Selective Induction of Apoptosis of Human Oral Cancer Cell Lines by Avocado Extracts Via a ROS-Mediated Mechanism

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INTRODUCTION

Cancer is the second leading cause of death in the United States, and cancer of the oral cavity is one of the 10 most common forms representing 2 to 3% of all cancers in men and women (1,2). Ninety percent of these cancers are squamous cell carcinomas, and the prognosis is poor, with an overall mortality rate of approximately 50%. Because current therapeutic protocols are relatively ineffective for oral cancer, it is important to identify and develop chemopreventive strategies to prevent this disease. The consumption of fruits and vegetables has been associated with a reduced risk for many different types of human cancer, including cancers of the oral cavity and pharynx (3–5). The protective effect of fruits and vegetables is thought to be related to multiple anticancer phytoneutrients. Hass avocado, *Persea americana* Mill. (Lauraceae), a widely consumed fruit, has been reported to exhibit medicinal effects toward many diseases including cancer (6). The health benefits of avocado may be due to its content of essential nutrients, monounsaturated and polyunsaturated fats, and potentially cancer-preventing phytochemicals (6). Additionally, avocados are devoid of sodium and are relatively low in calories, carbohydrates, and saturated fats. Although the health benefits of avocados have been known for many years, the cellular and molecular mechanisms of the phytochemicals responsible for cancer prevention are largely unknown. Studies by us and other research groups have shown that phytochemicals extracted from the avocado meat inhibit cancer cell growth (6–8). Among these many mechanisms, apoptosis appears as an important target by avocado phytochemicals.
in selectively eliminating cancer cells from normal tissues (6,9).

Our previous published data showed that a chloroform extract (D003) of phytoneutrients from the avocado meat selectively inhibited premalignant and malignant human oral cell growth and induced apoptosis (6). We further showed that this avocado extract increased the already high levels of reactive oxygen species (ROS) and induced apoptosis in the cancer-derived cell lines while not affecting the normal oral cell lines. The selective toxicity and its potency suggested further investigation into the underlying mechanisms disrupting the cellular balance of ROS leading to apoptosis. In the present study, we showed that the D003 extract initiates apoptosis via ROS activating both the intrinsic and extrinsic pathways.

MATERIALS AND METHODS

Human Oral Cell Lines

Normal (TE1177) and malignant (83-01-82CA) human oral epithelial cell lines have been previously described (10–13). The 83-01-82CA/GFP and 83-01-82CA/GFP/FADD-DN cell lines were established by transfecting the 83-01-82CA cell line with a plasmid containing dominant negative FADD (FADD-DN) or control vector according to a published procedure (14). The 83-01-82CA and transfected cell lines were maintained at 37°C in MEM containing 10% fetal bovine serum (HyClone, Logan, UT), 5 U/ml penicillin, and 5 µg/ml streptomycin. HPV16 E6 and E7 transformed TE1177 cell lines were described previously (15). The TE1177 and transformed cell lines were maintained at 37°C in an alpha modified MEM supplemented with epithelial growth factor, insulin, transferrin, hydrocortisone, and 10% FBS. Cells were seeded on the first day and treated from the second day for 3–24 h for apoptosis and 24 h for cytotoxicity assay.

Extracts and Reagents

Hass avocado fruit (Persea americana) was kindly provided by California Avocado Commission. Extracts were prepared from avocado meat following a previously published plant extraction scheme (16,17). Two different batches (b1 and b2) of avocados were used in these studies. There was a slight variation (15%) in the cellular and molecular responses to the D003 extracts, and concentrations were adjusted to 30 (D003b1) and 35 (D003b2) µg/ml to obtain the same end points in the cell lines. Because the normal cell line, TE1177, was resistant to D003, higher concentrations (30–100 µg/ml) were used for a comparison of sensitivity between normal TE1177 and HVP 16 E6- or E7-transformed TE1177 cell lines. JC-1 (50 µg/ml) was obtained from Molecular Probes (Eugene, OR). N-Acetyl-L-cysteine (NAC; 0–5 mM), thenoyltrifluoroacetone (TTFA; 10–50 µM), antimycin A (10–50 µM), rotenone (1–5 µM), and valinomycin (3.3 µM) were purchased from Sigma Chemical Co. (St. Louis, MO). Dichlorofluorescein diacetate (DCF-DA; 10 µM), z-IETD-fmk (caspase 8 inhibitor; 60 µM), z-LHTD-fmk (caspase 9 inhibitor, 80 µM), z-VAD-fmk (pancaspase inhibitor, 20 µM) were purchased from Calbiochem (San Diego, CA).

MTT Assay

Briefly, cells were treated in 96-well plates for 24 h with D003 extract. If required, 1 to 5 mM NAC was added 1 h before incubation with D003. Cells were then incubated with 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide for 1 to 4 h before harvesting (18). The color was measured in a microtiter plate reader. The relative number of cells was determined by comparing treated cells to solvent control cells as percent of control growth as (Abtreated/Abcontrol)× 100, where Ab represents the mean absorbance.

ROS Detection

Intracellular hydrogen peroxide and superoxide anion production were determined by flow cytometry using a fluorescent probe, 2′,7′-dichlorofluoresceindiacetate (DCFH-DA). H2O2 converts nonfluorescent DCFH to fluorescent DCF. Cells (3 × 103) were incubated with 1 to 5 mM NAC for 1 h or 1 to 2.5 µM rotenone for 30 min, D003 was added, and incubation continued for 3 h. Cells were then incubated with 10 µM DCF-DA for 30 min at 37°C before harvest and analyzed for fluorescence intensity using FACS Caliber (Becton-Dickinson, Rutherford, NJ) at the excitation and emission wavelengths of 488 nm and 538 nm. The median fluorescence intensity (Fl-2) was measured by CellQuest software (Becton-Dickinson) analysis of the recorded histograms.

Mitochondria Membrane Potential (MMP) Assay

Cells were processed as described previously (19). Briefly, cells (3 × 105) were treated with D003 for 24 h. As indicated, cells were preincubated with 1 to 5 mM NAC for 1 h or 1 to 2.5 µM rotenone for 30 min before addition of D003. JC-1 was added to the medium during the final 20 min of incubation at a final concentration of 50 µg/ml. Valinomycin at 3.3 µM was used as a positive control. After incubation, the medium was replaced with pre-warmed PBS. Cells were analyzed using FACS Caliber flow cytometer. The red (J-aggregates) and green (JC-1) fluorescence intensities on the FL-1 and FL-2 channels, respectively, were recorded. All samples were prepared in triplicate.

Western Blot Analysis

After treatment of cells, proteins were harvested and processed according to our previously published protocol (18). Briefly, cells were lysed in a SDS buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. Protein concentration was determined using the Bio-Rad assay kit (Bio-Rad, Hercules, CA). Equal amounts (30–50 µg) of protein were subjected to gel electrophoresis in 12% polyacrylamide slab gels (Invitrogen, Carlsbad, CA) in the presence of SDS. The protein
was electrophoretically transferred to a nitrocellulose membrane and dried. The membrane was preincubated with blocking buffer (5% nonfat milk, 500 mM NaCl, 100 mM Tris, and 0.1% Tween-20) and then incubated with primary antibody at room temperature for 1 h. The blots were probed with primary antibody specific for the following proteins: PARP; Asp 214, 19F4, 1:500 for cleaved band), caspase 3 (Asp 175, 1:500, for cleaved bands), and caspase 9 (1:500) from Cell Signaling Technology (Boston, MA); PARP (H-250, 1:500 for both full and cleaved band), caspase 3 (H-277, for both full and cleaved bands), caspase 8 (p20, C20, 1:500), and Lamin B (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA); and α-tubulin (Ab-1, 1:1000) from Oncogene Research Products (Darmstadt, Germany). After washing the membrane 3 times with TBS-T (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20) for 10 min, the membrane was incubated with mouse or rabbit secondary antibody labeled with horseradish peroxidase (HRP) at room temperature for 1 h. Western blots were developed using a chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was then exposed to X-ray film. α-Tubulin or Lamin B was detected on the same membrane and used as a loading control.

Statistical Analyses
Each experiment was repeated 3 to 4 times as indicated in the figure legends. Where appropriate, the data are expressed as the mean ± standard error of the mean (SEM) and were analyzed for significance using the SPSS software Student’s t-test. A P < 0.05 was considered to be statistically significant.

RESULTS

Avocado Extract D003 Induces Apoptosis by Activating Both Intrinsic and Extrinsic Pathways

In a previous study, we showed that avocado D003 extract selectively induced apoptosis in premalignant and malignant human oral cell lines (6). Consistent with our previous study, treatment of malignant human oral epithelial cell line 83-01-82CA with D003 resulted in apoptosis as demonstrated by PARP and caspase 3 cleavages at a concentration ≥30 μg/ml for 24 h (Fig. 1A). In contrast, the same batch and same concentration of D003 extract did not induce apoptosis of normal human oral epithelial cell line TE1177 even after 48-h treatment (Fig. 1B). To further investigate underlying mechanisms involved in D003-induced apoptosis, the 83-01-82CA cell line was treated with 30 μg/ml D003 extract for various times. As shown in Fig. 2A, D003 extract induced apoptosis following 12- and 18-h treatments as evidenced by cleavage of PARP and caspase 3. Caspases 8 and 9, upstream activators of caspase 3 and PARP, were activated, albeit weakly, as early as 6 h, with full activation at 12 and 18 h of treatment (Fig. 2A).

To explore the involvement of caspases in D003-induced apoptosis, cells were treated with either the z-VAD, a pan caspase inhibitor (data not shown), z-IETD-fmk, a caspase 8 specific inhibitor, or z-LHTD-fmk, a caspase 9 specific inhibitor (Fig. 2B). Pan-caspase inhibitor VAD completely abolished D003-induced PARP and caspase 3 cleavages (data not shown). When cells were treated in the presence of z-IETD-fmk, as expected, the cleavage of caspase 8 induced by D003 was completely inhibited. The inhibition of the cleavage of caspase 9 by this inhibitor suggests that caspase 8 may be upstream activator of caspase 9 (20). The z-LHTD-fmk inhibitor completely blocked, as expected, the cleavage of caspase 9 induced by D003. Caspase 8 cleavage was also partially inhibited probably due to caspase 9, indirectly via caspase 3, activating caspase 8 (21). Both of these inhibitors decreased the levels of the cleaved active 17 Kd protein band while increasing cleaved inactive 19 Kd band of caspase 3, confirming that caspase 3 requires activation of upstream both caspases 8 and 9. These data suggest that D003 activates both the intrinsic (caspase 9) and extrinsic (caspase 8) pathways leading to activation of caspase 3 and apoptosis in human oral cell cancer lines. To further determine the involvement of the intrinsic pathway, Bcl2 family member proteins were measured by Western blot after treatment of the 83-01-82CA cell line with D003. As shown in Fig. 2C, proapoptotic members Bad and Bak decreased and proapoptotic Bax and proapoptotic members Bad and Bak decreased and proapoptotic Bax and
FIG. 2. Involvement of both intrinsic and extrinsic pathways in D003 extract induced apoptosis. A: The malignant human oral epithelial cell line 83-01-82CA was treated with 30 µg/ml D003b1 extract for the indicated times. The cleaved protein fragments of PARP and caspase 3, caspase 8, and caspase 9 were determined by Western blot. B: The 83-01-82CA cell line was pretreated with the caspase 8 inhibitor z-IETD-fmk (60 µM) or the caspase 9 inhibitor z-LHTD-fmk (80 µM) for 1 h prior to treatment of cells with 30 and 35 µg/ml D003b2 extract for 18 h. Cleavage of PARP and caspases 3, 8, and 9 is indicated by Western blot. C: The 83-01-82CA cell line was pretreated with D003b1 for 18 h. Protein was harvested and PARP and Bcl-2 family proteins determined by Western blot. D: The role of FADD in D003 extract-induced apoptosis. 83-01-82CA/GFP and 83-01-82CA/GFP/FADD-DN cell lines were treated with D003b2 for the indicated times. The cleaved bands of PARP and caspases 3, 8, and 9 were determined by Western blot. Bad, Bcl-2 antagonist of cell death; Bak, Bcl-2 homologous antagonist killer; Bax, Bcl-2-associated x protein; Bcl-xl, Bcl-extra large.

antia apoptotic Bcl2 and Bcl-xl were not altered by the treatment. These data suggest that these members may not contribute to D003-induced intrinsic pathway activation. To further define the involvement of the extrinsic pathway in D003 extract-induced apoptosis, the 83-01-82CA cell line was stably transfected with dominant negative FADD (CA/GFP/FADD-DN) plasmid. Figure 2D shows that FADD-DN delayed the cleavage of both caspase 3 and PARP, suggesting a possible role of FADD in the initiating stage of apoptosis. It is interesting that caspase 8 cleavage was not affected in the presence of FADD-DN, implying that caspase 8 may not be the direct mediator of the FADD signaling pathway in the activation of downstream caspase 3 in avocado extract D003-induced apoptosis. FADD-DN also did not affect caspase 9 activation.

ROS Accumulation Is Critical for Avocado Extract D003 Activation of the Intrinsic and Extrinsic Apoptotic Pathways

In our previous study, we observed that ROS is important to avocado D003 extract induced apoptosis (6). As shown in Fig. 3A, treatment with the D003 extract resulted in significant ROS accumulation in the 83-01-82CA cell line. Pretreatment of cells with 1 to 5 mM NAC, a ROS scavenger, attenuated the apoptotic response of ROS, measured at 3 h in a concentration-dependent (Fig. 3A) and in a time-dependent manner (data not shown). Furthermore, the presence of 1 to 2 mM NAC during treatment with D003 significantly abated D003-induced cytotoxicity even at the non-apoptotic concentrations of D003 (Fig. 3B). To further delineate the effect of modulating ROS levels, apoptotic pathways were determined after treatment with D003 in the presence of NAC. As shown in Fig. 3C, pretreatment of cells with 1 and 2.5 mM NAC completely abolished D003-induced cleavage of PARP, caspase 3, caspase 8, and caspase 9. Flow cytometric analyses showed that the reduction of ROS by NAC protected cells from D003-induced loss of mitochondrial potential, a marker of the intrinsic apoptotic pathway (Fig. 3D). Taken together, these data suggest that ROS plays an important role in D003 extract-induced cell death by activating both the intrinsic and extrinsic apoptotic pathways. Because the data indicate an association between D003 extract induced ROS and cytotoxicity and apoptosis, we measure the levels of ROS in the normal human oral epithelial cell line TE1177 following the D003 treatment. As shown in Fig. 4A, TE1177 cell line contains much lower basic levels of ROS compared to the 83-01-82CA cell line. D003 treatment only increased ROS levels by 1.3-fold at the highest concentration (30 µg/ml) compared to twofold to threefold in the 83-01-82CA cell line. This indicates that the low toxicity
FIG. 3. The effects of antioxidants on avocado extract D003-induced apoptosis and reactive oxygen species (ROS) generation in the 83-01-82CA cell line. A, NAC reduced ROS levels generated by D003. The 83-01-82CA cell line was pretreated with various concentrations of NAC for 1 h followed by treatment with 30 µg/ml of D003b1 for 3 h. DCF-DA (10 µM) was added 30 min before harvesting cells. ROS content was analyzed by flow cytometry. Data represent the mean (±SD) from triplicate samples. *P < 0.05, **P < 0.01 compared to D003 no NAC control. B: NAC protected cells from D003-induced cytotoxicity. The 83-01-82CA cell line was treated for 24 h with D003b2 in the presence or absence of NAC. NAC (0, 1, and 2 mM) was added 1 h before treatment with D003 (12.5, 25, and 50 µg/ml). The relative number of cells was determined by a MTT assay. Data represent the mean (±SD) of triplicate samples. *P < 0.05, **P < 0.01 compared to D003 no NAC control. C: NAC protected cells from D003-induced apoptosis. The 83-01-82CA cell line was treated with 35 µg/ml D003b2 in the presence of NAC for 16 h. Cell lysates were harvested, and cleavage of PARP, caspase 3, 8, and 9 were determined using Western blot. D: NAC prevents MMP loss induced by D003 extract. The 83-01-82CA cell line was pretreated with 2 mM NAC for 1 h followed by treatment with 30 µg/ml of D003b1 for 16 h. Cells were stained with JC-1, and MMP was measured using flow cytometry. Data represent the mean (±SD) of triplicate samples. **P < 0.01 compared to D003 without NAC.

and lack of apoptosis exhibited by the D003 extract to primary normal human epithelial cell lines coincides with lower levels of ROS generation. These data suggest (a) a differential ROS response in the normal compared to malignant cell lines to the D003 extract, and (b) the higher ROS levels in the malignant cell line sensitizes them to apoptosis induced by the D003 extract.

Mitochondria Are Responsible for Avocado D003 Extract-Induced ROS Accumulation

ROS are produced during cellular metabolism via the mitochondrial respiratory chain, involving autoxidation of complexes I, II, and III (22,23). To further investigate the potential role of these pathways in the ROS generation in response to the treatment with avocado D003 extract, the 83-01-82CA cell line was treated with D003 in the presence of pharmacological inhibitors. Rotenone is a specific inhibitor of complex I, which interferes with the electron flow from NADH-linked substrates and NADH dehydrogenase to the ubiquinone pool. Pretreatment with 1 µM rotenone significantly (P < 0.05) reduced the intracellular ROS level in cells upon the treatment with D003 (Fig. 4B) while partially blocking D003-induced apoptosis (Fig. 4C). A higher, 2.5 µM, concentration of rotenone did not further reduce ROS levels over the 1 µM concentration but completely blocked D003 induced apoptosis. On the other hand, inhibition of complex II by TTFA (10–50 µM) or inhibition of complex III by antimycin A (10–50 µM) did not block the increased levels of ROS levels induced by D003 (data not shown).

Higher ROS Levels in the E6 and E7 Transfected TE1177 Cell Line Correlate With Higher Sensitivity to the D003 Extract

To further determine the role of ROS levels in their sensitivity of the cancerous human oral cell lines to the D003 extract, the primary normal oral cell line, TE1177, was infected with retrovirus containing the HPV16 E6 or HPV16 E7 gene. These oncogenes were selected, as they have been shown to be involved with human oral premalignant state and cancer (24). It was our expectation that primary normal oral epithelial cells infected with these oncogenes would harbor higher levels of ROS and be more sensitive to D003-induced apoptosis. All 7 transformed
FIG. 4. Role of mitochondria respiratory chain in D003-induced generation of ROS. A: Differential response of ROS of normal human oral epithelial cell line on treatment with D003. The TE1177 (normal) and 83-01-82CA (malignant) human oral epithelial cell lines were treated with various concentrations of D003b1 for 3 h. DCF-DA (10 \( \mu \)M) was added 30 min before harvesting cells. ROS content was analyzed by flow cytometry. Data represent the mean (±SD) from triplicate samples; * \( P < 0.05 \). B: The 83-01-82CA cell line was preincubated with various concentrations of rotenone (ROT) for 1 h followed by incubation with 30 \( \mu \)g/ml D003b1 for 2.5 h. ROS production was determined by flow cytometry. Results represent the mean (±SD) of triplicate samples; * \( P < 0.05 \). C: The 83-01-82CA cell line was treated with various concentrations of rotenone (ROT) for 1 h then followed by incubation with 30 \( \mu \)g/ml D003b1 for 16 h. Apoptosis was measured by PARP and caspase 3 cleavage using Western blotting.

cell lines (2 from E6 and 5 from E7) exhibited significantly higher basal levels of ROS compared to the parent TE1177 cell line and empty vector infected TE1177 cells (\( P < 0.05 \); data not shown). Empty vector infected TE1177 cells have similar levels of ROS to the parent cell line (data not shown). After treatment with D003, the levels of ROS markedly increased in all 7 transformed cell lines (Fig. 5A). To further investigate the role of the enhanced ROS in D003-induced apoptosis in these transformed cell lines, the TE1177, TE1177/vector, TE1177/E6, TE1177/E7 cell lines were treated with various concentrations of D003. As shown in Fig. 5B, the transformed cells were more sensitive to the treatment of D003 extract. As expected, ablation of ROS by NAC protected these transformed cell lines from D003-induced apoptosis (Fig. 5C). These data suggest increased ROS levels in transformed TE1177 cell lines at least partially contributed to selective cytotoxicity induced by D003.

**DISCUSSION**

A number of previous studies, including our own, have indicated that extracts obtained from avocado meat contain phytochemicals that have the ability to selectively inhibit cancerous cell growth (6, 7, 25). Cancer cells often show high levels of ROS and must maintain a delicate balance between prooxidants and antioxidants (26–28). As ROS may act as growth inhibitory and apoptotic signaling agents, perturbing the balance of ROS in cancer cells may offer an opportunity for prevention by phytochemicals (6, 28–33). Recent studies have suggested a prevention strategy with phytochemicals targeting ROS metabolism and raising the levels of ROS in cancer cells above a toxic threshold (28, 34, 35). In the present study, D003 extracts increased levels of ROS in oral cancer cell lines, activating both the extrinsic and intrinsic apoptotic pathways. As these cancerous cell lines contain high endogenous levels of ROS, the selective action of the D003 extract may raise the levels of ROS above a toxic threshold in the cancerous cell lines.

The role of ROS appears to be somewhat paradoxical (28). High levels of ROS generation, characteristic of tumor cells, may be attributable to their faster rate of growth and metabolism. However, ROS are also critical signaling molecules in apoptosis induced by chemotherapeutic and prevention drugs (35–40). Although cancerous cells are able to tolerate high levels of ROS, there appears to be a threshold above which ROS levels become toxic. Agents that increase ROS above this threshold can lead to their selective killing (28, 34, 35). A study by Trachootham et al. (27) showed that ROS stress created by \( \beta \)-phenylethyl isothiocyanate induced apoptosis of Bcr-AbI and H-RasV transformed cells (27). In the present investigation, it was found that increasing ROS levels in cancerous human oral cell lines by the D003 extract also induced apoptosis. The lack of apoptosis induced by D003 in the normal cell lines may be due to much lower levels of endogenous ROS than the cancer cell lines. This relationship between high endogenous levels of ROS and sensitivity to the D003 extract was confirmed by the fact that (a) normal oral
FIG. 5. Differential generation of ROS and sensitivity of primary cell line TE1177 and transformed primary cell lines TE1177/E6 and TE1177/E7 to D003. A: E6 and E7 transformed normal oral epithelial cell lines harbor higher levels of ROS. The TE1177/V, TE1177/E6, and TE1177/E7 cell lines were treated with various concentrations of D003b2 for 3 h. DCF-DA (10 \( \mu \)M) was incubated during the last 30 min. ROS content was measured by flow cytometry. Because all transformed cell lines have higher levels of basic ROS and increased to a much higher level after treatment with D003b2 than the parent TE1177 and TE1177/V cell lines (* \( P < 0.05 \)), only data from one E6 (TE1177/E6) and one E7 (TE1177/E7) transformed cell line are shown. B: Transformed TE1177 cell lines were more sensitive to D003. Two of each TE1177/V, TE1177/E6, and TE1177/E7 infected cell lines were treated with various concentrations of D003b2. Cytotoxicity was determined by the MTT assay after treatment for 24 h. Because results from 2 parallel cell lines from the same infection were similar, only data from one cell line from each infection are shown (** \( P < 0.01 \) compared to TE/V D003 control). C: addition of NAC protected the E6 and E7 cell lines from D003-induced cytotoxicity. NAC (2 and 5 mM) was added 1 h before D003b2 (75–100 \( \mu \)g/ml). Cytotoxicity was determined by the MTT assay after treatment for 24 h (** \( P < 0.01 \) compared to D003 no NAC control). Because results from pretreatment with 2 and 5 mM NAC exhibit a similar pattern of protection of both E6 and E7 cell lines, only data from E6 cell line with 2 mM NAC are shown as a representative.

cells with low endogenous levels of ROS are resistant to D003 induced apoptosis, and (b) transforming the normal cell line with HPV16 E6 and HVP16 E7 leading to cells having high endogenous levels of ROS sensitizes them to D003 extract. In the sensitized cancer and transformed normal cell lines, apoptosis was preceded by an elevation of ROS beyond a threshold necessary to induce apoptotic signaling pathways. As implicated by our data, low concentrations of D003 (10–20 \( \mu \)g/ml) enhance the ROS levels of malignant cells (Fig. 4A). Even though these concentrations do not induce apoptosis (Fig. 1A), ROS scavenger NAC significantly protected malignant cells from D003-induced cytotoxicity (Fig. 3B). We reason that malignant cells already have higher basal levels of ROS, and a slight increase in the generation of ROS may inhibit their growth or induce apoptosis. These low concentrations do not increase the ROS levels in normal primary cell lines to reflect clinical relevance.

ROS are physiological products generated by all mammalian cells in the mitochondria during aerobic metabolism. Intracellular ROS levels are kept in a balance by metabolism offset by cellular antioxidant enzymes and scavengers. Several possible signaling pathways have been described linking ROS to apoptosis. These include cell surface death receptors (extrinsic) and mitochondria (intrinsic) pathways (41). These data indicate that D003 extract-induced ROS activates both the extrinsic and intrinsic apoptotic pathways. Even though the intrinsic pathway is involved in D003-induced apoptosis supported by the loss of mitochondria membrane potential and activation of caspase 9, Bcl2 family proteins Bax, Bad, Bak, Bcl2, and Bcl-xl proteins do not appear to be involved. Also, overexpression of Bcl2 by stable transfection did not abate avocado D003 extract-induced cell death (data not shown). One possibility is that enhanced levels of ROS in mitochondria may oxidize cardiolipin leading to the release of proapoptotic factors from mitochondria into the
cytosol leading to the activation of caspase 9 (42,43). Among the 2 identified sources of ROS in the mitochondrial respiratory chain, our studies indicate that inhibition of complex I by rotenone significantly reduces D003-induced ROS generation. Neither inhibition of complex II by TTFA nor inhibition of complex III by antimycin A affects the content of ROS. These data suggest that D003-induced ROS generation is mainly processed through complex I of the mitochondrial respiratory chain. This site also contributes to ceramide-induced ROS production (23), which implies that D003 extract may share a similar mechanism with ceramide in the induction of ROS production. These data are consistent with other food and diet-derived chemopreventive agents disrupting the mitochondrial membrane to induce apoptosis (44,45).

In the extrinsic pathway, FADD/TRADD adaptor proteins recruit the initiator procaspase 8, leading to activation of caspase 3, Bid and/or Bim. Cleavage of Bid leads to changes in the mitochondrial membrane releasing cytochrome c and procaspase 9 to complex with Apaf-1 to form the apoptosome. ROS has been found to trigger the clustering of FAS receptor on the surface of Jurkat cells and caspase 8 dependent apoptosis (46). In our study, FADD-DN prevents early time-induced apoptosis, suggesting that the FADD-mediated extrinsic cell membrane death signal pathway is important to the initiation of D003-induced apoptosis. On the other hand, FADD-DN did not prevent caspase 8 cleavage, suggesting D003 may also activate other pathways leading to the activation of caspase 8. Cagnol et al. (47) showed that prolonged Erk activation-induced caspase 8 activation is FADD-independent. Further studies are required to define the underlying mechanism.

In conclusion, a selective anticancer effect of avocado extract on premalignant and malignant human oral epithelial cell lines has been observed. The potent anticancer activity is initiated by ROS generation, leading to the induction of both the intrinsic and extrinsic apoptotic pathways. The differential effect between normal, transformed normal, premalignant, and malignant cell lines appears dependent on the endogenous levels of ROS. These results provide further support for use of prooxidant agents, like those found in the D003 avocado extract, in chemoprevention of cancers that have high endogenous levels of ROS. The data also indicate the potential of using phytochemicals from avocado meat in cancer prevention.

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