Artemisinin: An Alternative Treatment for Oral Squamous Cell Carcinoma

EIKI YAMACHIKA[1,2], TEMESGEN HABTE[1] and DOLPHINE ODA[1]

[1]*Department of Oral and Maxillofacial Surgery, School of Dentistry,*
University of Washington, Box 357134, Seattle, WA 98195-7134, U.S.A.;
[2]*Department of Oral and Maxillofacial Reconstructive Surgery,*
Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho Okayama City 700-8525, Japan

Abstract. *Artemisinin (AR) is a widely used antimalarial drug. Recently, additional uses for AR as an anticancer drug were discovered. Using TUNEL, immunohistochemistry (IHS) markers and flow cytometry techniques, we evaluated the effect of AR and 5-FU on HPV 16 immortalized and transformed human gingival epithelial (IHGK) cells. The results of TUNEL showed that AR-treated IHGK cells consisted of 82% positive cells, while 5-FU-treated cells consisted of 18% positive cells. The IHS markers demonstrated positive staining with Bax, p53, CD40 and CD40L in AR-treated cells and negative staining with Bcl-2. 5-FU-treated cells demonstrated a profile similar to AR but with less intensity. Cell cycle by flow cytometry results showed that only 5-FU-treated cells demonstrated a significant S-phase rate increase to 45%. In conclusion, our results indicate that AR is cytotoxic to transformed oral epithelial cells through apoptosis, while 5-FU is cytotoxic primarily through cell toxicity.*

Artemisinin (AR) is a sesquiterpene lactone isolated from the plant *Artemisia annua* L. It is currently used in various countries as an antimalarial drug and it has a potent effect on choloroquine-resistant malarial parasites (1). The cancer cell cytotoxicity of AR was first reported by Woerdenbag *et al.* in 1993 (2). Using the microculture tetrazolium assay, they demonstrated that AR had an IC_{50} value of 29.8 μM on Ehrlich ascites tumor cells (2). Lai and Singh later demonstrated that 200 μM treatment over an 8-hour period using dihydroartemisinin (DAR) reduced the cell number of cultured molt-4 lymphoblastoid cells by 50% (3). Singh *et al.* further demonstrated a 28% reduction in breast cancer

Correspondence to: Dolphine Oda, Department of Oral and Maxillofacial Surgery, School of Dentistry, University of Washington, Box 357134, Seattle, WA 98195-7134, U.S.A. Tel: +1-206-616-4748, Fax: +1-206-685-7222, e-mail: doda@u.washington.edu

Key Words: Artemisinin, oral squamous cell carcinoma.

cells after 16-hour exposure to 200 μM of DAR (4). Recently, other derivatives of AR were tested *in vitro* and found to have cytotoxicity against leukemia, ovarian cancer cells and non-small cell lung cancer cells (5).

Squamous cell carcinoma is the most common malignant neoplasm in the oral cavity and tends to be aggressive if not discovered early. Tobacco and alcohol are the most common etiologies of this disease. However, human papillomavirus (HPV) has been implicated as a significant etiology, especially in younger and non-smoking patients. HPV type 16 is the most frequently identified type of HPV in oral squamous cell carcinoma (6, 7). Our laboratory successfully infected normal oral epithelial cells with the HPV 16 E6/E7 gene (IHGK), which led to immortalization of the normal cells. The cell line was passaged more than 380 times, as opposed to normal cells, which senesce and die at 7-9 passages (8, 9). In addition to immortalization, this line has consistently demonstrated histological features of carcinoma *in situ* and invasion (Figure 1B) of the underlying matrix using a three-dimensional organotypic culture system (9). Based on our studies, we believe that the IHGK cell line is a useful model for oral squamous cell carcinoma and we chose to use it in this manner.

The treatment of choice for oral squamous cell carcinoma is surgery. However, surgery with radiation and/or chemotherapy, or chemotherapy alone, is used at times. 5-Fluorouracil (5-FU) is a chemotherapeutic agent commonly used in conjunction with other agents such as cisplatin and methotrexate (10). 5-FU alone or in combination with other drug(s) is used to treat cancer in a variety of sites, including breast, cervix, ovaries, gastrointestinal tract and head and neck (11). Chemotherapy, regardless of the malignancy being treated, is always associated with unpleasantness and often causes serious complications and side-effects. Therefore, alternative chemotherapy with minimal or no side-effects should be carefully considered. AR is a relatively safe drug with few side-effects even at high doses (1, 12). It has no identifiable dose-related adverse effects in
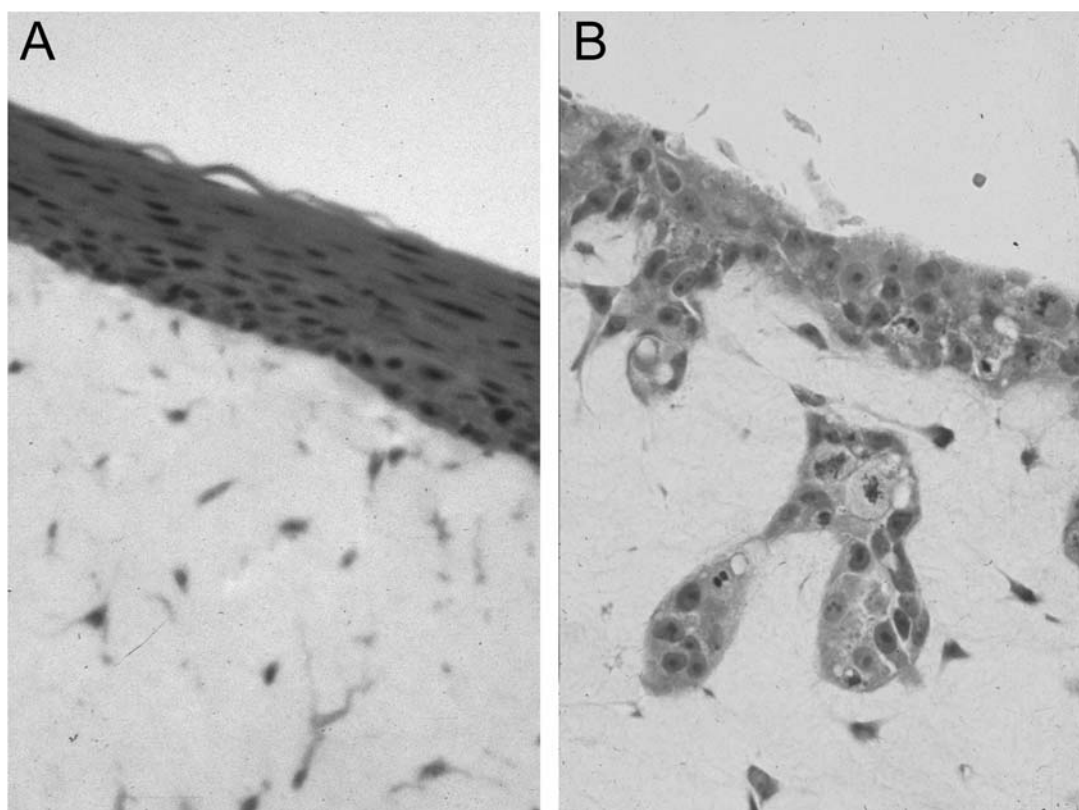
Figure 1. *The hematoxylin and eosin-stained section of normal (A) and IHGK cells (B) grown by organotypic culture system. Note the cellular and nuclear pleomorphism, prominent nucleoli and invasion of the matrix in IHGK cells (B).*

humans and only very rarely produces allergic reactions (13). The effect of 5-FU on cultured oral epithelial cells has been reported by our laboratory previously (14). This study is designed to focus on the effect of AR on oral malignant epithelial cells with 5-FU used as a control.

Materials and Methods

Cell culture. All experiments were performed using an IHGK cell line (Figure 1A and B). The cells were cultured in a Keratinocyte-SFM (Invitrogen Co., Carlsbad, CA, USA) with 4mM L-glutamine (Invitrogen Co.) and 1% Antibiotics/Antimycotic (Invitrogen Co.) at 37˚C in a 5% CO_2 air atmosphere and fed every 48 hours. The cells were seeded at approximately 1.5×10^5 cells/ml in 10ml medium using 75-cm^2 cell culture flasks (Corning Inc. Corning, NY, USA), or in 1ml medium using 1-cm^2 tissue culture chamber slides (Nalge Nunc International, Naperville, IL, USA).

Dose response. Time- and dose-response experiments for DAR (Calbiochem, San Diego, CA, USA) and 5-FU (Sigma, St. Louis, MO, USA) were performed. DAR was administered in concentrations ranging between 50 μM and 400 μM and 5-FU between 15 mM and 30 mM. Treatment periods were planned ranging from 6 to 96 hours.

TUNEL analysis for apoptosis. For the TUNEL analysis, IHGK cells with and without DAR and 5-FU were cultured in 1-cm^2 chamber slides at 1×10^4 cells per well for 24 hours. The cells were fixed with 4% paraformaldehyde at room temperature for one hour. They were then treated with a TUNEL mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP. Following this treatment, the fluorescein was detected by the anti-florescein antibody conjugated with alkaline phosphatase (*In situ* cell death detection Kit AP, Mannheim, Germany). The substrate reaction for alkaline phosphatase was performed by Alkaline phosphatase substrate kit I (Vector Laboratories, Inc., Burlingame, CA, USA). Cells were evaluated using Olympus BH-2 light microscopy.

Immunohistochemistry and antibodies. For the immunohistochemistry studies, cells were cultured in 1-cm^2 chamber slides similar to those used for TUNEL. Staining was performed using the avidin-biotin methods (ABC-Elite Kit; Vector). Five primary antibodies were used in this study: mouse monoclonal antibody to Bcl-2 (1:100 Santa Cruz, CA, USA sc-7382), mouse monoclonal antibody to p53 (1:500 Santa Cruz sc-263), rabbit polyclonal antibody to Bax (1:100 Santa Cruz sc-526), rabbit polyclonal antibody to CD40 (1:100 Santa Cruz sc-974) and rabbit polyclonal antibody to CD40L (1:400 Santa Cruz sc-978). After the treatment with 2 mM DAB, cells were evaluated using an Olympus BH-2 light microscopy.
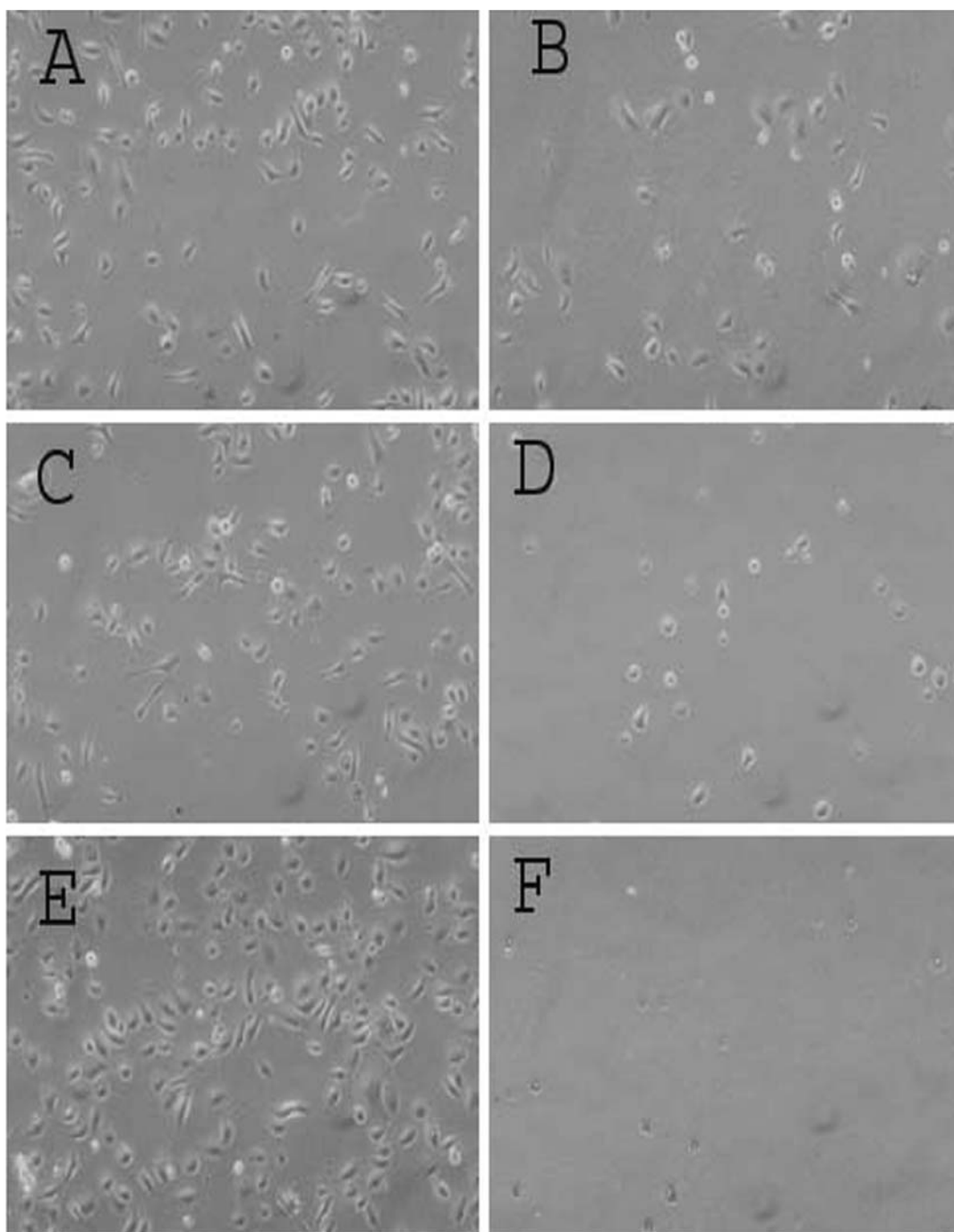
Figure 2. *Cell culture. (A) 8 h. Control, (B) 8 h. 400 mM DAR-treated, (C) 24 h. Control (D) 24 h. 400 μM DAR-treated, (E) 48 h. Control, (F) 48 h. 400 mM DAR-treated.*

Cell cycle distributions. The cell cycle studies were performed using a DNA-specific dye, 4,6-diamidino-2-phenylindole (DAPI), for flow cytometry as described by Rabinovitch (15). A total of five groups of IHGK cells were used. Two were treated with DAR, two with 5-FU and one was in medium alone as a control. The DAR-treated cells were at 200 μM and 400 μM and the 5-FU-treated cells were at 15

mM and 30 mM. They were all incubated at 37˚C for 24 hours. After treatment, about two million cells per group were trypsinized, centrifuged and re-suspended in ice-cold DAPI solution and analyzed using a Coulter ELITE cytometer (Coulter Corp, Maiami, FL, USA). DNA content and cell cycle were analyzed using the MultiCycle software program (Phoenix Flow System, San Diego, CA, USA).
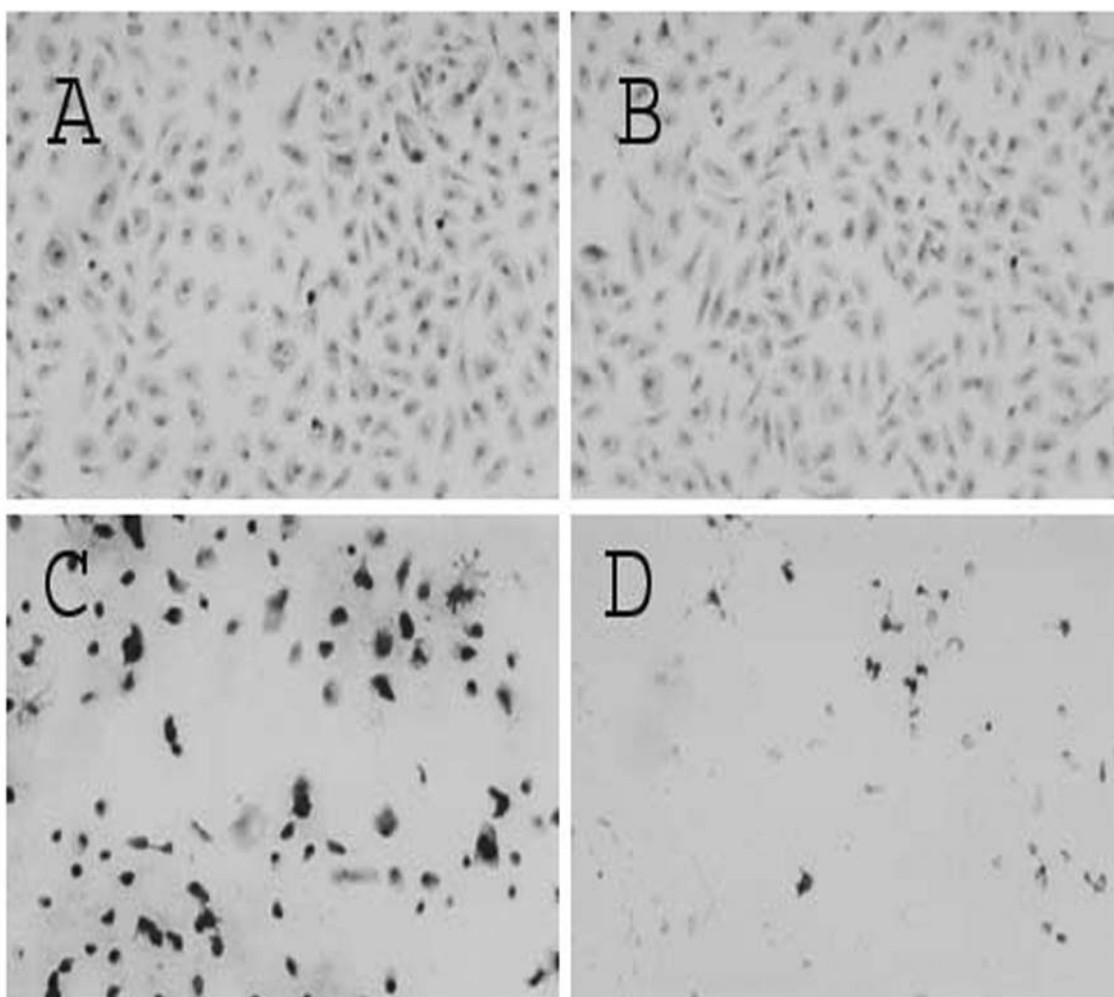
Figure 3. *(A) 24 h. Control, (B) 24 hr. Control, (C) 24 h. 400 μM DAR-treated, (D) 24 h. 15 mM 5-FU-treated.*

Results

Dose-response. A time- and dose-response for DAR was performed using the IHGK cells. As early as 8 hours after treatment with 400 μM of DAR, IHGK cells demonstrated evidence of detachment from the culture dish compared to the medium alone control cells (Figure 2 A and B). The cell cytolysis was both time- and dose-dependent. The cell death with 200 μM DAR concentration was less evident after 8 hours of treatment and slightly evident after 24 hours of treatment. However, with 400 μM of DAR almost 40% of the cells were dead by 24 hours and 63% by 48 hours (Figure 2 C, D, E, F). Morphologically, dead and dying cells appeared uniformly small, spherical in shape, golden in color and detached from the culture dishes when compared to the normal control cells, which were more cuboidal and flat (Figure 2 A, C, E). 5-FU-treated cells, on the other hand, demonstrated clear cytotoxic changes at the early stages of 8 and 16 hours, followed by 85% reduction after 24 hours of treatment. None of the cells survived 5-FU treatment at 48 hours. Based on our data, the optimal dose and treatment times for complete elimination of these cells are 400 μM over a 24-hour period for DAR and 15 mM over a 24-hour period for 5-FU.

TUNEL analysis. To differentiate between apoptosis and necrosis, we used TUNEL to stain cells treated separately with DAR and 5-FU at doses of 400 μM and 15 mM, respectively. The cells were washed and stained using the TUNEL technique as described above. The DAR-treated cells demonstrated 82% positive staining with TUNEL (Figure 3C) while only 18% of the 5-FU-treated IHGK cells
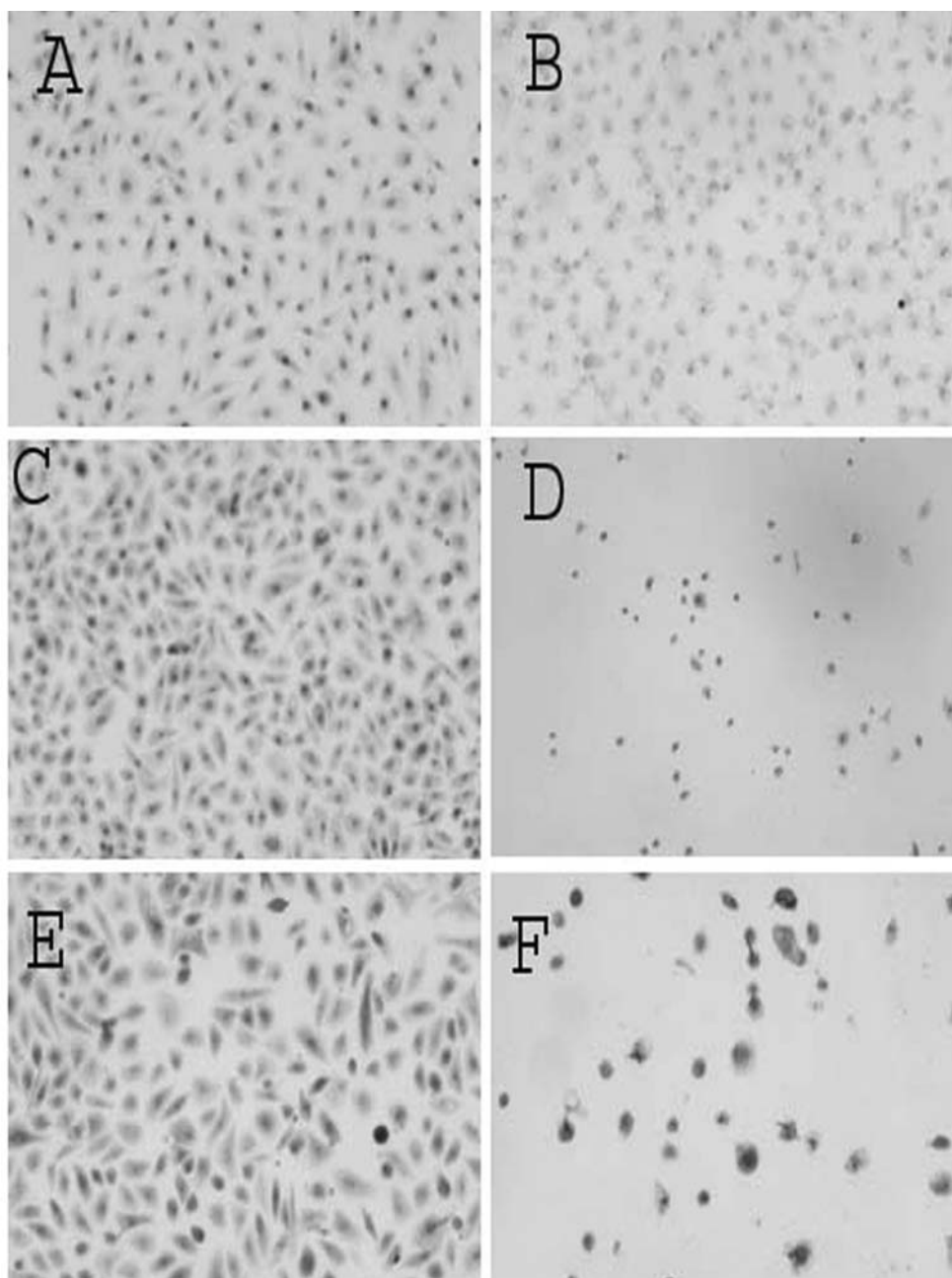
Figure 4. *Bcl-2, p53, Bax immunohistochemical analysis. (A) Bcl-2 - Control 24 h (B) Bcl-2 - 24 h 400 mM DAR-treated (C) p53 - Control 24 h (D) p53-24 h 400 mM DAR-treated (E) Bax-Control 24 h (F) Bax-24 h 400 mM DAR-treated.*

were positive (Figure 3D). Medium-alone control cells demonstrated about 1% positive cells (Figure 3A and B).

Bcl-2, p53, Bax, CD40, CD40L immunohistochemical analysis. DAR-, 5FU-treated cells and control IHGK cells were stained by immunohistochemistry with antibodies directed to apoptosis to determine the process of cell death. Control IHGK cells were focally-positive for Bcl-2 (Figure 4A), while the DAR-treated cells were negative (Figure 4B). The tumor suppressor gene p53 antibody was strongly and uniformly positive, affecting 85% of DAR-treated cells (Figure 4D) compared to the focally-positive control cells (Figure 4C). Like p53, Bax was strongly and uniformly positive, affecting 78% of the DAR-treated cells (Figure 4F) compared to 16% positive control
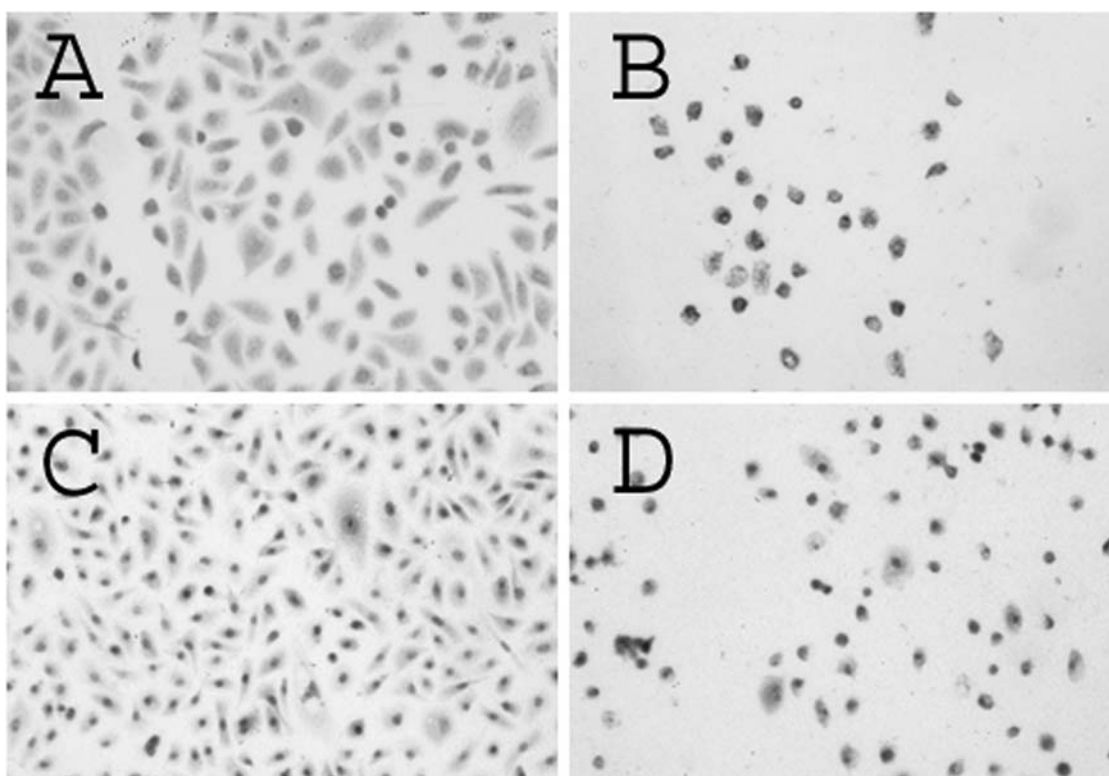
Figure 5. *CD40, CD40L immunohistochemical analysis. (A) CD40 - Control (B) CD40 – 400 μM DAR-treated (C) CD40L - Control. (D) CD40L – 400 μM DAR-treated.*

cells (Figure 4E). Both control and DAR treated cells were uniformly positive to the antibodies to ligands CD40 and CD40L. However, both antibodies were slightly more positive in the treated cells (Figure 5 A-D).

Cell cycle distributions. Flow cytometry analysis showed that cells treated with DAR and 5-FU varied in their cell cycle profile. The proportions of cells in the G1-, S- and G2-phases after 24 hours of treatment compared to the control cells are displayed in Figure 6. The cell cycle profile of the control cells was very similar to the cells treated with 400 μM DAR. Although the S-phase proportion among the DAR-treated cells was slightly more than that of the control cells (21% *vs.* 16%), the difference was not statistically significant. The 5-FU-treated cells, on the other hand, demonstrated a significant decrease in the number of cells in the G1- and G2-phase and a substantial increase in S-phase cells: 45.8% compared to 16% in the control group.

Discussion

Oral squamous cell carcinoma presents a significant health problem that affects over half a million people in the world each year (16). Treatment has traditionally consisted of surgery, with radiation and chemotherapy used as supportive measures (17). Recent advances in the understanding of chemotherapy have produced new strategies for the use of chemotherapeutic agents in a primary role. Some studies suggest that in 40% of patients with head and neck cancer, including oral cancer, chemotherapy followed by radiation therapy produces results comparable to treatment with surgery and radiation therapy (18). 5-FU is a commonly used chemotherapeutic agent (19), typically used in combination with cisplatin (20). However, conventional chemotherapy agents have been associated with numerous significant clinical complications including nausea, hair loss and pancytopenia, which makes alternative and less toxic chemical treatment of oral cancer highly desirable.

AR has been shown, through its wide use as an antimalarial drug, to be a non-toxic chemical with minimal or no side-effects. One clinical case report demonstrating the effectiveness of AR on laryngeal cancer has been reported in which a 71-year-old male from India with stage II well-differentiated squamous cell carcinoma responded with 30% reduction in the size of the lesion after two weeks of treatment with 60 mg of artesunate, an AR analog given intramuscularly (21). AR has been shown in both *in vitro* (22,23) and in animal models (24) to be cytotoxic to many malignant neoplasms.
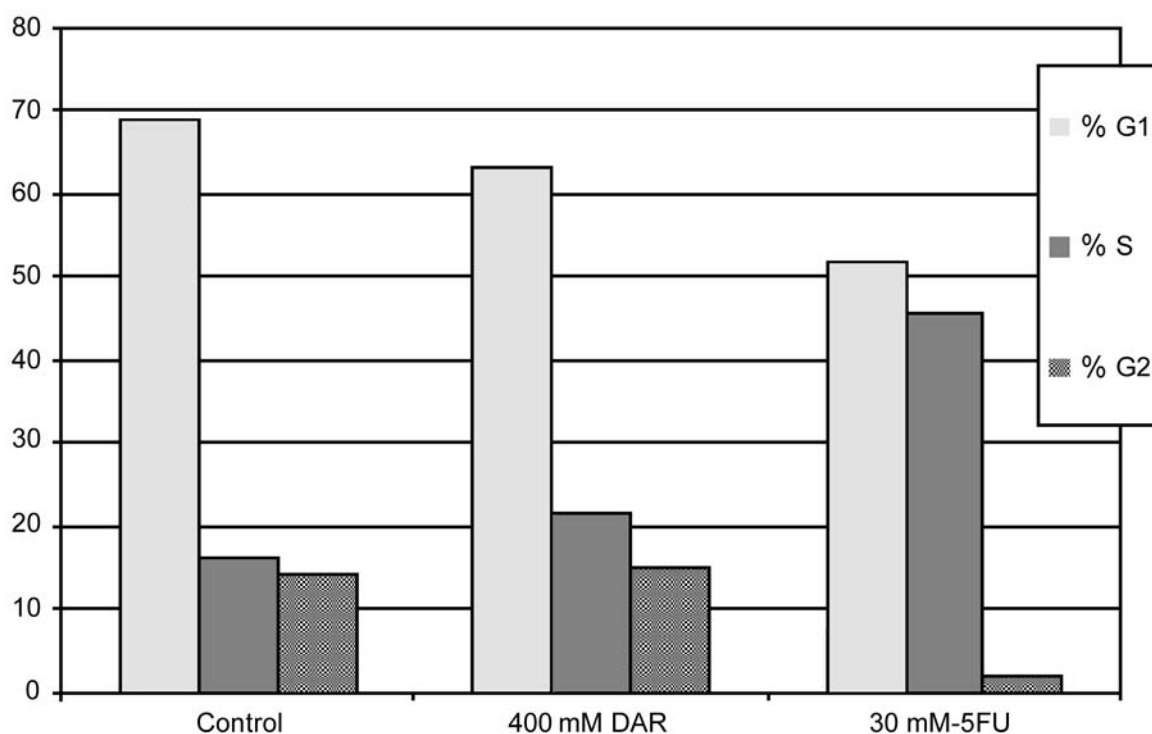
Figure 6. *Cell cycle distribution.*

Based on the cell number and morphology (Figure 2), our data demonstrate that DAR is cytotoxic to the IHGK cells; furthermore, we show that the effect of DAR is dependent on both dose and time. Our results also show that the effect of DAR on the IHGK cells was not as toxic as that of 5-FU: a small dose of 5-FU lysed 85% of the cells in a 24-hour period, compared to 40% cell death with 400 μM DAR in 24 hours and 63% in 48 hours. It is necessary to state that 15 mM 5-FU is less than the optimum dose of treatment for oral squamous cell carcinoma (14). The less intense cytotoxicity of DAR is also supported by the cell cycle distribution results (Figure 6), in which 45.8% of cells treated with 5-FU were in S-phase while only 21% of the DAR-treated cells were. The high rate of cells in S-phase is an indication of cell DNA damage, leading to increased cell proliferation.

The results of the TUNEL analysis clearly demonstrate that DAR-treated IHGK cells die by apoptosis. The low positive score (18%) of the 5-FU-treated cells is an indication that 5-FU kills malignant cells mostly by cell necrosis and partly by apoptosis. The high positive score (82%) in the DAR-treated cells is a clear indication that artemisinin kills cells through apoptosis.

To confirm the apoptotic effect of DAR using an immunohistochemistry technique, we stained treated and control cells with five antibodies: Bcl-2, p53, Bax, CD40 and CD40L. The p53 tumor suppressor gene has been reported to induce the up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 (25). Increased Bax and lowered Bcl-2 expression has been shown to reduce mitochondrial membrane potential (26), which has been defined as the early event in the process of apoptosis (27). It has been demonstrated that higher levels of p53 expression are necessary for the induction of apoptosis (28, 29), and CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily (30). It has been previously shown that, in common with other members of the TNFR family, CD40 stimulation sensitizes carcinoma cells *in vitro* to apoptosis induced by cytotoxic chemotherapy (31). CD40 ligand (CD40L), also known as CD154, functions as the natural ligand for CD40 (30, 32, 33). Our immunohistochemical staining results demonstrate an increased expression of p53 and Bax and a decreased expression of Bcl-2 after treatment with DAR. These results support our hypothesis that DAR kills IHGK cells by apoptosis. The increased expression of CD40 and CD40L in the DAR-treated cells also corroborates the apoptotic effect of DAR.

In conclusion, our results strongly suggest that DAR is cytotoxic to oral malignant epithelial cells and that it kills these cells by apoptosis rather than by necrosis, as is the case with the widely used chemotherapy agent 5-FU. It is also clear that DAR is both time- and dose-dependent. Our findings have potential clinical applications for the chemotherapeutic treatment of oral squamous cell carcinoma.

References

1 Klayman DL: Qinghaosu (artemisinin): an antimalarial drug from China. Science *228*: 1049-1055, 1985.

2 Woerdengab HJ, Moskal TA, Pras N, Malingre Th M, Elferaly FS, Kampinga HH and Konings AWT: Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumour cells. J Nat Prod *56*: 849-856, 1993.

3 Lai H and Singh NP: Selective cancer cell cytotoxicity from exposure to dihydroartemisinin and holotransferrin. Cancer Lett *91*: 41-46, 1995.

4 Singh NP and Lai H: Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. Life Sci *70*: 49-56, 2001.

5 Galal AM, Ross SA, ElSohly MA, ElSohly HN, El-Feraly FS, Ahmed MS and McPhail AT: Deoxyartemisinin derivatives from photooxygenation of anhydrodeoxydihydroartemisinin and their cytotoxic evatuation. J Nat Prod *65*: 184-188, 2002.

6 Syrjanen SM, Syrjanen KJ and Happonen RP: Human papillomavirus (HPV) DNA sequences in oral precancerous lesions and squamous cell carcinoma demonstrated by *in situ* hybridization. J Oral Pathol *17*: 273-278, 1988.

7 Kiyabu MT, Shibata D, Arnheim N, Martin WJ and Fitzgibbons PL: Detection of human papillomavirus in formalin-fixed, invasive squamous carcinomas using the polymerase chain reaction. Am J Surg Pathol *13*: 221-224, 1989.

8 Oda D, Bigler L, Lee P and Blanton R: HPV immortalization of human oral epithelial cells: a model for carcinogenesis. Exp Cell Res *226*: 164-169, 1996.

9 Oda D, Bigler L and Disteche C: Chromosomal abnormalities in HPV 16-immortalized human oral epithelial cells. Carcinogenesis *17*: 2003-2008, 1996.

10 Merlano M, Benasso M, Cavallari M, Blengio F and Rosso M: Chemotherapy in head and neck cancer. Eur J Cancer B Oral Oncol *30B*: 283-289, 1994.

11 Perry MC editor: The Chemotherapy Source Book. 2nd ed. Baltimore, Williams and Wilkins, 1996, pp 318-325.

12 Hien TT and White NJ: Qinghaosu. Lancet *341*: 603-608, 1983.

13 Taylor WR and White NJ: Antimalarial drug toxicity: a review. Drug Saf *27(1)*: 25-61, 2004.

14 Tong D, Poot M, Hu D and Oda D: 5-Fluorouracil-induced apoptosis in cultured oral cancer cells. Oral Oncol *36*: 236-241, 2000.

15 Rabinovitch P: DNA content histogram and cell cycle analysis. Methods Cell Biol *41*: 127-130, 1994.

16 Parkin DM, Pisani P and Ferlay J: Estimates of the worldwide incidence of 18 major cancers in 1985. Int J Cancer *54*: 594-606, 1993.

17 McAndrew PG: Oral cancer and precancer: treatment. Br Dent J *168*: 191-198, 1990.

18 Posner MR and Colevas AD: Induction chemotherapy in the management of squamous cell cancer of the head and neck. Cancer J Scient Amer *3*: 73-75, 1997.

19 MacConald JS ed: Manual of Oncologic Therapeutics. 3rd ed. Philadelphia, JB Lippincott, 1991, pp111-113.

20 Merlano M, Benasso M, Cavallari M, Blengio F and Rosso M: Chemotherapy in head and neck cancer. Eur J Cancer B Oral Oncol *30B*: 283-289, 1994.

21 Singh NP and Verma KB: Case report of a laryngeal squamous cell carcinoma treated with artesunate. Arch Oncol *10(4)*: 279-280, 2002.

22 Jeyadevan JP, Bray PG, Chadwick J, Mercer AE, Byrne A, Ward SA, Park BK, Williams DP, Cosstick R, Davies J, Higson AP, Irving E, Posner GH and O'Neill PM: Antimalarial and antitumor evaluation of novel C-10 non-acetal dimers of 10beta-(2-Hydroxyethyl)deoxoartemisinin. J Med Chem *47(5)*: 1290-1298, 2004.

23 Chen HH, Zhou HJ and Fang X: Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives *in vitro*. Pharmacol Res *48(3)*: 231-236, 2003.

24 Moore JC, Lai H, Li JR, Ren RL, McDougall JA, Singh NP and Chou CK: Oral administration of dihydroartemisinin and ferrous sulfate retarded implanted fibrosarcoma growth in the rat. Cancer Lett *98(1)*: 83-87, 1995.

25 Iwata E, Asanuma M, Nishibayashi S, Kondo Y and Ogawa N: Different effects of oxidative stress on activation of transcription factors in primary cultured rat neuronal and glial cells. Brain Res Mol Brain Res *50*: 213-220, 1997.

26 Pastrorino JG, Chen ST, Tafani M, Synder JW and Faber JL: The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J Biol Chem *273*: 7770-7775, 1998.

27 Levine AJ: p53 the cellular gatekeeper for growth and division. Cell *88*: 323-331, 1997.

28 Crochemore C, Michaelidis TM, Fisher D, Loeffler JP and Almedia OF: Enhancement of p53 activity and inhibition of neural cell proliferation by glucocorticoid receptor activation. FASEB J *16*: 761-770, 2002.

29 Chen X, Ko LJ, Jayaraman L and Prives C: p53 levels, functional domains and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev *10*: 2438-2451, 1996.

30 Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F and Saeland S: The CD40 antigen and its ligand. Annu Rev Immunol *12*: 881-922, 1994.

31 Eliopoulos AG, Dawson CW, Mosialos G, Floettmann JE, Rowe M, Armitage RJ, Dawson J, Zapata JM, Kerr DJ, Wakelam MJ, Reed JC, Kieff E and Young LS: CD40-induced growth inhibition in epithelial cells is mimicked by Epstein–Barr virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene *13*: 2243-2254, 1996.

32 Spriggs MK, Armitage RJ, Strockbine L, Clifford KN, Macduff BM, Sato TA, Maliszewski CR and Fanslow WC: Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J Exp Med *176*: 1543-1550, 1992.

33 Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, Anderson DM, Gimpel SD, Davis-Smith T and Maliszewski CR: Molecular and biological characterization of a murine ligand for CD40. Nature *357*: 80-82, 1992.