[CANCER RESEARCH 61, 6698–6702, September 15, 2001]

Advances in Brief

Evidence Supporting a Role for Mitochondrial Respiration in Apoptosis Induction by the Synthetic Retinoid CD437[1]

Numsen Hail, Jr., Emile M. Youssef, and Reuben Lotan[2]

Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030-4095

Abstract

Retinoids have been shown to modulate cell proliferation, differentiation, and apoptosis. It is thought that these effects mediate the chemopreventive and therapeutic effects of retinoids. Recently, some synthetic retinoids, including 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437), have been found to induce apoptosis even in tumor cell lines that are resistant to all-*trans* retinoic acid. The proapoptotic activity of CD437 has been attributed to mitochondrial dysfunction via the induction of mitochondrial permeability transition (P. Marchetti *et al.*, Cancer Res. *59*: 6257–6266, 1999). The mechanistic aspects pertaining to how CD437 promotes changes in mitochondrial function are unclear. This study investigated the role of mitochondrial respiration in CD437-induced apoptosis. Human cutaneous squamous cell carcinoma COLO 16 cells were chronically exposed to ethidium bromide to inhibit mitochondrial DNA synthesis and produce respiration-deficient clones. These clones were exposed to CD437 (≤10 μM) for 48 h and exhibited a marked resistance to mitochondrial permeability transition and apoptosis illustrating that mitochondrial respiration was required for these effects.

Introduction

CD437[3] (also known as AHPN) is a synthetic analogue of vitamin A belonging to a growing family of compounds known as retinoids. CD437 is unique among many retinoids because it has the ability to rapidly promote apoptosis in a variety of tumor cell lines that are refractory to archetypal retinoid ATRA (1–5). As such, CD437 and perhaps structurally related compounds could be important tools to prevent or treat cancer. In a recent elegant study, the acute cytotoxic effects of CD437 were shown to be directly associated with mitochondrial dysfunction via the induction of MPT (3). This study was significant because it linked the proapoptotic effects of CD437 to the mitochondria, which are now well established as playing a pivotal role in apoptosis (6, 7). The way in which CD437 promotes MPT has not been determined. The mitochondria are attractive targets for cancer chemotherapy. There is growing evidence, both in isolated mitochondria and in intact cells, illustrating that many cancer chemotherapeutic agents modulate or interfere with mitochondrial functions to promote MPT (8). Mitochondria constitute 15–50% of the total cytoplasmic volume in most cells and participate in more metabolic functions than any other organelles, especially in cellular energy production (9). In addition, the mitochondria consume ~90% of cellular oxygen and are a significant source of ROS, which if unchecked, are deleterious to mitochondrial and other cellular functions (10, 11). For these reasons, we hypothesized that CD437 disrupts mitochondrial respiration as a means of triggering MPT. To test this hypothesis, ρ^0 cells were generated. These clones were resistant to rapid MPT induction and apoptosis observed in parental cells exposed to CD437 indicating that mitochondrial respiration was required for these effects. Interestingly, the ρ^0 clones displayed peculiar morphological features after exposure to CD437 that are apparently unique with respect to other retinoids examined in human epidermal keratinocytes.

Materials and Methods

Cells and Reagents. The cutaneous SCC cell line COLO 16 was derived from a metastatic lesion in a female patient who succumbed to metastatic disease (12). COLO 16 cells were routinely cultured in keratinocyte growth medium consisting of keratinocyte basal medium supplemented with 100 ng/ml human recombinant epidermal growth factor and 0.4% bovine pituitary extract (BioWhittaker, Walkersville, MD) unless otherwise specified. Cell cultures were incubated at 37°C in humidified air containing 5% CO_2. Treatment with CD437 and other agents was performed on subconfluent cultures.

CD437 was synthesized at Galderma Research and Development and obtained from Dr. Braham Shroot (Sophia Antipolis, France). ATRA was obtained from Dr. Werner Bollag (F. Hoffman-La Roche, Basel, Switzerland). ATR, Vit-C (L-ascorbic acid), CsA, potassium cyanide, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). $DiOC_6(3)$ and dihydroethidium were purchased from Molecular Probes, Inc. (Eugene, OR).

Isolation and Characterization of ρ^0 Cells. Respiration-deficient SCC cells (ρ^0 clones) were isolated according to a method described previously (13) with limited modifications. Briefly, COLO 16 cells were cultured for 8 weeks in enriched medium consisting of DMEM containing 4.5 mg/ml glucose (Sigma Chemical Co.), 110 mg/ml pyruvate (Sigma Chemical Co.), 50 mg/ml uridine (Sigma Chemical Co.), 100 ng/ml EtBr (Sigma Chemical Co.), and 2% dialyzed (M_r 3,500 cutoff) fetal bovine serum (Life Technologies, Inc., Rockville, MD). Subcloning was done by limiting dilution. The effects of chronic exposure to EtBr on mitochondrial DNA and respiration in isolated clones were assessed by three methods. First, when cultured in enriched medium without uridine the clones exhibited greatly diminished cell survival (not shown). ρ^0 cells have been reported to become pyrimidine auxotrophs, suggesting respiration is defective in these cells (13). Second, a PCR procedure was used to detect mitochondrial DNA sequences in DNA samples from COLO 16 cells and the isolated clones. The PCR reaction was conducted as specified for 40 cycles in a thermal cycler using a primer set (L1–5′AACAT-ACCCATGGCCAACCT3′ and H1–5′GGCAGGAGTAATCAGAGGTG3′) designated for the detection of total mitochondrial DNA (14). Using this method, PCR products derived from mitochondrial DNA were detected in parental cells but were absent in the isolated clones (not shown). Finally, oxygen consumption rates in the isolated clones were ~15% of those observed in parental cells (see Fig. 3*A*), illustrating that the ρ^0 clones were functionally deficient in respiration. Treatment of ρ^0 clones was conducted 24 h after culture in enriched medium without EtBr.

Measurement of Oxygen Consumption in Cultured Cells. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode and YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instruments

Received 4/12/01; accepted 7/26/01.

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.

[1] Supported in part by the USPHS Project Grant PO1 CA68233 from the National Cancer Institute, and by a Cancer Prevention Fellowship sponsored by the National Cancer Institute Grant R25 CA57780 (Robert M. Chamberlain, Principal Investigator).

[2] To whom requests for reprints should addressed, at Department of Thoracic/Head and Neck Medical Oncology, Box 432, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4095. Phone: (713) 792-6363; Fax: (713) 794-0209; E-mail: rlotan@mdanderson.org.

[3] The abbreviations used are: CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; ATRA, all-*trans* retinoic acid; EtBr, ethidium bromide; ATR, atractyloside; CsA, cyclosporin A; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; $\Delta\Psi_m$, mitochondrial inner transmembrane potential; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; ρ^0, respiration-deficient cells lacking mitochondrial DNA; SCC, squamous cell carcinoma; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; Vit-C, vitamin C.

Inc., Yellow Springs, OH). Cells ($\sim 6 \times 10^6$) suspended in 3 ml of their respective culture medium were added to a 3-ml respiration chamber in a circulating water bath at 37°C. Oxygen consumption was measured over a 10-min period after equilibration of the electrode in the respiration chamber. Respiration rates for cells were normalized (nmol O_2/min/10^6 cells) assuming an O_2 concentration of 220 μM in air-saturated medium at 37°C (15). In certain determinations, CD437 was added directly to cell suspensions (final concentration 1 μM) in the respiration chamber for a 10-min exposure. Control cells received an equal volume of the vehicle DMSO. For longer exposures, CD437 and/or other agents were added directly to the medium of cells cultured in 10-cm plastic tissue culture plates. The cells were harvested by trypsinization, pelleted by centrifugation, resuspended at a density of $\sim 2 \times 10^6$ cells/ml in fresh medium at 37°C, and 3 ml of the cell suspension were placed in the respiration chamber. Oxygen consumption rates were obtained during the final 10 min of exposure. Cell viability was routinely checked via trypan blue exclusion after oxygen consumption determinations.

Cytofluorometric Analysis. Evaluations of $\Delta\Psi_m$ dissipation, ROS production, and DEVDase activity were adapted from methods published previously (3) with limited modifications. For determinations of $\Delta\Psi_m$ dissipation and ROS production, cells in 10-cm plastic tissue culture plates were treated with agents alone or in combination for various periods. Twenty min before the cells were harvested, $DiOC_6(3)$ or dihydroethidium was added directly to the culture medium to a final concentration of 30 nM or 5 μM, respectively. The cells were harvested by trypsinization, pelleted by centrifugation, resuspended in 1 ml PBS at 37°C, and analyzed immediately by flow cytometry. DEVDase-like caspase activity was determined with PhiPhiLux-G_1D_2 (OncoImmunin Inc., Gaithersburg, MD), which is a cell-permeant fluorogenic substrate (DEVD-rhodamine) that is cleaved in a DEVD-dependent manner (3). Cells were treated in 10-cm plastic tissue culture plates as described above. After treatment, the cells were harvested by trypsinization, incubated in 10 μM PhiPhiLux-G_1D_2, and washed according to the manufacture's recommendations. The resulting cell suspension was analyzed immediately by flow cytometry. Intranucleosomal DNA fragmentation was evaluated by the TUNEL technique using a flow cytometry apoptosis detection kit (Phoenix Flow Systems, Inc., San Diego, CA; Ref. 5). Cells were treated for the appropriate times in 10-cm plastic tissue culture plates as described above. The cells were harvested by trypsinization, fixed, and stained for TUNEL analysis. All of the flow cytometric procedures were performed using an EPICS Profile II Analyzer (Coulter Corp., Miami, FL). Cells ($\sim$10,000 events) were evaluated for each sample. In all of the cytofluorometric determinations, cell samples were gated to exclude cell debris and clumps.

Results and Discussion

CD437-induced Apoptosis Is Associated with MPT in COLO 16 Cells. CD437 (0.5–1 μM) promotes intranucleosomal DNA fragmentation as soon as 2 h after exposure in SCC cell lines including the COLO 16 cell line (5). To determine whether MPT was linked to this process, the effects of CD437 alone or in combination with the prototypical MPT inhibitor CsA (16) were examined in various parameters associated with apoptosis. Retention of the cationic dye $DiOC_6(3)$ can be used as an indicator of $\Delta\Psi_m$ in intact cells (3). A 3-h exposure to 1 μM of CD437 resulted in a dramatic reduction in $DiOC_6(3)$ retention indicating loss of $\Delta\Psi_m$. This loss could be inhibited by pretreating the cells for 30 min with 5 μM of CsA (Fig. 1*A*). CsA alone slightly enhanced $\Delta\Psi_m$ (not shown), a phenomenon we have observed previously (17). Enhanced production of ROS resulting from impairment of mitochondrial electron transport is also a feature of MPT (3). A 3-h exposure of COLO 16 cells to CD437 greatly enhanced the production of ROS (primarily superoxide; Ref. 18) as indicated by the oxidation of dihydroethidium to ethidium (Fig. 1*B*). The CD437-induced ROS production was also inhibited by CsA (Fig. 1*B*), which alone had no detectable effect on ROS production (not shown). MPT induction results in the release of cytochrome C and other soluble mitochondrial factors capable of inducing caspase activity (7). Indeed, CD437 treatment promoted DEVDase-like caspase activity after a 3-h exposure that was inhibited by CsA (Fig. 1*C*). CsA

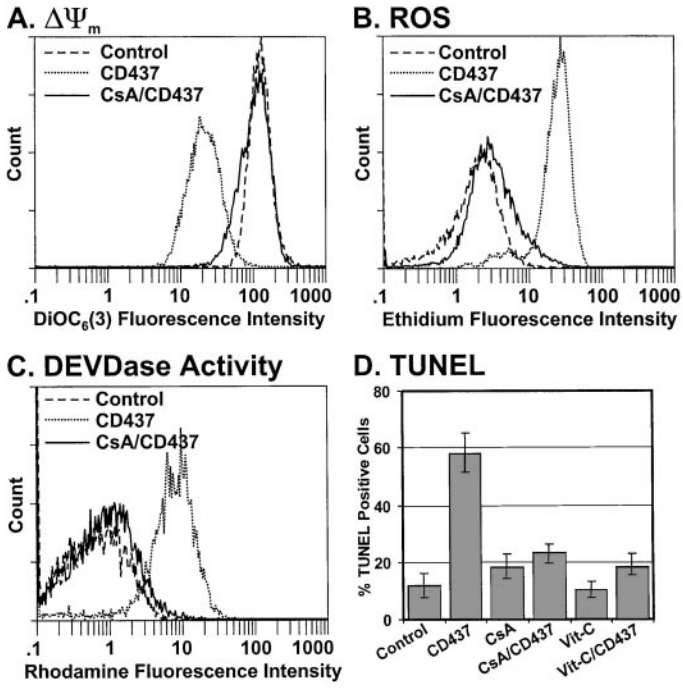

Fig. 1. CD437-induced apoptosis is MPT-dependent. *A*, COLO 16 cells were treated for 3 h with DMSO (*control*) or 1 μM of CD437, or 30 min with 5 μM of CsA followed by a 3-h exposure to 1 μM of CD437. Twenty min before the cells were harvested, $DiOC_6(3)$; (final concentration 30 nM) was added directly to the culture medium. Cells were harvested and analyzed by flow cytometry. *B*, COLO 16 cells were treated as described in *A*. Twenty min before the cells were harvested, dihydroethidium (final concentration 5 μM) was added directly to the culture medium. Cells were harvested and analyzed by flow cytometry. *C*, COLO 16 cells were treated as described in *A*. Cells were harvested, incubated in PhiPhiLux-G_1D_2 (final concentration 10 μM) and subsequently analyzed by flow cytometry. Data in *A*, *B*, and *C* are representative of at least three independent experiments. In *A*, *B*, and *C* the histograms for CsA are omitted to simplify the figures (see text for description). *D*, COLO 16 cells were treated for 6 h with DMSO (*control*), 1 μM of CD437, 5 μM of CsA, or 500 μM of Vit-C; or for 30 min with CsA or Vit-C followed by a 6-h exposure to 1 μM CD437. Cells were harvested, fixed, and stained for TUNEL analysis. Data are expressed as an average of triplicate samples; *bars*, $\pm$ SD.

alone had no effect on DEVDase-like caspase activity (not shown). After a 6-h exposure to CD437, $\sim$60% of COLO 16 cells were TUNEL positive (Fig. 1*D*). This process was also blocked by CsA, which alone slightly increased the number of TUNEL-positive cells.

Because CD437 treatment enhanced ROS production in COLO 16 cells, we were interested in the potential effects of the antioxidant Vit-C on CD437-induced apoptosis. We have reported recently that Vit-C could block the pro-oxidant and proapoptotic effects of *N*-(4-hydroxyphenyl)retinamide in these cells (17). Surprisingly, pretreating the cells for 30 min with Vit-C was as effective as CsA in inhibiting the DNA fragmentation promoted by a subsequent 6-h exposure to CD437 (Fig. 1*D*). Vit-C was later found to inhibit $\Delta\Psi_m$ dissipation, ROS production, and DEVDase-like activity promoted by CD437 (not shown). These results indicate that CD437 promotes MPT, which is linked to apoptosis in COLO 16 cells. The ability of Vit-C to block MPT and apoptosis indicates that ROS production and oxidative stress are intimately involved in this process.

CD437 Rapidly Decreases Oxygen Consumption in COLO 16 Cells. CD437 rapidly promotes permeability transition-dependent colloidosmotic swelling in isolated mitochondria (3). If this process was to occur in mitochondria of intact cells respiration should be impaired considering the mitochondria consume $\sim$90% of cellular oxygen (10). To test this hypothesis, COLO 16 cells were treated with CD437 resulting in $\sim$30% reduction in cellular oxygen consumption within the first 10 min of exposure (Fig. 2*A*). This inhibition reached $\sim$60% after 1 h illustrating that CD437 could rapidly modulate mitochondrial function in intact cells.

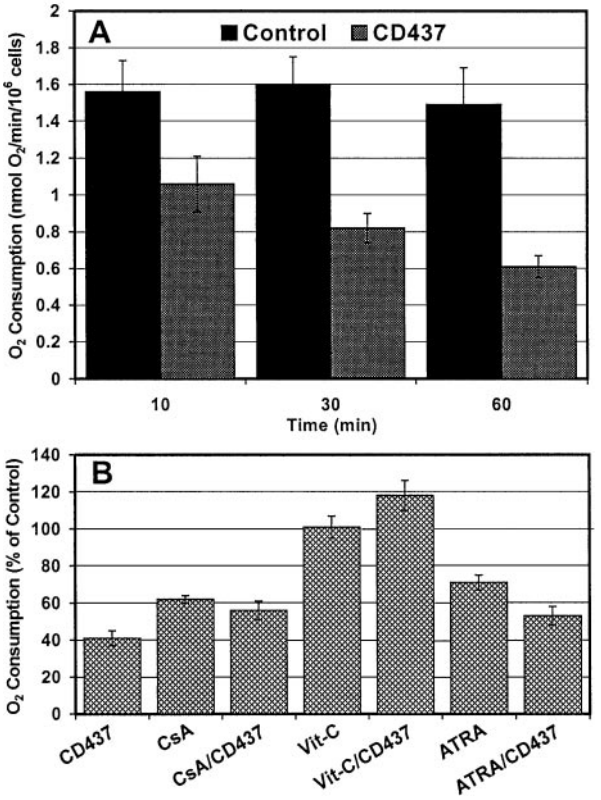

Fig. 2. CD437 modulates mitochondrial function. *A*, COLO 16 cells were exposed for the indicated times to 1 μM of CD437 or the vehicle DMSO. Rates of oxygen consumption were determined during the final 10 min of exposure (average of triplicate samples); *bars*, ± SD. *B*, COLO 16 cells were treated for 1 h with 1 μM of CD437; 1.5 h with 5 μM of CsA, or 500 μM of Vit-C; 2 h with 10 μM of ATRA; 30 min with CsA or Vit-C followed by a 1-h exposure to 1 μM of CD437; or 2 h with ATRA followed by a 1-h exposure to 1 μM of CD437. Rates of oxygen consumption were determined during the final 10 min of exposure and expressed as a percentage of rates obtained for cells treated for equivalent times with DMSO (*control*; average of triplicate samples); *bars*, ± SD.

Both CsA and Vit-C could inhibit CD437-induced MPT and apoptosis in COLO 16 cells. This prompted examination of the effects of these agents on the decrease in cellular oxygen consumption promoted by a 1-h exposure to CD437. Interestingly, short-term exposure (1.5 h) to CsA also inhibited respiration and only moderately reversed the effects of CD437 (Fig. 2*B*). The decrease in respiration promoted by CsA can potentially be explained as follows. CsA binds to cyclophilin D to stabilize the closed matrix conformation of the adenine nucleotide translocator (7, 16). This action could conceivably inhibit coupled respiration by limiting ADP/ATP exchange and/or reducing proton conductance into the mitochondrial matrix. The adenine nucleotide translocator is a highly specific transporter of ADP and ATP and is postulated to have a dual function as an ion conductance channel (8, 16).

Exposure to Vit-C, whereas it had no considerable impact on oxygen consumption, was able to block the effects of CD437. This treatment combination resulted in ~20% higher respiration than observed in DMSO-treated control cells. This would indicate that ROS production was a major factor influencing the rapid decrease in respiration promoted by CD437. It is plausible that CD437 could function by inhibiting electron transport. The rotenone- and antimycin A-sensitive sites of the respiratory chain can be attacked by a variety of hydrophobic xenobiotics (19, 20). Depending on the electron transport complex affected, ROS production could occur via redox cycling of reduced electron carriers upstream of the site of inhibition (11). There is also the possibility that CD437 could be converted to a radical species capable of undergoing redox metabolism to promote oxidative stress and MPT. In both scenarios Vit-C, as a potent reductant capable of scavenging ROS (21), should inhibit these effects.

We have reported that the natural retinoid ATRA can transiently inhibit CD437-induced apoptosis in COLO 16 cells (5). A 2-h exposure to 10 μM ATRA resulted in ~35% reduction in oxygen consumption and, like CsA, only slightly reversed the effects of a 60-min exposure to CD437. Evidently, the decrease in respiration promoted by CsA and ATRA was linked to CD437 resistance. This is concluded primarily because ATRA, unlike CsA, is not known as a MPT inhibitor. Inhibiting respiration is believed to block MPT by keeping the matrix pyridine nucleotide pool in a more reduced state (16), principally by inhibiting electron flux through complex I of the electron transport chain (22). Short-term exposure to CsA did not promote MPT or apoptosis in COLO 16 cells (Fig. 1), and ATRA is not a potent apoptogenic agent in these cells (5). Therefore, the ability of these agents to inhibit respiration is apparently mechanistically different from the effects triggered by CD437. Together, these results indicate that ROS production and respiration are associated with CD437-induced MPT and apoptosis.

ρ^0 Clones Exhibit Marked Resistance to CD437-induced MPT and Apoptosis. To evaluate the role of mitochondrial respiration in CD437-induced cell death, parental COLO 16 cells were chronically exposed to EtBr to inhibit mitochondrial DNA synthesis and produce ρ^0 clones. These clones typically exhibited reduced rates of oxygen consumption that were ~15% of the rate observed in parental COLO 16 cells (Fig. 3*A*). These rates were also comparable with that obtained when parental COLO 16 cells were exposed for 30 min to the complex IV inhibitor cyanide (11), indicating that cellular oxygen consumption was predominantly of mitochondrial origin.

In cells depleted of mitochondrial DNA, maintenance of $\Delta\Psi_m$ is dependent on a functional F1-ATPase that hydrolyzes ATP to pump protons of the mitochondrial matrix (23). In the ρ^0 clones generated in

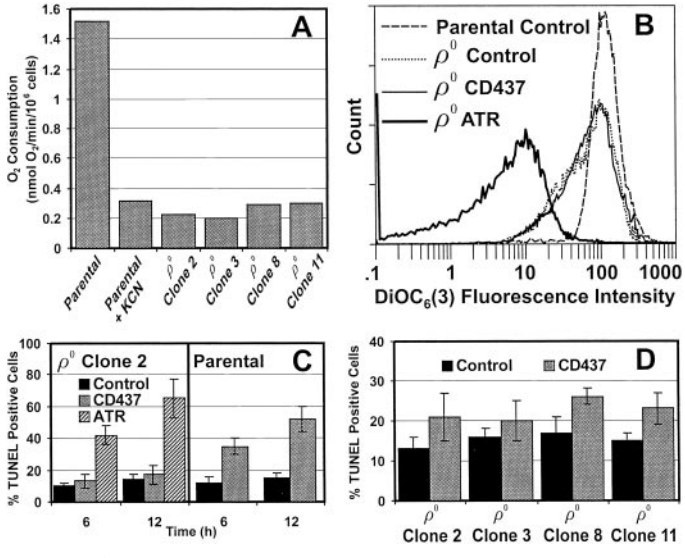

Fig. 3. ρ^0 clones are resistant to CD437-induced MPT and apoptosis. *A*, oxygen consumption rates were determined for parental COLO 16 cells untreated or treated for 30 min with 2.5 mM of potassium cyanide and several ρ^0 clones. *B*, parental COLO 16 and ρ^0 Clone 2 cells were evaluated for $\Delta\Psi_m$ dissipation as described in Fig. 1*A*. ρ^0 Clone 2 cells were exposed for 12 h to 10 μM of CD437 or 10 mM of ATR. Controls for parental COLO 16 and ρ^0 Clone 2 cells were treated with DMSO. Data are representative of at least three independent experiments. *C*, Parental COLO 16 and ρ^0 Clone 2 cells were evaluated for DNA fragmentation using the TUNEL technique. ρ^0 Clone 2 cells were exposed to 10 μM of CD437, 10 mM of ATR, or DMSO (*control*) for the indicated times. Parental COLO 16 cells were exposed for the indicated times to 1 μM of CD437 or DMSO (*control*). Data are expressed as an average of triplicate samples; *bars*, ± SD. *D*, several ρ^0 clones derived from parental COLO 16 cells were exposed for 48 h to 10 μM of CD437 or DMSO (*control*) and evaluated using the TUNEL technique. Data are expressed as an average of triplicate samples; *bars*, ± SD.

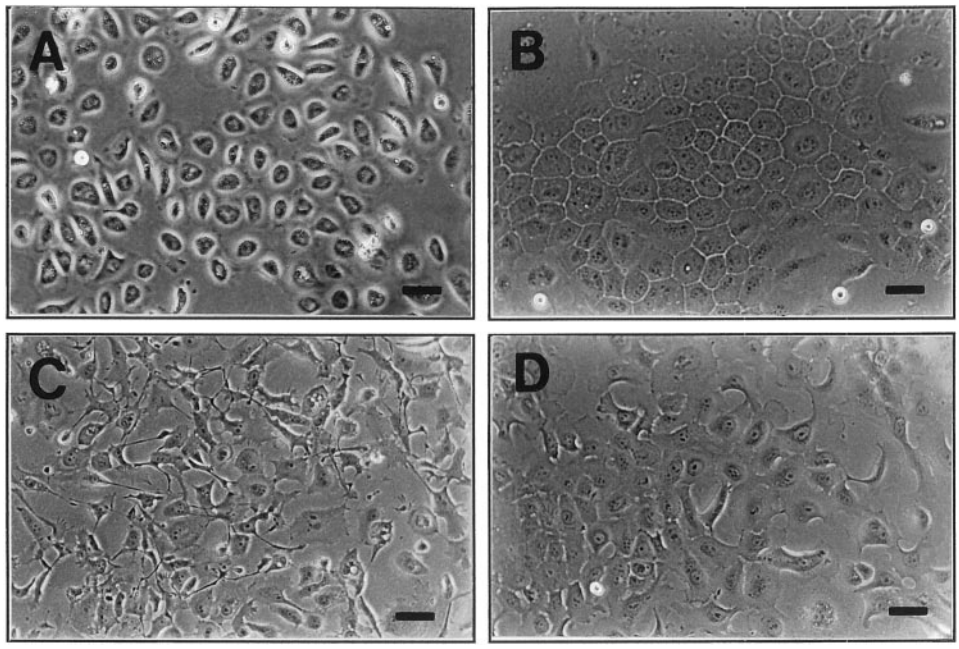

Fig. 4. Morphological changes in EtBr- and CD437-treated cells. Parental COLO 16 cells (*A*) were chronically exposed to EtBr to produce ρ^0 clones (*B*). *C*, cells pictured in *B* after a 48-h exposure to 10 μM of CD437. *D*, cells pictured in *C* ~6 h after replacement of the culture medium. Cells were photographed using a Nikon camera and microscope. *Scale bars*, 32 μm.

this study, the retention of $DiOC_6(3)$ was slightly less than that observed in parental cells indicating slightly lower $\Delta\Psi_m$ (Fig. 3*B*). Exposures (12 h) to 1, 5, or 10 μM of CD437 had no effect on $\Delta\Psi_m$ (only the histogram representing the exposure to 10 μM of CD437 is presented) indicating that MPT was not induced. In stark contrast, a 12-h exposure to the adenine nucleotide translocator ligand and MPT promoter ATR (16) greatly reduced $DiOC_6(3)$ retention indicative of MPT.

Exposures of $\leq$10 μM of CD437 had no short-term impact on $\Delta\Psi_m$ in ρ^0 cells. Therefore, these cells were cultured for various periods ($\leq$48 h), and the TUNEL assay was conducted to determine whether intranucleosomal DNA fragmentation could be induced by CD437 in a MPT-independent fashion. Exposures (6 and 12 h) to CD437 were unable to promote extensive intranucleosomal DNA fragmentation indicative of apoptosis (Fig. 3*C*). Longer exposures (24 and 48 h) to CD437 were also examined, and the TUNEL values were not markedly different from controls (not shown). Exposure to ATR was very effective in promoting intranucleosomal DNA fragmentation indicating that these cells could still undergo MPT-dependent apoptosis.

To determine whether the enriched medium could possibly protect ρ^0 clones from CD437-induced apoptosis, parental COLO 16 cells were cultured for 24 h in enriched medium without EtBr and exposed to 1 μM of CD437. The enriched medium contains a higher glucose concentration than the keratinocyte growth medium and is supplemented with uridine, which is absent in keratinocyte growth medium (see "Materials and Methods"). Interestingly, the kinetics of intranucleosomal DNA fragmentation observed under these conditions were slower than that reported previously for cells cultured in keratinocyte growth medium (5). As such, cells cultured in enriched medium required ~12 h to obtain similar levels of TUNEL-positive cells after exposure to CD437 (Fig. 3*C*) as observed for a similar treatment in the same cells cultured in keratinocyte growth medium (Fig. 1*D*).

The presumed reason for this difference may be related to the glucose concentration in the enriched medium, which is approximately four times higher than that of the keratinocyte growth medium. As such, the higher glucose concentration in the enriched medium could offer some protection from MPT induction, because glucose is known as a MPT inhibitor (8). Oxygen consumption rates were determined for parental COLO 16 cells cultured in the keratinocyte growth medium and enriched medium. For cells cultured in keratinocyte growth medium, the rates of oxygen consumption were ~1.54 nmol O_2/min/10^6 cells compared with 1.07 nmol O_2/min/10^6 cells obtained for cells cultured in enriched medium. Consequently, this 30% decrease in respiration in cells cultured in the enriched medium could also account for the inhibition of CD437-induced MPT and apoptosis considering the data presented for CsA and ATRA (Fig. 2*B*).

Several ρ^0 clones were derived from parental COLO 16 cells and exposed for 48 h to 10 μM of CD437. As indicated by the results of the TUNEL analysis (Fig. 3*D*), all of the clones exhibited resistance to CD437-induced apoptosis. Together, these results illustrate that ρ^0 clones are refractory to CD437 by virtue of their respiration deficiency and support a role for mitochondrial respiration in CD437-induced apoptosis in parental COLO 16 cells.

CD437 Promotes Morphological Changes in ρ^0 Clones. We have reported previously that normal human epidermal keratinocytes undergo G_1 arrest and adopt peculiar morphological features after exposure to CD437 (5). Interestingly, morphological changes were also promoted in parental COLO 16 cells (Fig. 4*A*) after chronic exposure to EtBr (Fig. 4*B*). The ρ^0 clones exhibited a greatly flattened appearance with definite demarcations between cells indicating cell-to-cell attachments. After a 48-h exposure to 10 μM of CD437, ρ^0 clones exhibited dendritic morphology (Fig. 4*C*). These features appeared ~6 h after exposure to CD437 and disappeared ~6 h after the culture medium was replaced indicating a reversible effect (Fig. 4*D*). It required ~24 h for the demarcations characteristic of cell-to-cell attachments (Fig. 4*B*) to be reestablished after CD437 was removed (not shown). The morphological changes promoted in normal human epidermal keratinocytes (5) and the ρ^0 clones are apparently unique to CD437. The ρ^0 clones were exposed for $\leq$48 h to 10 μM concentrations of the retinoids ATRA, 9-*cis* retinoic acid, and TTNPB, none of which triggered any discernable alterations in morphology (not shown).

In conclusion, the acute cytotoxic effects of CD437 are contingent on mitochondrial respiration. We speculate that CD437 promotes these effects in parental COLO 16 cells by inhibiting some segment of the electron transport chain. This inhibition results in a rapid decrease in respiration, dissipation of $\Delta\Psi_m$, and ROS production that trigger

MPT and apoptotic cell death. However, determining the exact mechanism involved in the process of MPT induction is outside of the scope of this study and would be better suited for examination in isolated mitochondria rather than intact cells. In the ρ^0 clones, which were selected by their ability to survive via glycolytic energy production (13), the apoptogenic effects of CD437 were lost. This would imply that CD437 and perhaps other structurally related compounds would be more appropriate for cancer prevention or early intervention in the process of carcinogenesis considering that many malignant tumors *in vivo* exhibit high rates of glycolysis independent of oxygen concentration characteristic of the Warburg effect (8, 24). The ability of CD437 to promote phenotypic changes as evidenced by morphological alterations observed in the ρ^0 clones and normal human epidermal keratinocytes (5) points out another possible clue to the activity of CD437. Perhaps studying these changes in the above mentioned cells would be more informative with respect to understanding the molecular biology of CD437. This is presumed, because most of the cancer cell lines examined to date rapidly undergo apoptosis after exposure to CD437 without the involvement of the nucleus (3). Finally, with increasing knowledge connecting mitochondrial manipulation to the death of cancer cells, agents like CD437, which target the mitochondrion, appear to be potentially useful to prevent or treat skin as well as other cancers by exploiting endogenous apoptosis-inducing mechanisms (8).

Acknowledgments

We thank Karen Ramirez in the Department of Immunology at M. D. Anderson Cancer Center for her assistance in the acquisition of the flow cytometry data presented in this study; Rodney Baty in the Toxicology laboratory of Dr. Mary Ann Smith at the University of Texas/Houston School of Public Health for his advice and assistance in the polarographic determinations; Dafna Lotan for her invaluable assistance in retinoid preparation; and Dr. Janet Price for the COLO 16 cell line used in this study.

References

1. Oridate, N., Higuchi, M., Suzuki, S., Shroot, B., Hong, W. K., and Lotan, R. Rapid induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl)hydroxyphenyl]-2-naphtalene carboxylic acid (CD437). Int. J. Cancer, *70:* 484–487, 1997.
2. Hsu, C. A., Rishi, A. K., Su-Li, X., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, U., Shroot, B., Poirer, G. C., and Fontana, J. A. Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. Blood, *89:* 4470–4478, 1997.
3. Marchetti, P., Zamzami, N., Joseph, B., Schraen-Maschke, S., Méreau-Richard, C., Costantini, P., Métivier, D., Susin, S. A., Kroemer, G., and Fromstecher, P. The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. Cancer Res., *59:* 6257–6266, 1999.
4. Sun, S-Y., Yue, P., Shroot, B., Hong, W. K., and Lotan, R. Induction of apoptosis in human non-small cell lung carcinoma cells by the novel synthetic retinoid CD437. J. Cell. Physiol., *173:* 279–284, 1997.
5. Hail, N., Jr., and Lotan, R. The synthetic retinoid CD437 promotes rapid apoptosis in malignant human epidermal keratinocytes and G_1 arrest in their normal counterparts. J. Cell. Physiol., *186:* 24–34, 2001.
6. Susin, S. A., Zamzami, N., and Kroemer, G. Mitochondria as regulators of apoptosis: doubt no more. Biochim. Biophys. Acta, *1366:* 151–165, 1998.
7. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. Science (Wash. DC), *281:* 1309–1312, 1998.
8. Costantini, P., Jocotot, E., Decaudin, D., and Kroemer, G. Mitochondrion as a novel target of anticancer chemotherapy. J. Natl. Cancer Inst., *92:* 1042–1053, 2000.
9. Modica-Napolitano, J. S., Steele, G. D., and Chen, L. B. Aberrant mitochondria in two human colon carcinoma cell lines. Cancer Res., *49:* 3369–3373, 1989.
10. Chance, B., Sies, H., and Boveris, A. Hydroperoxide metabolism in mammalian organs. Physiol. Rev., *59:* 527–605, 1979.
11. Turrens, J. F. Superoxide production by the mitochondrial respiratory chain. Biosci. Rep., *17:* 3–7, 1997.
12. Moore, G. E., Merrick, S. B., Woods, L. K., and Arabasz, N. M. A human squamous cell carcinoma cell line. Cancer Res., *35:* 2684–2688, 1975.
13. King, M. P., and Attardi, G. Isolation of human cell lines lacking mitochondrial DNA. Methods Enzymol., *264:* 304–313, 1996.
14. Wei, Y-H., Kao, S-H., and Lee, H-C. Simultaneous increase of mitochondrial DNA deletions and lipid peroxidation in human aging. Ann. N. Y. Acad. Sci., *786:* 24–42, 1996.
15. Estabrook, R. W. Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. Methods Enzymol., *10:* 41–47, 1967.
16. Zoratti, M., and Szabò, I. The mitochondrial permeability transition. Biochim. Biophys. Acta, *1241:* 139–176, 1995.
17. Hail, N., Jr., and Lotan, R. Mitochondrial permeability transition is a central coordinating event in *N*-(4-hydroxyphenly)retinamide-induced apoptosis. Cancer Epidemiol. Biomark. Prev., *9:* 1293–1301, 2000.
18. Buxser, S. E., Sawada, G., and Raub, T. J. Analytical and numerical techniques for evaluation of free radical damage in cultured cells using imaging cytometry and fluorescent indicators. Methods Enzymol., *300:* 256–274, 1999.
19. Degli Esposti, M. Inhibitors of NADH-ubiquinone reductase: an overview. Biochim. Biophys. Acta, *1364:* 222–235, 1998.
20. Yaguzhinski, L. S., Smirnova, E. G., Ratnikova, L. A., Kolesova, G. M., and Krasinskaya, I. P. Hydrophobic sites of the mitochondrial electron transfer system. Bioenergetics, *5:* 163–174, 1973.
21. Rose, R. C., and Bode, A. M. Biology of free radical scavengers: an evaluation of ascorbate. FASEB J., *7:* 1135–1142, 1993.
22. Fontaine, E., and Bernardi, P. Progress on the mitochondrial permeability transition pore: regulation by complex I and ubiquinone analogs. J. Bioenerg. Biomembr., *31:* 335–345, 1999.
23. Buchet, K., and Godinot, C. Functional F1-ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted ρ^0 cells. J. Biol. Chem., *273:* 22983–22989, 1998.
24. Brand, K. Aerobic glycolysis by proliferating cells: protection against oxidative stress at the expense of energy yield. J. Bioenerg. Biomembr., *29:* 355–364, 1997.

Evidence Supporting a Role for Mitochondrial Respiration in Apoptosis Induction by the Synthetic Retinoid CD437

Numsen Hail, Jr., Emile M. Youssef and Reuben Lotan

Cancer Res 2001;61:6698-6702.