Park SC, Kim JH, et al. The involvement of oxidative stress in tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in HeLa cells. Cancer Lett 2002;182:75–82.
28. Burkle A. PARP-1: a regulator of genomic stability linked with mammalian longevity. Chembiochem 2001; 2:725–8.
29. Wesierska-Gadek J, Schloffer D, Kotala V, Horky M. Escape of p53 protein from E6-mediated degradation in HeLa cells after cisplatin therapy. Int J Cancer 2002; 101:128–36.
30. Nicholls PK, Stanley MA. Canine papillomavirus—a centenary review. J Comp Pathol 1999;120:219–33.