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Mitochondrial Contributions to Cancer Cell Physiology: Redox Balance, Cell Cycle, and Drug Resistance

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Alterations in the biochemistry of mitochondria have been associated with cell transformation and the acquisition of drug resistance to certain chemotherapeutic agents, suggesting that mitochondria may play a supportive role for the cancer cell phenotype. Mitochondria are multifunctional organelles that contribute to the cellular adenosine triphosphate (ATP) pool and cellular redox balance through the production of reactive oxygen intermediates (ROI). Our laboratory has focused on these mitochondrial functions in the context of cancer cell physiology to evaluate the potential role of mitochondria as controllers of tumour cell proliferation. Low concentrations of ROI have been implicated as messengers in intracellular signal transduction mechanisms; thus an imbalance of ROI production from the mitochondria may support cancer cell growth. In addition, suppression of mitochondrial ATP production can halt cell cycle progression at two energetic checkpoints, suggesting that the use of tumor-selective agents to reduce ATP production may offer a therapeutic target for cancer growth control.

KEY WORDS: Mitochondria; redox balance; cell cycle; membrane potential; reactive oxygen intermediates; adenosine triphosphate; resistance.

INTRODUCTION

The focus of cancer research in recent years has been toward the identification and categorization of proteins as either promoters or suppressors of cancer evolution. Rapid progress to define the protein machinery that controls genome integrity, cell cycle progression, and pathways connecting extracellular stimuli to nuclear gene expression has placed candidate cancer-associated proteins onto an expanding map that defines the biochemistry of a cell. To determine the significance of any given protein to cancer development, the basic experimental strategy has been to identify critical gene mutations that influence protein quantity or activity. Although this represents a logical and necessary step in cancer research, it does not address the gaps in our knowledge of cancer in the context of the whole cell (or tissue) as a network, influenced not only by individual proteins but by intracellular physiology in general. Thus, to understand cancer cell evolution, it is also necessary to understand how proteins are regulated by their cellular environment which is defined by the activity of all proteins, protein assemblies, and organelles present within the cell. In an attempt to address one aspect of cancer physiology, we have focused our research efforts toward the potential role that mitochondria play in the malignant cell phenotype. Currently, investigations in our lab address distinct aspects of mitochondrial function: (i) the role that mitochondria play in cellular redox balance, (ii) the energetic role of mitochondria as a requisite for cell cycle progression, and (iii) the relationship between mitochondrial proton gradients and development of resistance to various chemotherapeutic modalities.

REDOX BALANCE AND CANCER

If cancer is considered a biological disease of multicellular species that are dependent on oxygen for

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survival, then it is logical to consider the role that oxygen and its derivatives play in the evolution of cancer. The fundamental role for molecular oxygen (O_2) is to participate in cellular respiration as the ultimate electron acceptor during the mitochondrial synthesis of adenosine triphosphate molecules (ATP). Oxygen is also utilized as a substrate for lipid oxidation reactions at the plasma membrane to generate intracellular signals, in peroxisomes during fatty acid oxidation, during xenobiotic metabolism in the endoplasmic reticulum, and by specialized oxidases of phagocytic cells to generate oxygen radicals useful in pathogen destruction (Chance *et al.*, 1979 for review). One repercussion of oxygen metabolism is the generation of incompletely reduced metabolites which are highly reactive and potentially detrimental to numerous cellular components, a biochemical phenomenon that is classically termed the "oxygen paradox" (Chance *et al.*, 1979). Reactive oxygen intermediates (ROI) are eliminated by substrate-specific enzymes and general antioxidant molecules to combat unwanted intracellular oxidation reactions with the cytoplasm generally maintained as a highly reducing environment (Derman *et al.*, 1993). The term "oxidative stress" is commonly mentioned in the literature to imply a state where ROI outnumber the antioxidant defences of a cell such that irreversible cellular injury is incurred in the form of DNA damage or lipid peroxidation preceding a loss of cell viability. Given this, we would like to qualify our use of the term "oxidative stress" to imply a milder shift in the intracellular antioxidant/oxidant balance (redox balance) which has the potential to modify cell physiology. Increasing evidence suggests that shifts in intracellular redox balance may be a mechanism by which protein activity is controlled in the cell. A growing list of proteins that seem to be controlled by redox balance (at least *in vitro*) contain free sulfhydryl groups primarily supplied by cysteine residues (Hwang *et al.*, 1992). This group of proteins that are either directly or indirectly responsive to shifts in redox balance includes several that are implicated in cancer progression such as transcription factors (AP-1 and NFkB), kinases of the mitogenic signalling pathways (MAPK, BMK1), and cell cycle regulators (p53) (see Powis *et al.*, 1995 for review). These *in vitro* data suggest that redox balance does have the potential to regulate cancer cell growth.

ROI have generally been considered as noxious metabolites that are capable of damaging essential macromolecules; however, with the confirmation that small metabolites like nitric oxide are genuine mediators of biological processes such as maintenance of vascular tone, there is renewed interest in the biological roles of other oxygen derivatives (see Wolin, 1996 for review). Of specific interest to our laboratory is the metabolite hydrogen peroxide (H_2O_2) and the more reactive radical superoxide (O_2^-). Both are by-products of normal cell metabolism; O_2^- is generated by single-electron-transfer reactions to oxygen with predominant intracellular production believed to result from electron transport activity at the mitochondrion (Boveris *et al.*, 1972). H_2O_2 can be generated directly by two electron transfers to oxygen or through the catalytic conversion of O_2^- to H_2O_2 by superoxide dismutases (SOD) localized either in the cytosol (Cu/Zn-SOD) or the mitochondrial matrix (MnSOD) and subsequently neutralized by glutathione peroxidase or catalase (Chance *et al.*, 1979). The role of O_2^- as a defensive toxin utilized by phagocytic cells to kill invading pathogens has been well described, but beyond this, there is also evidence for a role of these metabolites in growth control at nonlethal concentrations (nanomolar to picomolar range) (Murell *et al.*, 1990; Burdon, 1995). Secretion of relatively high concentrations of H_2O_2 by cancer cells in culture has been observed by both our laboratory and others (Szatrowski *et al.*, 1991; Burdon, 1995). In our laboratory we have observed that the addition of catalase but not SOD to the culture medium of cancer cell lines drastically reduces the growth rate of these cells, suggesting that H_2O_2 is a necessary factor for growth (unpublished data). Inhibition of cell growth *in vivo* has also been observed with Walker tumors treated with a chemical agent that mimics SOD activity (Kariya *et al.*, 1995). This suggests that O_2^- may have a similar role in the growth regulation of certain cancer cells.

H_2O_2 treatment of cultured cells has been shown to stimulate mitogen-activated protein kinases (MAPK) that are usually stimulated by growth factor binding to extracellular receptors (Rao, 1996; Guyton *et al.*, 1996). Investigations of the pharmacologic activity of H_2O_2 suggest that it behaves as a reversible inhibitor of phosphatases that leads to enhanced phosphorylation of tyrosine residues on growth factor receptors (Sullivan *et al.*, 1994; Sundaresan *et al.*, 1995; Knebel *et al.*, 1996). Given the influence of H_2O_2 over growth control, identification of the predominant cellular source of H_2O_2 may represent an ideal therapeutic target to reduce the uncontrolled growth that is characteristic of cancer cells. It has been estimated that mitochondria are the major source of ROI from normal tissues such as liver and kidney (Boveris *et*

al., 1972). Localized H_2O_2 production at plasma membrane sites is possible, however, and may reflect the mechanism of H_2O_2 action on nearby phosphatases and growth factor receptors. A novel plasma membrane NADH oxidase that is similar to the NADPH oxidase of phagocytic cells has been shown to produce H_2O_2 in both fibroblasts and liver cells although no similar enzyme has yet been identified in cancer cells (Brightman *et al.*, 1992; Meier *et al.*, 1993; Thannickal and Fanburg, 1995). Our laboratory is currently investigating the predominant sources of H_2O_2 generation in cancer cells with a special interest in cells that show H_2O_2-dependent growth. Reduction of H_2O_2 production from these sources may represent therapeutic targets for the control of cancer cell proliferation.

MITOCHONDRIAL ENERGY PRODUCTION AND CELL CYCLE PROGRESSION

Cells are able to generate chemical energy in the form of nucleotide triphosphates. These molecules are required substrates for key cellular functions like the maintenance of ionic gradients, remodelling of cell shape, and control of intracellular signalling pathways that involve protein phosphorylation and dephosphorylation reactions. ATP is the primary utilizable source of high-energy phosphate within the cell and can be derived from both cytosolic glycolysis and mitochondrial oxidative phosphorylation.

We are currently investigating the relationship between mitochondrially derived ATP and progression through the cell cycle in an attempt to define the energetic dependence of proliferation since most cell cycle research to date has focused on protein interactions that mediate cell division. Our specific hypothesis is that cell cycle progress is sensitive to changes in the energy status of the cell, in particular, to the mitochondrial contribution to overall ATP availability. We propose a model wherein a sensory mechanism exists that has the capacity to detect changes in the amount or availability of ATP and then signals the proteins of the cell cycle machinery to alter the rate of progression through the cell cycle. This would result in a redistribution of the population following energetic stress with an observable accumulation of cells at the proposed energetic checkpoints.

To first define the ATP profile of a cycling population, HL-60 human leukemic cells were separated by elutriation according to cell cycle phase and compared for total mitochondrial mass, mitochondrial membrane potential, and ATP content. Our observations suggest that phase-specific fractions maintain different steady-state levels of cellular ATP relative to mitochondrial mass, suggesting that different cell cycle phases have different energetic characteristics. The observed cycle-dependent differences in ATP content may highlight points during cycle transition which may be sensitive to ATP inhibition, particularly those points where steady-state levels are relatively low, as they are in cells making the transition from G_1 to S (unpublished data).

Further data from our lab suggests that an energy-sensitive sensory mechanism exists and may be an additional property of the known cell cycle checkpoints which monitor changes in environmental factors and integrity of the genome. The HL-60 cell cycle was studied following disruption of mitochondrial energy production by oligomycin, a potent and specific agent that inhibits ATP production at the site of the ATP synthase in the inner mitochondrial membrane. Increasing doses of oligomycin will reproducibly lower total cellular ATP and concurrently induce a significant redistribution of the cell population in a single generation. Using nontoxic doses of oligomycin to ensure that the observed redistribution is not a result of selective loss of cells from a given cycle phase, we were able to demonstrate an accumulation of cells in G_1 with the smallest measurable decrease in ATP (approx. 12%) and further decreases in ATP (20–30%) led to increases in the G_2M population (Fig. 1) (Sweet and Singh, 1995). We propose that energetic checkpoints exist at G_1-S and G_2M boundaries which ensure that cellular ATP content is at or above the critical threshold required for successful passage through that cell cycle phase.

To further explore energetic cell cycle control within the context of cancer therapy we chose to exploit an alteration that is commonly found in tumor mitochondria. It has been observed that the membrane potential component of the overall electrochemical gradient is increased in tumor mitochondria (Chen, 1988 for review). This property is defined by the ability of tumor cells to accumulate and preferentially retain lipophilic, cationic agents (Chen, 1988; Rahn *et al.*, 1991). As prototype compounds, we chose Rhodamine 123 (a monovalent cation) and dequalinium chloride (a divalent cation), which accumulate in the mitochondrion and dissipate the electrochemical gradient that is required for ATP synthesis (Modica-Napolitano *et al.*, 1984; Chen, 1988; Singh and Shaughnessy, 1988). Each agent induced cell cycle effects similar to those of oligomycin in accordance with their ability to reduce

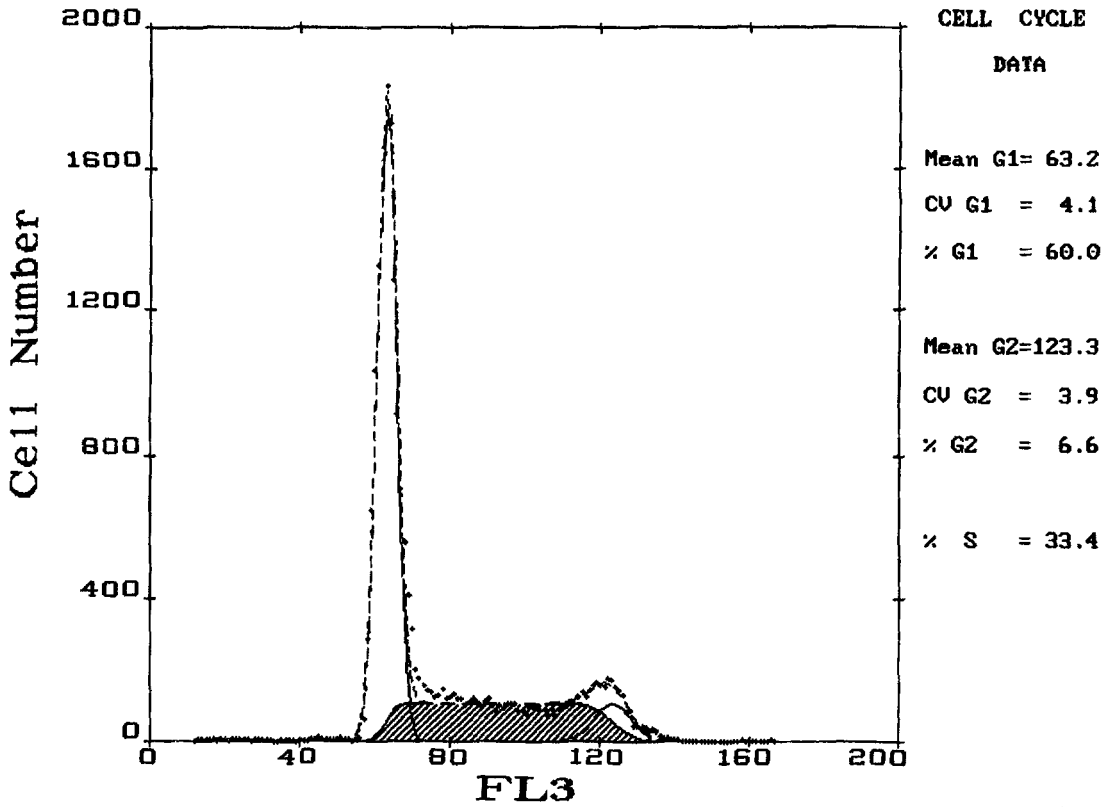

Fig. 1a. Normal cell cycle distribution of an HL-60 population in logarithmic phase of growth. DNA content is represented on the *x*-axis by ethidium bromide fluorescence.

whole cell ATP content (Sweet and Singh, 1995). Considering the mitochondrial selectivity of these agents and newer cationic/lipophilic agents like MKT-077, the potential for control of cancer cell proliferation through the disruption of mitochondrial functions represents a novel therapeutic approach that has already reached early phases of clinical trials (Koya *et al.*, 1996; Weisberg *et al.*, 1996).

The sensitivity of cells to arrest in G_1 with even small reductions in cellular ATP suggests that an active sensing mechanism is in operation to prevent cells from progressing through the cycle without a sufficient supply of ATP substrate. We are currently examining the effect of ATP modulation on cell proliferation at the level of the cell cycle proteins, with the intention of further characterizing the molecular sensing mechanism for ATP depletion. An integral part of cell cycle control involves the formation of protein complexes between cyclins and their respective cyclin-dependent kinases (cdk's). Association of D-type cyclins with cdk4/6 is crucial for the initiation of a new cycle of proliferation while cyclin E/cdk2 complexes are essential for the transition from G_1 into S phase (Ohtsubo and Roberts, 1993; Matsushime *et al.*, 1991). The best characterized substrate for phosphorylation by these complexes is the protein product of the Retinoblastoma tumor suppressor gene (Rbp) (Weinberg, 1995). When this protein is hypophosphorylated, it binds and inactivates the E2F transcription factor, thereby inhibiting its influence on the transcription of genes necessary for progression into S phase. Overexpression of these G_1 cyclins results in hyperphosphorylation of Rbp and rapid acceleration through G_1 while microinjection of antibodies against them causes cells to arrest prior to the onset of DNA replication (Resnitsky *et al.*, 1994; MacLachlan *et al.*, 1995). More recently, a new family of small regulatory proteins has been described which inhibit the kinase activity of cyclin/cdk complexes and so have potential roles as tumor suppressors (Biggs and Kraft, 1995 for review). These cyclin-dependent kinase inhibitors (cdki's: p16, p21, p27) which interact directly with the proteins controlling G_1 progression into S are of particular interest to us as they may represent the final mediators of G_1 accumulation in response to energetic stress.

Our particular interest lies in the identification of changes in the rate/degree of interactions between components of the cell cycle engine in response to a

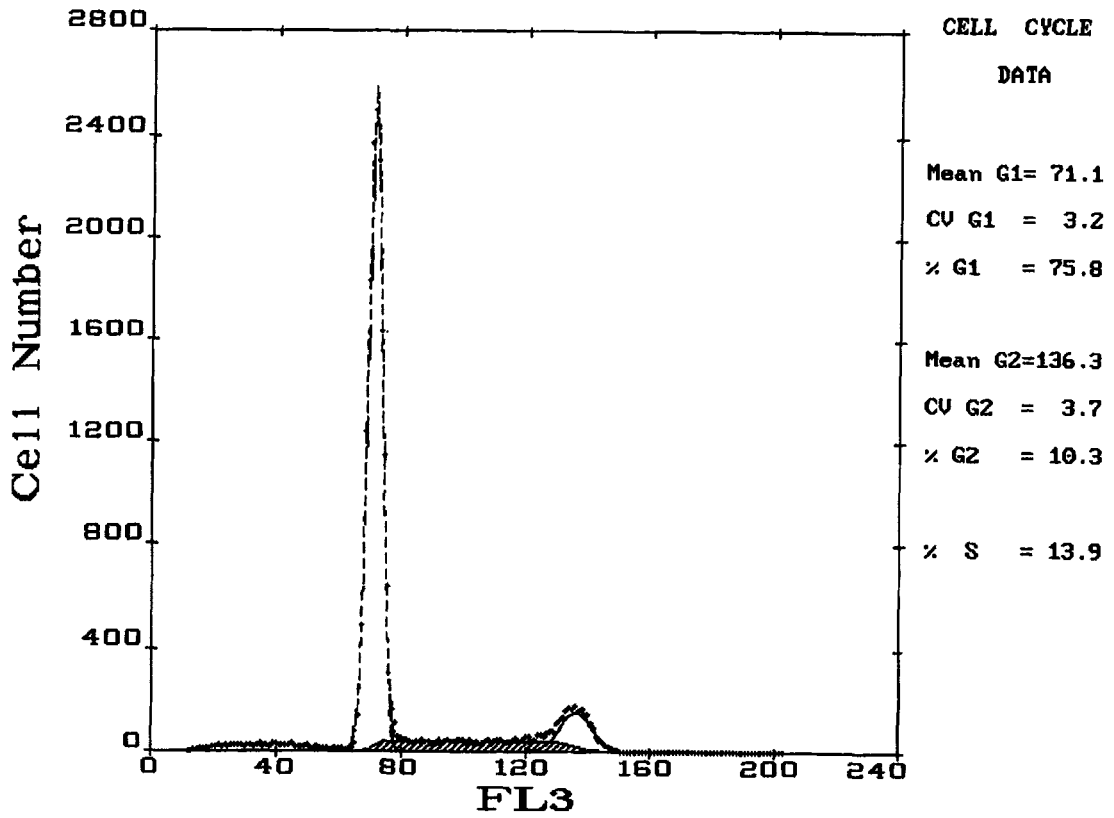

Fig. 1b. The observed cell cycle redistribution following continuous incubation in 0.5 ng/ml oligomycin for 24 hours. Increases in both G1 and G2M fractions are apparent. Cell cycle profiles were generated on a Coulter EPICS IV profile flow cytometer and analyzed by the MCYCLE program for cell cycle distribution histograms (Phoenix Flow Systems Inc., 1991).

decreased supply of ATP fuel. Given the observed increase in the proportion of cells in the G_1 phase of the cell cycle following inhibition of mitochondrial ATP production, we are currently studying associations and activity of the cdk's and their potential inhibition by cdki's. Our preliminary results in p53 (−/−) human leukemic cells suggest that recruitment of the cdki's may not be responsible for the observed G_1 arrest (unpublished data), suggesting that the energetic checkpoint is mediated through another mechanism. Other alternatives that are being investigated in our lab include decreased cyclin synthesis and/or decreased association of cyclins with their appropriate cdks.

MITOCHONDRIAL ALTERATIONS IN DRUG-RESISTANT CANCER CELLS

Our specific interest in the mitochondria as a contributor to cancer cell physiology has developed somewhat from observations that mitochondria have altered properties in cancer cells with acquired resistance to various drug modalities. We have developed and characterized a murine fibrosarcoma cell line that is resistant to photodynamic therapy (PDT) and have observed increased staining density of the mitochondrial cristae under the electron microscope and an apparent elevation in electron transport chain activity and ATP production compared to the parental cell line (Sharkey *et al.* 1993). PDT requires the simultaneous interaction of a photosensitizer (Photofrin II in this case) with energy from visible light of an appropriate wavelength and O_2 to generate highly reactive singlet oxygen (O_2^1) intermediates that damage nearby cellular components. Mitochondria have been implicated as the primary target for PDT based on the localization of specific photosensitizers to this organelle; in this scenario it may be expected that resistance to PDT involves alterations of mitochondrial characteristics (Salet and Moreno, 1990; Henderson and Dougherty, 1992; van Hillegersberg *et al.*, 1994). The significance of these mitochondrial changes, however, to the physi-

ological workings of the cell is a current interest of our laboratory.

Mitochondrial alterations have also been associated with resistance to the chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (cisplatin). Andrews and Albright (1992) observed that cisplatin resistance in a human ovarian carcinoma cell line was accompanied by mitochondrial changes like densely stained cristae under the electron microscope and an elevation in mitochondrial and plasma membrane potentials. Selection of revertants from the cisplatin-resistant population which had lower mitochondrial membrane potentials also lost their cisplatin-resistant characteristic, suggesting a link between the mitochondrial changes and the resistant phenotype (Zinkewich-Peotti and Andrews, 1992b). When the revertant cell population was evaluated for other properties related to cisplatin resistance like glutathione levels, metallothionein levels, and drug accumulation kinetics, again the reduction in mitochondrial membrane potential was the only parameter which correlated with restored cisplatin sensitivity. Our pharmacologic investigations with both PDT-resistant and cisplatin-resistant cells further implicate mitochondrial alterations as an important determinant of drug sensitivity: (i) the cisplatin-resistant variants are cross-resistant to PDT (Sharkey *et al.*, 1993), (ii) the PDT-resistant variants are cross-resistant to cisplatin (Moorehead *et al.*, 1994), and (iii) the cisplatin-resistant variant seems to be more dependent on mitochondrial ATP production given its enhanced sensitivity to oligomycin, an inhibitor of oxidative phosphorylation (Dorward and Singh, 1996; Moorehead and Singh, 1995). Zinkewich-Peotti and Andrews (1992a) have also suggested that the energy-producing role of mitochondria has an influence on cisplatin resistance since elimination of mitochondrial DNA and the encoded electron transport chain proteins restores cisplatin sensitivity in previously resistant cells. Precise identification of the mitochondrial functions which have the potential to influence drug sensitivity may pose novel targets for combination therapies to combat drug resistance.

MITOCHONDRIAL ALTERATIONS AS A PRIMARY TARGET FOR CANCER THERAPY

An increase in the mitochondrial membrane potential is associated with both cell transformation and acquired drug resistance, suggesting a pivotal role in cancer cell evolution. As a general characteristic of cancer cells it may also represent a selective target for chemotherapeutic intervention. As previously mentioned, an elevation in the mitochondrial membrane potential leads to enhanced accumulation and retention of lipophilic cations which can dissipate the electrochemical gradients of mitochondria if accumulated to high concentrations. The antitumour activity of Rhodamine 123 has been demonstrated *in vitro* in human breast and pancreatic cancer cells and in murine melanoma and hepatoma cells (Lampidis *et al.*, 1983; Singh and Moorehead, 1992; Krag *et al.*, 1989; Steichen *et al.*, 1991). *In vivo* studies with mice bearing Ehrlich ascites carcinoma or MB49 bladder carcinoma cells have also shown significant therapeutic benefits in terms of reduced tumor growth when Rhodamine 123 was administered as the sole chemotherapeutic agent (Bernal *et al.*, 1983; Herr *et al.*, 1988). Another lipophilic cation, dequalinium chloride (Deca), has also shown promise as an antitumour agent *in vitro* and *in vivo* (Weiss *et al.*, 1987). We have demonstrated that a cisplatin-resistant cell line which possessed an elevated mitochondrial membrane was hypersensitive to Deca alone as a result of the increased accumulation and retention of this lipophilic cation (Moorehead and Singh, 1995).

Investigations of a new lipophilic cation, MKT-077, are currently at the Phase I/II clinical trial stage. In cultured cells this agent selectively inhibits mitochondrial respiration in tumor cells but not in normal cells; in addition, it was observed that there was a loss of mitochondrial DNA (but not nuclear DNA) in tumor cells, an effect that was not observed in normal cells (Modica-Napolitano *et al.* 1996). MKT-077 has been shown to inhibit tumor cell growth both *in vitro* and *in vivo* and appeared relatively nontoxic to normal tissues (Koya *et al.*, 1996; Weisberg *et al.*, 1996). If these new lipophilic cations become approved clinically, treatment modalities which utilize these agents alone or in combination with other chemotherapeutics may improve the selectivity of tumor therapy with equivalent therapeutic efficacy.

Christman *et al.* (1990) and our laboratory have evaluated the drug combination of Deca with cisplatin as a chemotherapeutic regime. In nude mice injected with human ovarian carcinoma cells, the combination of Deca with cisplatin was capable of extending mouse survival from 50 days to 200 days (Christman *et al.*, 1990). *In vitro,* we have shown that the combination of Deca and cisplatin induces synergistic cell kill in a murine hepatoma cell line and in a human ovarian

carcinoma cell line (Singh and Moorehead, 1992; Moorehead and Singh, 1995). The combination of Deca and cisplatin was also synergistic in a cell variant that was previously resistant to cisplatin alone, suggesting that combinations of these drugs could be useful to overcome drug resistance. Possibly the most important observation, however, was that the combination of Deca and cisplatin reduced the amount of cisplatin required by approximately 90-fold without compromising cell kill. This observation has significant clinical relevance since cisplatin has limited selectivity for tumor cells versus normal cells and a reduction in the cisplatin dose would reduce the side-effects of cisplatin-based therapy while maintaining chemotherapeutic efficacy if a combination of lipophilic cation and cisplatin was administered.

SUMMARY

Our interest in mitochondria and its contribution to tumor cell physiology stems from observations that mitochondria have altered characteristics between normal and tumor cells, and that mitochondria may also contribute to the drug-resistant phenotype of cancer cells. Specifically, we are investigating the role of reactive oxygen intermediates in tumor cell signalling and expect that mitochondria may be a major source of these metabolites. Mitochondria also produce ATP which is essential for many cellular processes including cell cycle progression. Understanding the mechanisms whereby ATP and ROI operate to regulate proliferation may reveal novel targets for cancer growth control.

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