The Intrinsic Mitochondrial Membrane Potential ($\Delta \psi_{\text{m}}$) Is Associated with Steady-State Mitochondrial Activity and the Extent to Which Colonic Epithelial Cells Undergo Butyrate-mediated Growth Arrest and Apoptosis

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ABSTRACT

Transformation of colonic epithelial cells is characterized by decreased mitochondrial activity, increased mitochondrial membrane potential ($\Delta \psi_{\text{m}}$), and disruptions in the equilibrium between cell proliferation and death by apoptosis. We have previously shown that an intact $\Delta \psi_{\text{m}}$ is essential for growth arrest and apoptosis induced by butyrate, a physiological regulator of maturation in these cells, suggesting a role for the $\Delta \psi_{\text{m}}$ in the initiation and integration of proliferation and apoptotic pathways. To extend this work, we have generated isogenic cell lines, from SW620 human colonic carcinoma cells, which exhibit significant differences in intrinsic $\Delta \psi_{\text{m}}$. These differences in $\Delta \psi_{\text{m}}$ are not linked to alterations in viability, Bcl-2 levels, or the differentiation status of the cells. However, compared with parental cells and those with increased $\Delta \psi_{\text{m}}$, cells with decreased intrinsic $\Delta \psi_{\text{m}}$ exhibit significantly higher levels of steady-state mitochondrial mRNA and butyrate-induced p21$^\text{WAF1/CIP1}$ and G0-G1 arrest. Moreover, despite butyrate-mediated translocation of proapoptotic Bak to the mitochondria, fewer cells with elevated intrinsic $\Delta \psi_{\text{m}}$ exhibit concomitant cytochrome $c$ release, and cells with elevated $\Delta \psi_{\text{m}}$ undergo significantly lower levels of $\Delta \psi_{\text{m}}$ dissipation and apoptosis than parental cells, or cells with decreased $\Delta \psi_{\text{m}}$. Homeostasis of the colonic mucosa depends on balancing cell proliferation with apoptosis, and mitochondrial abnormalities are associated with disruptions in this balance.

Thus, by affecting steady-state mitochondrial activity and the extent to which cells enter growth arrest and apoptotic cascades, these data establish a role for the intrinsic $\Delta \psi_{\text{m}}$ in contributing to the probability of colonic tumorigenesis and progression.

INTRODUCTION

The epithelial lining of the colonic mucosa is renewed approximately every 6 days (1, 2). Proliferating cells, localized to the lower two-thirds of the crypt, produce $\sim 10^7$ cells every hour (3, 4); these cells continuously migrate along the crypt-luminal axis, undergoing maturation, differentiation, and, finally, apoptosis and extrusion into the colonic lumen (5–8). Homeostasis of the colonic mucosa is contingent on preserving the equilibrium between proliferation and apoptosis of colonic epithelial cells throughout an individual’s lifetime. Colonic tumorigenesis, on the other hand, is linked to disruption in this balance (2).

We have previously identified depressed expression of mitochondrial genes in the flat mucosa from two high-risk populations (9, 10), thereby implicating alterations in mitochondrial function as an early event in the transformation of colonic epithelial cells, regardless of the etiology. Consistent with a role for mitochondria in the transition to malignancy, decreased mitochondrial gene expression (11), mutations and deletions in the mitochondrial genome (12, 13), and alterations in mitochondrial enzymatic activity have been reported in colonic tumors (14, 15). In addition, compared with the adjacent mucosa, the majority of colonic tumors exhibit elevations in the mitochondrial membrane potential ($\Delta \psi_{\text{m}}$; Refs. 16–20).

The successful progression through many apoptotic cascades includes the translocation of the proapoptotic Bcl-2 family members Bak and/or Bax from the cytosol to the mitochondria, dissipation of the $\Delta \psi_{\text{m}}$, and the release of mitochondrial-associated apoptogenic factors, including cytochrome $c$ (21–27). In the cytosol, cytochrome $c$ drives the recruitment and processing of pro-caspase-9, which, in turn, activates effector caspases-3 and -7 (28–30), ultimately leading to terminal apoptosis. Interfering with $\Delta \psi_{\text{m}}$ dissipation through its elevation, stabilization, or collapse results in delayed, decreased, or blocked apoptosis (21–24). Consistent with this, we have shown that, in SW620 human colonic carcinoma cells, initiation of an apoptotic cascade induced by the short-chain fatty acid NaB, a physiological regulator of maturation pathways in colonic epithelial cells (31–34), is blocked by collapse of the $\Delta \psi_{\text{m}}$ (21, 22, 35). Moreover, we found that $\Delta \psi_{\text{m}}$ collapse also blocked initiation of a NaB-mediated G0-G1 arrest pathway (21, 22, 35), suggesting a role for the $\Delta \psi_{\text{m}}$ in integrating proliferation and apoptotic cascades and, as a consequence, in contributing to the probability of colonic tumor formation and progression (36).

To address this role, we established five isogenic cell lines from SW620 human colonic carcinoma cells. Two of these lines exhibited modest, but highly significant, decreases in intrinsic $\Delta \psi_{\text{m}}$, two exhibited significantly increased in $\Delta \psi_{\text{m}}$, and the $\Delta \psi_{\text{m}}$ of the fifth line was comparable with that of parental cells. Here we report that variations in the intrinsic $\Delta \psi_{\text{m}}$ of the magnitude exhibited by these cell lines are not linked to alterations in cell viability, Bcl-2 levels, or cellular differentiation. However, compared with the parental cells, steady-state mitochondrial mRNA levels are significantly increased in the isogenic lines with decreased intrinsic $\Delta \psi_{\text{m}}$. Moreover, cells with decreased $\Delta \psi_{\text{m}}$ undergo significantly higher levels of NaB-induced p21$^\text{WAF1/CIP1}$ and G0-G1 arrest than parental cells, whereas NaB-mediated cell cycle arrest is significantly lower in cells with elevated $\Delta \psi_{\text{m}}$. Finally, despite increased levels of NaB-mediated mitochondrial translocation of Bak and Bax, significantly fewer cells with elevated intrinsic $\Delta \psi_{\text{m}}$ exhibit concurrent cytochrome $c$ release, and cells with elevated intrinsic $\Delta \psi_{\text{m}}$ undergo significantly lower levels of $\Delta \psi_{\text{m}}$ dissipation and apoptosis than do parental cells and cells with decreased $\Delta \psi_{\text{m}}$.

These data suggest that, by affecting steady-state mitochondrial activity and the initiation and integration of growth arrest and apoptotic cascades, the intrinsic mitochondrial membrane potential plays a role in influencing the probability of colonic tumorigenesis and progression.

MATERIALS AND METHODS

Treatment of Cells with Agents That Target the Mitochondria. SW620 human colonic carcinoma cells (37), obtained from the American Type Culture
Collection, were treated for 24 h with rotenone, antimycin A, or oligomycin B, each at 12.5 μM; azide at 500 μM; valinomycin or nigericin, each at 5 μM (all agents obtained from Sigma, St. Louis, MO) as we have described previously (21, 22, 35).

Quantitation of Cellular Shedding and Apoptosis in Shed Cells. Cellular shedding and DNA fragmentation were quantitated as we have described previously (38). Briefly, confluent cultures of SW620 cells were treated with the agents described above, the conditioned medium was removed, and the cells that had been shed into the conditioned medium were harvested by centrifugation. Shed cells and corresponding adherent cells from the same flask were lysed, and the percentage of nonrandom DNA fragmentation in each cell population, as well as the percentage of DNA recovered in the conditioned medium (an index of shedding), were determined using the diphenylamine (DPA) reaction.

Quantitation of Active Caspases-3 and -9, and Annexin V Staining. Cells were incubated with phycoerythrin (PE)-conjugated anti-active caspase-3 (PharMingen, San Diego, CA). The percentage of stained cells was determined by flow cytometry (Becton Dickinson FACScan; Becton Dickinson Immunocytochemistry Systems, San Jose, CA) as we have described previously (22).

Caspase-9 activity was measured by staining cells with a fluorescent marker, Red-LEHD-FMK (Oncogene Research Products, San Diego, CA), according to the manufacturer’s protocol. Cells were analyzed by flow cytometry (Becton Dickinson FACScan; Becton Dickinson Immunocytochemistry Systems), determining log fluorescence in the FL-2 channel in a minimum of 10,000 cells.

Surface expression of phosphatidyl serine was determined by annexin V staining. The percentage of cells that stained positive for annexin V, but negative for PI, was determined by flow cytometry, using an Annexin V–FITC apoptosis detection protocol (PharMingen).

Generation of Isogenic Cell Lines. Confluent cultures of SW620 cells were treated with the mitochondria-targeted agents as described above (and Refs. 21, 22, and 35). Cells that were shed during treatment were recovered from the conditioned medium by centrifugation, washed with sterile PBS at 37°C, and resuspended in fresh medium supplemented with 1 mM pyruvate and 5 μg/ml uridine. The cells were then seeded into standard tissue-culture dishes. Twenty-four h later, the conditioned medium, containing cells that had not attached, was removed. Fresh medium was added back to the wells and was replaced at 3-day intervals, each time containing 50% less pyruvate and uridine. Thus, by day 12, supplemental pyruvate and uridine were omitted from the medium.

Within 14 days, adherent clones had been established from the cells that had been recovered from the conditioned medium of cultures treated with each of the agents, except for those recovered from valinomycin-treated cultures. Clones were selected, expanded, and the resulting isogenic cell lines were then maintained in MEM plus 10% fetal bovine serum. The lines are referred to as res, AntA, Az, OliB, or Nig, according to the agents to which the parental cells were originally exposed. It is critical to note that all of the experiments using these cell lines were done in the absence of the mitochondria-targeted agents.

Imaging and Quantitative Analysis of the Mitochondrial Membrane Potential (ΔΨm). Parental and isogenic cells were stained with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarboxylic acid iodide; Molecular Probes, Eugene, OR), analyzed by flow cytometry, and imaged as we have described previously (21, 22).

Quantitation of Bel-2 Protein. Bel-2 protein levels were determined using whole cell lysates and isolated mitochondria by ELISA (Oncogene, Boston, MA) according to the manufacturer’s protocol. Mitochondria were isolated as we have described previously (22).

Quantitation of Steady-State mRNA. Real-Time RT-PCR was used to quantify steady-state mRNA levels. RNA was prepared from parental and isogenic cell lines using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 3 μg total RNA with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using the manufacturer’s protocol. cDNA was amplified using SYBR Green PCR Master Mix and the ABI PRISM 7900HT Sequence Detection System Real Time PCR system (Applied Biosystems, Foster City, CA). Primers were designed using Primer Express software (Applied Biosystems) and supplied by Sigma-Genosys (The Woodlands, TX). Human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) Endogenous Control (Applied Biosystems) was used as an internal reference.

Human primer sequences were as follows: COI: forward, CCCACCG-GGTCTAAAGTAT, and reverse, TGCAGCACTTATCGTGTC; COII: forward, GCTGTCGCCACATATTGCTT; and reverse, GCGTGG- GAAAGTGGTGTGGT; ND6 forward, TCCCTAAAGCCATCTCGAA, and reverse, GCCGCCTAGTCTCAAGAGTACTG.

Dissociation curve analysis was performed on PCR products to confirm amplification specificity. Steady-state expression was determined in triplicate using GAPDH as a reference. Relative levels of expression were quantified by calculating 2−ΔΔCT, where ΔΔCT is the difference in CT (the cycle number at which the amount of amplified target reaches a user-defined threshold) between the gene of interest and the reference (GAPDH).

Quantitation of ALP Activity. Sigma Diagnostics Procedure No. 104 (Sigma) was modified to a 96-well format. Briefly, cells were seeded into 96-well plates and were allowed to grow to approximate confluence. Cells were washed once with ice-cold PBS, 0.25% sodium deoxycholate was added to each well, and cells were lysed by agitation for 15 min followed by freezing at −20°C. Plates were thawed and agitated again, and a portion of the lysate was removed from each well for protein determination. The remaining lysate was used for the determination of ALP activity as described in the manufacturer’s protocol.

Quantitation of Cell Cycle Arrest and Apoptosis. Confluent cultures of parental SW620 cells and the isogenic cell lines were exposed to 5 mM NaB (Sigma) for 16–24 h. Cell cycle parameters and terminal apoptosis were analyzed by staining cells with PI and determining the percentage of cells localizing in each phase of the cell cycle and in the subdiploid region, by flow cytometry, as we have described previously (21, 22, 35).

Levels of p21WAF1/CIP1 were quantified by ELISA according to the manufacturer’s instructions (Calbiochem-Novabiochem, San Diego, CA).

Immunofluorescence Analyses of Bax and Bak Translocation, and Cytochrome c Release. Cells were cultured overnight on preferred glass coverslips (Fisher, Pittsburgh, PA) followed by treatment with 5 mM NaB for 20 h. The cells were then fixed for 15 min in 4% paraformaldehyde (Electrode Microscopy Services, Ft. Washington, PA), permeabilized with 0.5% Triton X-100/PBS for 5 min, and incubated for 1 h in 1% BSA/PBS blocking solution. To detect Bax or Bak, cells were incubated for 3 h with their respective rabbit polyclonal antibodies recognizing the NH2 terminus of the protein (both Upstate Biotechnology, Lake Placid, NY; 1:100 dilution), followed by exposure to a goat Cy3-conjugated antirabbit secondary antibody (Amersham Biosciences, Piscataway, NJ). Cytochrome c was detected using a mouse monoclonal anti-cytochrome c IgG (PharMingen; 1:200 dilution), the binding of which was detected with a goat antimouse FITC-conjugated secondary antibody (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis IN). Mitochondria were detected with a mouse monoclonal Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution), and the same goat antimouse FITC-conjugated secondary antibody was used (Roche Diagnostics/Boehringer Mannheim). All of the secondary antibodies were used at a dilution of 1:200 with incubation for 1 h. To visualize nuclei, cells were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

Cells were visualized with a BX60 Olympus fluorescence microscope (Olympus, Melville, NY), and all images captured with a SPOT RT Diagnostic Instruments charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI). The percentage of cells exhibiting Bax or Bak localization to the mitochondrial membrane, with and without concurrent cytochrome c release, was determined by counting 200 cells/coverslip. Counts, performed blinded with regard to the cell line and treatment, were made on triplicate coverslips in each of three independent experiments. Therefore, a total of 1800 cells were scored for each cell line and each treatment.

Statistical Analyses. Data from at least three independent determinations were analyzed using two-sample Student’s t tests. Mean data were compared using linear regression analysis.

RESULTS AND DISCUSSION

Generation of Isogenic Cell Lines Using Mitochondria-targeted Agents. Our earlier work has shown that neither valinomycin nor nigericin, agents that directly target the mitochondrial membrane potential (ΔΨm), nor rotenone, antimycin A, azide, or oligomycin B, which indirectly alter the ΔΨm, affect baseline cell

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cycle parameters or induce apoptosis of SW620 human colonic carcinoma cells (21, 22, 35, 38). However, we report here that, compared with untreated cultures, treatment of SW620 cells with each of these agents, except azide, induces significant shedding of cells into the conditioned medium (Fig. 1a).

Previous studies have demonstrated that colonic carcinoma cells harvested from conditioned medium of untreated cultures are apoptotic, as are the cells shed during exposure to various inducers of apoptosis (38, 39). Consequently, we were surprised to find that the SW620 cells that were shed during treatment with the mitochondria-targeted agents exhibited less nonrandom DNA fragmentation than the cells recovered from the conditioned medium of untreated cultures.

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**Fig. 1. Generation of isogenic cell lines.** Mitochondria-targeted agents enhance cell shedding, but the shed cells are not apoptotic, the loss of adhesion is not a signal for anoikis, and the shed cells can reattach to culture dishes to produce adherent clones. 

- 1a, cellular shedding expressed as the percentage of DNA recovered in conditioned medium; 
- 1b, percentage of DNA fragmentation in shed cells; 
- 1c, percentage of shed cells staining positive for active caspase-3; 
- 1d, percentage of shed cells staining positive for annexin V but negative for PI; 
- 1e, percentage of DNA fragmentation in cells recovered from conditioned medium after reseeding of shed cells. Data are expressed as the means of at least three determinations ± SD; * $P \leq 0.01$; # $P \leq 0.05$, compared with untreated cells.
Moreover, compared with cells harvested from the conditioned medium of untreated cultures, the levels of active caspase-3 and annexin V staining were significantly decreased in the cells shed in cultures treated with the mitochondrial agents. Therefore, despite enhanced shedding, the majority of shed cells recovered from the media of cultures treated with mitochondria-targeted agents did not exhibit characteristics typical of apoptosis.

Survival of colonic epithelial cells in vivo depends on matrix adhesion, with the loss of cellular attachment triggering a form of apoptosis termed anoikis (40, 41). To determine whether the shedding associated with exposure to the mitochondria-targeted agents was a signal for anoikis, we replated cells recovered from conditioned medium. Sixteen and 24 h later, the medium was removed, the unattached cells were harvested by centrifugation, and the percentage of DNA fragmentation was determined as an index of apoptosis. After both time points, DNA fragmentation levels in the unattached cells were not elevated above those of the cells originally plated (Fig. 1e; 0 h). Thus, for a period of 24 h, the loss of adhesion that accompanies exposure of SW620 cells to the mitochondria-targeted agents used in this study was not a signal for anoikis.

Although the mitochondria-targeted agents used here enhanced cell shedding, the shed cells did not exhibit characteristics typical of apoptosis or anoikis. Moreover, the shed cells had the ability to form adherent clones. Cell lines were established from these clones and are referred to as Rot, AntA, Az, OliB, or Nig. It is essential to note that, although these isogenic lines were established after treatment with mitochondria-targeted agents, maintenance of the cell lines and the experiments reported here were in the absence of the agents.

Isogenic Cell Lines Exhibit Significant Variations in Intrinsic $\Delta \psi_{m}$ That Are Not Associated with Coincident Variations in Bcl-2 Levels. Because the agents used to establish the isogenic cell lines directly or indirectly affect the $\Delta \psi_{m}$ (21, 22), we asked whether the cells exhibited alterations in $\Delta \psi_{m}$. Parental SW620 and isogenic cells were incubated with JC-1, a fluorescent dye that is taken up by the mitochondria where it forms $\Delta \psi_{m}$-dependent complexes, or “J-aggregates.” J-aggregates exhibit emission at 590 nm, within the orange range of visible light (42), and the intensity of this emission is a reflection of the $\Delta \psi_{m}$ (21, 22).

Compared with parental cells, the isogenic cell lines exhibited a range of alterations in $\Delta \psi_{m}$. The intrinsic $\Delta \psi_{m}$ exhibited by the Rot line was comparable with that of parental cells. However, modest but highly significant increases in $\Delta \psi_{m}$ were detected in the AntA and Nig lines, whereas the Az and OliB lines exhibited significant decreases in $\Delta \psi_{m}$ (Fig. 2a; *, $P \leq 0.001$). Moreover, the $\Delta \psi_{m}$ of the AntA and Nig lines was approximately double that of the Az and OliB lines.

Representative micrographs corroborate these alterations in intrinsic $\Delta \psi_{m}$. Compared with parental cells, the Az cell line showed a decrease in JC-1 emission, or less extensive J-aggregate formation. In
The isogenic cell lines have been maintained in culture for >1 year. The data presented in Fig. 2a represent the mean ± SD of at least five independent determinations of Δψm (each in triplicate), made at intervals of ~8 weeks. Among these determinations, the coefficient of variation for JC-1 staining of the isogenic cell lines was comparable with that seen in the parental line, suggesting the absence of substantial drift in intrinsic Δψm over this time period.

Because overexpression of Bcl-2 has been linked to the elevation of the Δψm (23, 24), we quantified Bcl-2 protein in whole cell lysates and in isolated mitochondria by ELISA. As shown in Fig. 3, Bcl-2 levels in either whole cells (Fig. 3, filled bars) or in association with mitochondria (Fig. 3, open bars) were comparable among the isogenic and parental SW620 cells. Therefore, it is unlikely that the variations in intrinsic Δψm exhibited by the isogenic cell lines were linked to coincident variations in Bcl-2 levels.

**Intrinsic Δψm Is Linked to Steady-State Mitochondrial mRNA Levels.** Decreased mitochondrial gene expression is an early marker of colonic cancer risk and tumorigenesis (9, 43), and the Δψm is elevated in colonic tumors (16–19, 20). Therefore, we asked whether the variations in intrinsic Δψm exhibited by the isogenic cell lines were accompanied by alterations in the steady-state expression levels of mitochondrial mRNA.

The double-stranded, 16,569-bp, human mitochondrial genome is configured into a heavy and a light strand, which are polycistronically transcribed from a single promoter region to generate 2 rRNA species and 22 tRNAs that are used in protein synthesis with the genome’s 13 encoded mRNAs (44). Each of the mitochondrial mRNAs encodes a protein that is an integral component of an enzyme complex directly involved in electron transport/oxidative phosphorylation. With the exception of ND6, which encodes a component of NADH dehydrogenase (enzyme Complex I) and is situated on the light strand, genes for the other mitochondrial encoded proteins are located on the heavy strand of mitochondrial DNA (44).

RNA was extracted from parental SW620 and the isogenic cell lines and steady-state levels of ND6, COI and COII (the first and second subunits of cytochrome c oxidase, Complex IV), and nuclear-encoded GAPDH were quantified by real-time RT-PCR. As shown in Fig. 4, the levels of GAPDH mRNA were comparable among the isogenic and parental cell lines. However, compared with parental cells, steady-state levels of the sequences encoded by the mitochondrial genome were significantly increased in the Az and OliB lines, the lines that also exhibited significant decreases in Δψm (Fig. 2).

**Differences in the Intrinsic Δψm Are Not Linked to Differentiation Along the Absorptive Lineage.** Increased activity of the brush boarder enzyme ALP is frequently used as an index of differentiation of colonic epithelial cells along the absorptive lineage (38). Because we have previously reported that induced expression of mitochondrial genes is associated with the elevated activity of ALP (45), we next quantitated steady-state ALP activity levels in the parental and isogenic cell lines.

As shown Fig. 5, ALP activity in each of the isogenic cell lines was comparable with that of parental cells. Therefore, it is unlikely that the differences in intrinsic Δψm, or in steady-state mitochondrial mRNA levels, of the magnitude exhibited in these cell lines have an impact on colonic absorptive cell differentiation.

**Differences in the Intrinsic Δψm Are Linked to Sensitivity of Cells to Enter NaB-mediated Growth Arrest and Apoptotic Cascades.** Although the mechanisms that normally regulate proliferation, growth arrest, and apoptosis of colonic epithelial cells are intrinsically programmed, they are also subject to modulation by external factors. The unbranched short chain fatty acid NaB (butyrate) is a natural constituent of the colonic lumen, produced during the fermentation of fiber by endogenous intestinal bacteria (46, 47). NaB is rapidly taken up by cells where it enters the mitochondria and is metabolized by β-oxidation (48). In addition to functioning as the primary energy source for colonic epithelial cells (46), NaB mediates colonic epithelial cell maturation pathways in vivo and in vitro (31–34) and has been linked to chemoprevention (49–53).
The Intrinsic $\Delta \psi_m$ Affects the Mitochondrial Translocation of Bax and Bak, and the Release of Cytochrome c. Many apoptotic cascades depend on the translocation of proapoptotic Bax and/or Bak from the cytoplasm to the mitochondria. Insertion of these proteins into the outer mitochondrial membrane is associated with dissipation of the $\Delta \psi_m$ and the release of apoptogenic factors, including cytochrome c (28). Cytosolic cytochrome c drives the assembly of the apoptosome, which recruits and processes procaspase-9 which, in parental cells (Fig. 6, ○) or in cells with decreased $\Delta \psi_m$ (Az and OliB). Consistent with the accumulation of cells in G0-G1, there was a reciprocal loss of cells from S phase (Fig. 6c), which was also less extensive in the cell lines with elevated intrinsic $\Delta \psi_m$ than it was in parental cells or in cells with decreased $\Delta \psi_m$ ($*, P \leq 0.01$; $\#$, $P \leq 0.05$ versus parental cells).

The magnitude of NaB-induced $\Delta \psi_m$ dissipation and subsequent apoptosis was also less extensive in the cells with elevated intrinsic $\Delta \psi_m$ than it was in parental cells or in cells with decreased $\Delta \psi_m$ (Fig. 7, a and b). $*, P \leq 0.01$; $\#$, $P \leq 0.05$ versus parental cells).

The data shown in Figs. 6 and 7 are plotted as a linear regression, revealing not only the differences in the levels of p21$^{WAF1/Cip1}$ induction, cell cycle arrest, $\Delta \psi_m$ dissipation, and apoptosis among the cell lines, but also the highly significant relationship between intrinsic $\Delta \psi_m$ and extent to which the cells enter NaB-initiated growth arrest and apoptotic cascades ($P$ values between 0.002 and 0.008).

Our previous work has shown that within 24 h, 5 mM NaB induces significant p21$^{WAF1/Cip1}$ induction in SW620 cells, loss of cells from S phase and their accumulation in G0-G1, dissipation of the $\Delta \psi_m$, activation of caspase-3 and subsequent apoptosis. Furthermore, we have previously established that the initiation of the NaB-induced cell cycle arrest and apoptotic cascades is dependent on the presence of a $\Delta \psi_m$ (21, 35).

To determine whether variations in the level of the intrinsic $\Delta \psi_m$ had an impact on NaB-mediated growth arrest and/or apoptotic cascades, parental and isogenic cell lines were exposed to 5 mM NaB for 16–24 h. After 16 h, the levels of p21$^{WAF1/Cip1}$ induction (Fig. 6a) and the arrest of cells in G0-G1 (Fig. 6b) were less extensive in the cell lines with elevated intrinsic $\Delta \psi_m$ (Nig and AntA) than they were in
turn, activates effector caspases, resulting in apoptotic cell death (reviewed in Ref. 29).

To dissect the relationship between intrinsic $\Delta\psi_m$ and the extent to which the cells enter the NaB-mediated apoptotic cascade, we next investigated mitochondrial translocation of Bax and cytochrome $c$ release. Parental, Az and Nig cell lines were treated with 5 mM NaB for 20 h, stained with anti-Bax and anti-cytochrome $c$, and evaluated by immunofluorescence.

Fig. 8. Variations in the intrinsic $\Delta\psi_m$ have a significant impact on NaB-mediated mitochondrial translocation of Bax and the release of cytochrome $c$. Parental, Az (decreased intrinsic $\Delta\psi_m$), and Nig (elevated intrinsic $\Delta\psi_m$) cells were treated with 5 mM NaB for 20 h and were stained with anti-Bax and anti-cytochrome $c$, followed by appropriate secondary antibodies (described in “Materials and Methods” section). Mitochondrial association of Bax was validated by costaining with Hsp60 (not shown). a, representative immunofluorescence micrographs of Nig cells showing NaB-mediated Bax translocation to the mitochondria with and without concurrent cytochrome $c$ release; top panel, untreated cells; middle panel, NaB-treated cells, mitochondrial-associated Bax with coincident cytochrome $c$ release; bottom panel, NaB-treated cells, mitochondrial-associated Bax without coincident cytochrome $c$ release. The distribution of cells with these staining patterns was then quantified blinded, with regard to cell line and treatment, from a total of 1800 untreated and NaB-treated cells from each cell line. b, NaB-mediated Bax translocation to the mitochondria expressed relative to untreated cells. c, the percentage of NaB-treated cells that stained positive for mitochondrial-associated Bax with and without concurrent cytochrome $c$ release. Data are expressed as means ± SD; *, $P \leq 0.01$, compared with parental cells.
As expected, in untreated cells, Bax was restricted to the cytoplasm, depicted by diffuse cytoplasmic staining, whereas cytochrome c was confined to the mitochondria, demonstrated by its punctate staining (Fig. 8a, top panel). As indicated by the overlay, there was no overlap of cytoplasmic Bax and mitochondrial cytochrome c in these untreated cells.

After treatment with NaB, Bax translocation from the cytoplasm to the mitochondria was readily detected, indicated by its intense punctate staining (Fig. 8a, middle and bottom panels). Mitochondrial Bax localization was validated by costaining for the mitochondrial-specific heat shock protein Hsp60 (not shown).

The translocation of Bax to the mitochondria either was or was not associated with cytochrome c release. Release of cytochrome c in conjunction with Bax translocation was detected as punctate Bax staining, diffuse cytoplasmic cytochrome c staining, and the absence of overlap of cytochrome c and Bax staining (Fig. 8a, middle panel). Mitochondrial retention of cytochrome c, despite NaB-mediated translocation of Bax to the mitochondria, was identified by the punctate staining patterns of both Bax and cytochrome c, and the substantial overlap of Bax and cytochrome c staining (Fig. 8a, bottom panel).

The distribution of cells that exhibited these different staining patterns was then quantified. Levels of mitochondrial-associated Bax in untreated cells were negligible and comparable among the cell lines (>0.5% of cells in each line; \( P > 0.09 \), compared with parental cells). As expected, NaB treatment of each cell line significantly increased the number of cells staining positive for mitochondrial-associated Bax (\( P \leq 8.5 \times 10^{-6} \) compared with untreated cells). Moreover, NaB-induced mitochondrial translocation of Bax was detected in significantly more Az cells than in parental cells (Fig. 8b; *, \( P \leq 0.01 \), consistent with the increase in NaB-mediated \( \Delta \psi_m \) dissipation and apoptosis in cells with decreased intrinsic \( \Delta \psi_m \) (Fig. 7). However, despite the lower levels of NaB-induced dissipation of the \( \Delta \psi_m \) and apoptosis seen in cells with elevated intrinsic \( \Delta \psi_m \), the number of Nig cells that exhibited Bax translocation was also significantly higher than that detected in parental cells (Fig. 8b; *, \( P \leq 0.01 \)).

Cytochrome c was released in conjunction with Bax translocation in the majority of parental and Az cells, with ~70% of the mitochondrial Bax positive cells in each cell line also showing cytosolic cytochrome c (Fig. 8c; hatched bars). However, only ~45% of mitochondrial Bax-positive Nig cells showed coincidental cytochrome c release, with ~55% of Nig cells that exhibited Bax translocation showing no release of cytochrome c (Fig. 8c; solid bars; *, \( P \leq 0.01 \) versus parental).

Similarly, Bax was also efficiently translocated from the cytoplasm to the mitochondria in NaB-treated cells irrespective of intrinsic \( \Delta \psi_m \), but again, cells with elevated \( \Delta \psi_m \) exhibited less frequent release of cytochrome c than did parental cells or those with decreased \( \Delta \psi_m \) (not shown).

Consistent with the decrease in cytosolic cytochrome c release in cells with elevated intrinsic \( \Delta \psi_m \), despite mitochondrial-associated Bax or Bk, the levels of active caspase-9 in Nig cells treated with NaB for 20 h were significantly lower than those in identically treated Az cells (\( P = 0.015 \); data not shown).

Apoptosis is regulated at the level of the mitochondrial membrane by the molecular interactions between pro- and antiapoptotic Bcl-2 family members. Bax and Bak promote \( \Delta \psi_m \) dissipation and the release of factors from the mitochondria that activate caspases, which results in apoptosis. Bcl-2 antagonizes the activities of Bax and Bak, inhibiting apoptosis. We have shown that the proapoptotic activities of Bax and Bak are diminished in cells with elevated \( \Delta \psi_m \), even although Bcl-2 levels in isolated mitochondria are comparable among the cell lines, and despite the efficient translocation of Bax and Bak to the mitochondria.

Although the reasons for the relative inefficiency of Bax and Bak in promoting NaB-induced cytochrome c release, dissipation of the \( \Delta \psi_m \) and apoptosis in cells with increased \( \Delta \psi_m \) are unclear, they may include defects in achieving the conformational modifications in Bax and Bak that promote their insertion into the outer mitochondrial membrane (54, 55). An alternative reason may be that elevations in the \( \Delta \psi_m \) are reflected in, or paralleled by, alterations in the composition of mitochondrial membranes themselves. Bax has been reported to directly interact with VDAC, inducing conformational changes in VDAC that result in cytochrome c-permeable conduits in the outer mitochondrial membrane (26, 27). Although blocking Bax-mediated changes in VDAC conformation does not inhibit the association of Bax with the mitochondria, or its interaction with VDAC, it prevents Bax-mediated cytochrome c release and apoptosis (26). Thus, it is noteworthy that we have recently found significant differences in mitochondrial membrane components, including VDAC, among the isogenic cell lines with alterations in intrinsic \( \Delta \psi_m \).

Furthermore, a number of other factors interact with the mitochondrial membrane (25, 30, 56), modulating mitochondrial and cellular function. The role of these interactions in generating and maintaining the differences in intrinsic \( \Delta \psi_m \) and their impact on mitochondrial activity and the initiation and integration of growth arrest and apoptotic pathways, is under investigation.

In summary, although the mechanisms involved in generating and maintaining the alterations in intrinsic \( \Delta \psi_m \) of the magnitude exhibited by the isogenic cell lines described here do not affect the viability or differentiation status of the cells, and are not associated with coincident variations in Bcl-2 levels, these variations in \( \Delta \psi_m \) have a significant impact on mitochondrial activity and the extent to which cells enter growth arrest and apoptotic cascades.

Combined with previous work that link transformation of colonic epithelial cells to decreased mitochondrial activity, increased \( \Delta \psi_m \), and defects in the equilibrium between proliferation and apoptosis (11, 14–19, 20), these data implicate the intrinsic \( \Delta \psi_m \) in influencing the probability of colonic tumor formation and progression. In this regard, it is notable that we have recently found that the intrinsic \( \Delta \psi_m \) also has a significant effect on the viability of cells grown under adherence compromised and hypoxic conditions.

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ΔΨm, mitochondrial membrane potential, and sensitivity to NaB


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