Growth Properties of Colonic Tumor Cells Are a Function of the Intrinsic Mitochondrial Membrane Potential

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Abstract

Development of malignant transformation in the colonic mucosa includes disruption in the equilibrium between proliferation and apoptosis, decreased expression and deletions of the mitochondrial genome, alterations in mitochondrial enzymatic activity, and elevations in the mitochondrial membrane potential (Δψm). Focusing on the role of the Δψm in tumor development and progression, we generated novel isogenic colonic carcinoma cell lines that exhibit highly significant, stable differences in their intrinsic Δψm. Using these cell lines, we have recently shown that the intrinsic Δψm has a significant influence on steady state mitochondrial activity and the extent to which cells enter butyrate-mediated growth arrest and apoptotic cascades. Here, we report that the Δψm is also profoundly linked to important tumorigenic properties of the cells. Compared with cells with lower Δψm, cells with elevated intrinsic Δψm have an enhanced capacity to (a) respond to hypoxia by avoiding apoptosis and initiating angiogenesis, (b) escape anoikis and grow under anchorage-independent conditions, and (c) invade the basement membrane. Combined with our previous work, these data implicate the intrinsic Δψm of colonic carcinoma cells in determining the probability of tumor expansion and progression. (Cancer Res 2006; 66(3): 1591-6)

Introduction

The colonic mucosa has one of the highest proliferative rates of any adult tissue, with stem cells near the base of the crypt producing ~ 10⁷ new cells every hour (1, 2). As a consequence of this rapid proliferation, over a life span of six to seven decades, >10¹² cell divisions will have taken place in the colonic mucosa. For >90% of patients with colon cancer, the initiation of tumorigenesis is linked to only one of these cell divisions. Therefore, it is likely that subtle shifts in biochemical pathways and/or cell composition have the potential of dramatically affecting the probability of tumor formation.

Our previous work has shown that regardless of the etiology, early steps in the malignant transformation of colonic epithelial cells include depressed expression of the mitochondrial genome (3, 4). Other studies have shown that the subsequent development and progression of colonic tumors are linked to mutations and deletions in the mitochondrial genome (5, 6), alterations in mitochondrial enzymatic activity (7, 8) and elevations in the mitochondrial membrane potential (Δψm; refs. 9–13).

Focusing on the Δψm, we have shown the necessity for an intact Δψm in the initiation of both cell cycle arrest and apoptosis of colonic carcinoma cells induced by the short-chain fatty acid butyrate (NaB), a product of fiber fermentation that is found at high concentrations in the colonic lumen and used by colonic epithelial cells as their principle source of energy (14, 15). More recently, we developed novel isogenic colonic carcinoma cell lines that exhibit subtle, stable differences in their intrinsic Δψm, and showed that these differences in Δψm significantly affect steady state mitochondrial gene expression and the extent to which cells enter short-chain fatty acid butyrate–mediated growth arrest and apoptotic cascades (15).

We have dissected the functional effect of differences in the Δψm, asking whether they were associated with cellular properties that characterize the enhanced probability of colonic tumor expansion and progression. We report here that the modest, but significant, stable differences in the intrinsic Δψm exhibited by the isogenic cell lines are linked to the cell’s response to hypoxia, the ability to survive and grow under anchorage-independent conditions, and the capacity to invade the basement. These data show that differences in the intrinsic Δψm of colonic carcinoma cells are likely tied to the subtle shifts in biochemical pathways and/or cell phenotype that play fundamental roles in determining the probability of colonic tumor progression (15, 16).

Materials and Methods

Generation of isogenic cell lines that exhibit significant stable differences in intrinsic Δψm. We have previously described the establishment of the isogenic cell lines used in this study (15). Briefly, confluent cultures of SW620 human colonic carcinoma cells (17), obtained from American Type Culture Collection (Manassas, VA) were treated for 24 hours with rotenone, antimycin A, or oligomycin B, each at 12.5 μmol/L; or nigericin at 5 μmol/L; or at 500 μmol/L; or at 5 μmol/L (all agents obtained from Sigma-Aldrich, St. Louis, MO) as we have previously described (14, 18, 19). Shed cells were recovered from the conditioned medium, washed, and plated in the absence of the agent. Individual clones that arose from the plated cells were selected and expanded. The resulting isogenic cell lines were maintained in MEM plus 10% fetal bovine serum. The lines are referred to as B, AA, Az, OB, or N, abbreviations of the agent to which parental cells were exposed. It is critical to note that the isogenic cell lines were maintained, and that all of the experiments using the cell lines were done in the absence of the mitochondria-targeted agents.

Determination of Δψm. Cells were stained with the Δψm-dependent fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazol carbocyanineiodide; Molecular Probes, Eugene, OR) and analyzed by flow cytometry in FL-2 as we have previously described (14, 15, 19, 20).

Cell cycle variables and quantitation of apoptosis. Cells were stained with propidium iodide and analyzed by flow cytometry, as we have previously described (14, 18, 19).
Quantitation of active caspase-3 and caspase-9. Active caspase-3 was quantified using phycoerythrin-conjugated anti-active caspase-3 (PharMingen, San Diego, CA) followed by flow cytometry, as we have previously described (19). Active caspase-9 was quantified by staining cells with Red-Mingen, San Diego, CA) followed by flow cytometry, as we have previously quantified using phycoerythrin-conjugated anti-active caspase-3 (PharMingen, San Diego, CA) according to the manufacturer's protocol.

Response to hypoxia. Cells in replicate 96-well plates were grown to ~80% confluence for 6 days before CoCl₂ was added to a final concentration of 100 μmol/L. Twenty-four hours later, one of the plates was used to determine the number of viable cells by the spectrophotometric quantitation of reduction of the tetrazolium salt methylthiazol tetrazolium (Sigma-Aldrich) to formazan (21), and a standard curve was generated by serial dilution of parental SW620 cells. Cells collected from replicate plates were stained with JC-1 for determination of Δψm, or lysed for quantitation of p21 WAF1/Cip1 or Bcl2 by ELISA (Oncogene Research Products, San Diego, CA) according to the manufacturer's protocol. Conditioned medium was collected from replicate plates and used for quantitation of vascular endothelial growth factor (VEGF) by ELISA (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol.

Cell invasion. Cell invasion was determined employing a system that uses a matrix of reconstituted basement membrane protein, derived from the Engelbreth Holm-Swarm mouse tumor, overlaying an 8-μm pore size polycarbonate membrane (Chemicon, Temecula, CA). SW620 and isogenic cell lines were seeded on top of the basement membrane layer, as described by the manufacturer, and incubated at 37 °C for 24 hours. The cells that migrated through, and adhered to the bottom of the polycarbonate membrane, were stained and quantified by absorbance.

Evaluation of growth properties. To evaluate the effect of differences in Δψm on anchorage-independent growth, parental SW620 and isogenic cells were seeded at 1.5 × 10⁵ cells/well into 96-well UltraLow adhesion plates (ULA; Costar, Corning, Inc., Corning, NY), plates that we coated with polyHEMA as described (22), or standard tissue culture treated plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Viable cell number was determined at 2-day intervals for 8 days by the spectrophotometric quantitation of methylthiazol tetrazolium reduction to formazan (21) based on a standard curve generated by parental cells. Growth was confirmed by determination of total cellular protein/well using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Statistical analyses. Data from at least three independent determinations were compared by Newman-Keuls and Bonferroni's multiple comparison tests. Mean data were also evaluated as a function of intrinsic Δψm, determined by JC-1 staining, using linear regression analyses.

Results

Isogenic cell lines with differences in intrinsic Δψm as a system to study the effect of the Δψm on colonic carcinoma cell function. These studies used five isogenic cell lines, referred to as R, Az, OB, AA, and N, which were generated as previously described (15). Cells stained with the Δψm-sensitive dye JC-1 (23–25) and analyzed by flow cytometry in fluorescence detection channel 2 (FL-2), has established that compared with the parental SW620 cells, the intrinsic Δψm of two of these lines, the Az and the OB lines, is significantly decreased; the Δψm of two lines, the AA and the N lines, is significantly elevated; and the Δψm of the R line is comparable to that of parental cells (Fig. 1). Furthermore, whereas the intrinsic Δψm of the AA and N lines are similar to one another, as are the Δψm of the Az and OB lines, the differences in the intrinsic Δψm between the isogenic lines with high (AA and N) and low (Az and OB) Δψm are ~2-fold, and highly significant (Table 1).

Whereas the differences in Δψm among the cell lines are highly significant, they are modest. It is important to appreciate that greater differences in the intrinsic Δψm would likely promote the generation and accumulation of reactive oxygen species, and disrupt electron transport/oxidative phosphorylation as well as the mitochondrial import and subsequent processing of nuclear encoded peptides (26–29). The consequence of such perturbations in essential mitochondrial functions would be loss of viability. Therefore, these isogenic cell lines provide an excellent system with which to dissect the relationship between differences in the intrinsic Δψm that are compatible with cell viability and colonic carcinoma cell function and phenotype.

Differences in the intrinsic Δψm are linked to the cellular response to hypoxia and nonadherent conditions. Expansion and progression of solid tumors inevitably results in areas within the tumor in which the demand for oxygen by growing neoplastic cells exceeds the amount that can be obtained by diffusion from existing blood vessels. As a result, regions of the tumor become hypoxic. Although cells that are unable to tolerate an oxygen-poor microenvironment become necrotic or apoptotic, other cells survive by decreasing their growth rate, and/or activating pathways that block apoptosis and initiate the formation of new blood vessels. Thus, the cellular response to hypoxia could act as a selective pressure, favoring the expansion of cells that can adapt to an oxygen-poor environment.

Similar to hypoxia, CoCl₂ inhibits the degradation of the α subunit of the hypoxia-inducible factor 1 (HIF-1), resulting in

Table 1. Comparison of Δψm of isogenic cell lines

<table>
<thead>
<tr>
<th>Cell lines compared</th>
<th>Fold difference</th>
<th>Newman-Keuls multiple comparison test (P values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R vs. AA</td>
<td>1.14</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>R vs. Az</td>
<td>0.58</td>
<td>&lt;0.001</td>
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<tr>
<td>R vs. OB</td>
<td>0.60</td>
<td>&lt;0.001</td>
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<tr>
<td>R vs. N</td>
<td>1.21</td>
<td>&lt;0.010</td>
</tr>
<tr>
<td>AA vs. Az</td>
<td>0.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AA vs. OB</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AA vs. N</td>
<td>1.06</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>Az vs. OB</td>
<td>1.05</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>Az vs. N</td>
<td>2.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OB vs. N</td>
<td>2.01</td>
<td>&lt;0.001</td>
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HIF-1α accumulation (30). HIF-1α controls the cellular responses to hypoxia, activating the transcription of a number of genes, including those involved in modulation of proliferation, apoptosis, and angiogenesis (31–38). To address the question of whether differences in the intrinsic Δψm were linked to the ability of colonic carcinoma cells to adapt and respond to hypoxic conditions, the isogenic and parental cell lines were exposed to CoCl2-simulated hypoxia for 24 hours. Levels of cellular p21WAF/Cip1 and Bcl2, as well as secreted VEGF, were then quantified, expressed per viable cell, and plotted as a function of the intrinsic Δψm of the cell line (i.e., linear regression; Fig. 2).

Consistent with it being a HIF-1α-responsive gene (39), p21WAF/Cip1 was induced in each of the cell lines following exposure to CoCl2 (Fig. 2; closed circles). However, compared with parental cells, the levels of induction were higher in cells with decreased intrinsic Δψm, lower in cells with elevated Δψm, and inversely linked to the intrinsic Δψm (P = 4 × 10⁻⁴ from linear regression).

Unlike p21WAF/Cip1, the antiapoptotic protein Bcl2 is not transcriptionally regulated by HIF-1α (40). Nonetheless, compared with untreated cells, Bcl2 protein levels were increased in each of the cell lines following exposure to CoCl2-mediated hypoxia. Moreover, whereas the levels of Bcl2 in the cell lines with decreased Δψm were similar to that induced in parental cells, the levels in cells with increased intrinsic Δψm were significantly higher, and there was a significant correlation between the intrinsic Δψm and Bcl2 levels (P = 0.021).

As expected, levels of VEGF, a major HIF-1α target gene which stimulates the migration of endothelial cells into tumors to form new microvessels, increased in each of the cell lines following exposure to CoCl2-mediated hypoxia. However, compared with parental cells, the levels of induction were significantly higher in the cells with elevated Δψm, lower in cells with decreased Δψm and there was a significant relationship between the intrinsic Δψm and VEGF production (P = 0.003). Importantly, the level of VEGF secreted from untreated cells with the highest intrinsic Δψm, the N cell line, was significantly higher than that of parental cells (P < 0.001). We have recently reported that in subclones derived from untreated SW620 cells, similar significant elevations in steady state VEGF production are detected in cell lines with elevated intrinsic Δψm (41).

Because tumor progression could also lead to the selection of cells that have the ability to survive and grow under anchorage-independent conditions, cells were seeded into standard tissue culture wells, a substrate that promotes cell adhesion, or
into ULA wells, which block adherence. The number of viable cells was determined 2, 4, and 8 days after seeding and analyzed as a function of the intrinsic $\Delta\psi$m of the cell line (Fig. 3).

When grown under adherent conditions, the differences in the $\Delta\psi$m had no effect on cell number or growth rates at either 2, 4, or 8 days. In marked contrast, when grown under anchorage-independent conditions, there was a significant association between the intrinsic $\Delta\psi$m and cell number and growth rates. Compared with parental cells, there was less extensive and slower growth of cells with decreased intrinsic $\Delta\psi$m, but more extensive and faster growth in cells with increased $\Delta\psi$m. Moreover, the relationship between the intrinsic $\Delta\psi$m and the ability of cells to undergo anchorage-independent growth increased over time ($P$ values from linear regression analyses range from 0.019 at 2 days to 0.004 at 8 days). The relationship between the $\Delta\psi$m and the ability of cells to grow under anchorage-independent conditions was confirmed using a different source of ULA plates, those that we coated with polyHEMA, and total cellular protein as an index of cell number (Supplemental Figs. S1 and S2, respectively).

To further investigate variations in the ability of cells with different intrinsic $\Delta\psi$m to survive and grow under anchorage-independent conditions, cells were seeded into ULA plates and allowed to grow for 4 days. Levels of p21$\text{WAF/Cip1}$, Bcl2, and secreted VEGF were then quantified, expressed per viable cell, and plotted as a function of the intrinsic $\Delta\psi$m of the cell line (Fig. 2; closed squares).

With the exception of the cell line with the highest intrinsic $\Delta\psi$m, p21$\text{WAF/Cip1}$ levels in cells grown under nonadherent conditions were higher than those in cells grown under adherent conditions, but not as high as in CoCl2-treated cells, and inversely linked to the intrinsic $\Delta\psi$m ($P = 0.019$). In contrast, levels of Bcl2 and VEGF in viable nonadherent cells were comparable with those of adherent cells, and were not associated with differences in the intrinsic $\Delta\psi$m ($P = 0.119$ and $P = 0.241$, respectively). However, it is noteworthy that, as seen in the adherent cells and consistent with our recent work (41), the level of VEGF secreted from anchorage-independent cells with the highest $\Delta\psi$m was significantly higher than that of nonadherent parental cells ($P < 0.01$).

We next asked whether the $\Delta\psi$m-associated modulations in p21$\text{WAF/Cip1}$ levels in cells subjected to CoCl2-mediated hypoxia or nonadherent conditions were linked to growth arrest. For these studies, we focused on the cell lines with the lowest and highest intrinsic $\Delta\psi$m (the Az and N lines, respectively; Fig. 1; Table 1).

As shown in Fig. 4A, compared to cells with higher $\Delta\psi$m, fewer cells with low $\Delta\psi$m levels were in G0-G1, but more cells were in G2-M, after 24-hour CoCl2 treatment. In contrast, 4 days after seeding into ULA plates, more cells with low $\Delta\psi$m levels were in G0-G1, but fewer in G2-M, than cells with higher $\Delta\psi$m. p21$\text{WAF/Cip1}$ could bind to G1 cyclin-CDK complexes inducing arrest of cells in G0-G1, and interact with proliferating cell nuclear antigen resulting in G2-M arrest, depending on the relative abundance of p21$\text{WAF/Cip1}$, CDKs and proliferating cell nuclear antigen (42). Therefore, although the mechanism by which p21$\text{WAF/Cip1}$ induces inhibition of cell cycle progression in CoCl2-mediated hypoxia or anchorage-independence may differ, our data show that the magnitude of growth arrest in cells with low $\Delta\psi$m is significantly greater than that in cells with higher intrinsic $\Delta\psi$m, consistent with higher levels of p21$\text{WAF/Cip1}$ protein in these cells.

There are two main pathways to cell death by apoptosis; one triggered by death receptors on the plasma membrane, the other activated by diverse intracellular stresses. In the first pathway, ligation of cell surface receptors typically initiates the cleavage and activation of procaspases, such as procaspase-8, which can directly activate other caspase members, including caspase-3, ultimately producing apoptosis. In the second pathway, cytochrome $c$, released from the inner mitochondrial membrane, forms a complex with procaspase-9 and Apx1, the "apoptosome," which mediates the cleavage and activation of caspase-9. Active caspase-9 then processes other caspase members, including caspase-3, to initiate a caspase cascade that leads to apoptosis.

Because Bcl2 and other antiapoptotic family members could prevent cytochrome $c$ release and, consequently, caspase-9 activation (reviewed in ref. 43), we investigated how $\Delta\psi$m-associated modulations in Bcl2 protein levels were linked to caspase activation and apoptosis. For these studies, we again focused on the cell lines with the lowest and highest intrinsic $\Delta\psi$m and quantified caspase-9 and caspase-3 activation, and apoptosis following exposure to 24-hour CoCl2-mediated hypoxia or 4-day anchorage-independent growth.

As shown in Fig. 4B, consistent with increased Bcl2, activation of caspase-9 and caspase-3, and apoptosis were not detected in cells with high intrinsic $\Delta\psi$m levels following treatment with CoCl2.
Differences in the intrinsic $\Delta \psi_m$ are linked to the ability of cells to cross the basement membrane. Cells were seeded on top of a matrix of reconstituted basement membrane protein. Cells that migrated through, and attached to the bottom of a supporting polycarbonate membrane, were stained and quantified by absorbance at 560 nm. Mean $\pm$ SEM of at least three determinations were plotted as a function of intrinsic $\Delta \psi_m$ of each cell line as determined by mean emission of JC-1 in FL-2. JC-1 emission by parental cells is indicated by ''p'' on the horizontal axis. $P = 0.007$ from linear regression analysis. Significant differences in invasiveness of isogenic cell lines, compared with that of parental cells, as determined by Bonferroni’s multiple comparison tests: $^*$, $P < 0.01$.

Caspase-9 activation was also not detected in cells with high $\Delta \psi_m$ following nonadherent growth, but caspase-3 activation and apoptosis were slightly induced, but importantly, at significantly lower levels than in cells with low intrinsic $\Delta \psi_m$. In accord with the more robust anchorage-independent growth and survival of cells with high $\Delta \psi_m$ than those with low $\Delta \psi_m$. This caspase-3 activation and apoptosis in the absence of activation of caspase-9 suggests that, in cells with increased $\Delta \psi_m$, anchorage-independence may lead to low level activation of a receptor-mediated apoptotic pathway.

In cells with low $\Delta \psi_m$, the levels of caspase-9 activation were similar following CoCl$_2$ exposure or nonadherent growth, and higher than those in cells with elevated $\Delta \psi_m$. However, caspase-3 activation and apoptosis were significantly higher in cells seeded in ULA plates, suggesting that CoCl$_2$-mediated hypoxia might interrupt the caspase cascade in colonic carcinoma cells with low intrinsic $\Delta \psi_m$, possibly due to increased expression of an inhibitor of apoptosis (44).

**The intrinsic $\Delta \psi_m$ affects the ability of cells to invade the basement membrane.** Finally, tumor expansion and progression could also result in the selection of cells with the ability to invade. Successful invasion requires cells to sever interactions with surrounding cells, cross the basement membrane, and migrate through the extracellular matrix—all the while escaping anoikis (31). Because we found that cells with increased intrinsic $\Delta \psi_m$ had an increased capacity to survive under anchorage-independent conditions, and the $\Delta \psi_m$ of migrating cells (26, 45) and cells at the leading edge of a colony outgrowth in culture (12, 46) is elevated, we reasoned that differences in the intrinsic $\Delta \psi_m$ were likely also linked to the ability of cells to cross the basement membrane.

Cells were seeded onto a thin layer of extracellular matrix protein that covered a polycarbonate membrane, and 24 hours later, the cells that had migrated through the extracellular matrix layer and attached to the bottom of the polycarbonate membrane, were stained and quantified by absorbance. These absorbance data were then plotted as a function of the intrinsic $\Delta \psi_m$ of the cell line, determined by mean JC-1 emission in FL-2. As shown in Fig. 5, cells with elevated $\Delta \psi_m$ were significantly more invasive than those with lower $\Delta \psi_m$, and the intrinsic $\Delta \psi_m$ was associated with invasion ($P = 0.007$).

**Discussion**

In summary, using novel isogenic colonic carcinoma cell lines that have modest, but highly significant and stable differences in $\Delta \psi_m$, we had previously shown the influence of the intrinsic $\Delta \psi_m$ on steady state mitochondrial activity and the extent to which cells enter butyrate-mediated growth arrest and apoptotic cascades (15). Here, we showed the profound relationship between differences in the $\Delta \psi_m$ and important tumorigenic properties. Cells with higher intrinsic $\Delta \psi_m$ have an enhanced capacity to (a) respond to hypoxia by avoiding apoptosis and initiating angiogenesis, (b) escape anoikis and grow under anchorage-independent conditions, and (c) invade the basement membrane. Therefore, these data establish that differences in the intrinsic $\Delta \psi_m$ of colonic carcinoma cells are likely linked to shifts in biochemical pathways and/or cell composition that play fundamental roles in determining the probability of colonic tumor progression (15, 16).

Although the mechanisms involved in producing and maintaining the differences in $\Delta \psi_m$ exhibited by these isogenic cell lines are unclear, they may reflect modulations in the composition of mitochondrial membranes. The $\Delta \psi_m$, apoptosis and metastatic potential are each affected by alterations in mitochondrial membrane phospholipids (47–51), and we have recently found a significant correlation between the expression levels of the outer mitochondrial membrane protein VDAC and the intrinsic $\Delta \psi_m$. Thus, it is likely that variations in $\Delta \psi_m$ are associated with alterations in molecular and/or biochemical processes, regulated by the nuclear genome and/or environmental factors, that affect the generation, regulation, and maintenance of mitochondrial membranes (52, 53). Defining these processes, and dissecting their effect on colonic tumor cell growth properties, are important areas for future investigation.

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