[CANCER RESEARCH 55, 5164-5167, November 15, 1995]

Advances in Brief

Accumulation of Human Promyelocytic Leukemic (HL-60) Cells at Two Energetic Cell Cycle Checkpoints[1]

Susan Sweet and Gurmit Singh[2]

Ontario Cancer Treatment and Research Foundation, Hamilton Regional Cancer Centre and Department of Pathology, McMaster University, Hamilton, Ontario, L8N 3Z5 Canada

Abstract

Agents that disrupt mitochondrial function were used to monitor the contribution of ATP to cell cycle progression. Following nontoxic exposure to these agents, flow cytometric analysis of the cell population showed a significant increase in the proportion of cells in G_1 at low doses of the agent and in G_2-M at higher doses, in accordance with the degree of ATP reduction induced by the compound. These data indicate that cycling cells must maintain a minimal ATP content to satisfy the energy requirement of the checkpoint that allows passage through G_1 into S phase. Once committed, successful passage through G_2 into mitosis is also conditional upon maintenance of a critical ATP content sufficient to satisfy the second energy-sensitive checkpoint that exists at this transition. These data establish a foundation for future investigations into the energy dependence of cell cycle events and propose novel means for cell cycle intervention.

Introduction

Investigations into the mechanisms of cell cycle control have provided insight into the means through which a cell transfers its genetic information to its progeny. In doing so, the importance of the competence of the cell cycle has been stressed, where the slightest loss of integrity can result in the initiation of a cell death program or uncontrolled cell proliferation. The focus in cell cycle research has centered largely around elucidating the molecular events that drive the cell cycle engine. The importance of extracellular influences (1), the role of the cyclin proteins and the cyclin-dependent kinases (2–6) at various stages of the cell cycle, the phosphorylation status of the retinoblastoma tumor suppressor gene (6–8), the influence of p53 (9–12), and the various molecular events that must occur to initiate and end the characteristic processes of mitosis (4, 13, 14) have constituted the major advances in this area of research. Although these molecular events have been well described, there has been no consensus on the energetic dependence of the cell cycle machinery or any description of the link between decreased ATP availability and the resultant cell cycle effects. The effect of inhibiting mitochondrial protein synthesis on the cell cycle distribution has been studied (15, 16) with a result similar to that induced by nutrient deprivation (*i.e.*, G_1 accumulation). However, we examined the effect on cell cycle progression when mitochondrial ATP production is directly inhibited. Oligomycin, an inhibitor of the F_0F_1 ATPase located in the inner mitochondrial membrane, was used first as a positive control for reducing mitochondrially derived ATP. Rhodamine-123 and dequalinium chloride were subsequently chosen due to their lipophilic cationic properties that result in mitochondrial accumulation (17, 18). Many tumor cells are known to maintain a higher mitochondrial membrane potential than their nontransformed counterparts (17), rendering them an attractive target for induction of cytostasis or cytotoxicity in cancer cells. The results of our investigation show that agents that result in decreased cellular ATP content induce either a G_1 or a G_2-M phase accumulation, depending on the degree of ATP reduction. Specifically, a small reduction (approximately 15%) in the level of whole-cell ATP will induce a significant increase in the G_1 portion of the population while further decreases (up to 35%) will elicit a G_2-M accumulation, followed by the onset of cytotoxicity. These data suggest that the checkpoints that regulate passage through those cell cycle positions are sensitive to alterations in the ATP status of the cell. This study, the first to describe G_1 and G_2 energetic checkpoints, presents a novel means for both the analysis and intervention of the cell cycle.

Materials and Methods

Drugs, Cell Lines, and Cell Culture. Oligomycin, rhodamine-123, and dequalinium chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Oligomycin was dissolved in ethanol, with final experimental ethanol concentrations not exceeding 0.1%. Rhodamine-123 was prepared fresh daily in PBS, while dequalinium chloride was dissolved in DMSO prior to dilution in PBS. The HL-60 cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in α-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Counting and Viability. Cells were cultured in media containing the appropriate concentration of drug for 24 or 48 h. After the specified incubation time, cells were counted on a hemocytometer, and viability was assessed using trypan blue exclusion.

ATP Assay. Whole-cell ATP levels were measured using the luciferin/luciferase assay (19). Briefly, cells were rinsed with HBSS before being resuspended in deionized water and boiled for 5 min. Samples were cooled to room temperature prior to the addition of luciferin substrate and luciferase enzyme. Bioluminescence was assessed on a BioOrbit 1253 Luminometer, and whole-cell ATP content (in picograms/cell) was determined using the equation of the line achieved by luminescence of standard dilutions of ATP.

Cell Cycle Analysis. Cell cycle profiles were obtained using a variation of the procedure of Vindelov (20). A cell pellet of 10^6 cells was suspended in Tris buffer (0.10 M Tris, 0.10 M NaCl in deionized H_2O, pH 7.6). Ice-cold lysis solution was added (0.01 M glycine, 0.3 M NaCl, 0.10% v/v Triton-X, pH 10), followed by RNase A (100 μl of 1 mg/ml) and ethidium bromide (50 μl of 0.1 mg/ml, 0.013 mM). The samples were incubated for 10 min at 4°C and warmed to room temperature prior to analysis. Cell cycle profiles of no less than 20,000 cells were generated on a Coulter EPICS IV Profile flow cytometer. Data are expressed as number of cells *versus* the amount of DNA indicated by fluorescence intensity of ethidium bromide. Data are analyzed by the MCYCLE program for cell cycle distribution histograms (Phoenix Flow Systems, Inc.).

Elutriation Protocol. No fewer than 10^7 cells were suspended in 10 ml of supplemented PBS (containing 5 mM glucose, 10 mM sodium citrate, and 5% w/v albumin) and loaded into a Beckman J2-21 centrifuge equipped with a JE-6B elutriation system and rotor. The initial loading speed was 4000 rpm with a continuous flow rate of 17 ml/min. Samples of 100 ml were eluted, starting at 2500 rpm with the rotor speed decreasing by 100 rpm for each consecutive sample. A total of 15 samples was collected, and their DNA profiles were analyzed by flow cytometry.

Received 9/5/95; accepted 10/5/95.

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] This work was supported by Grant MA-8509 from the Medical Research Council of Canada (to G. S.) and a Medical Research Council of Canada Studentship (to S. S.).

[2] To whom requests for reprints should be addressed: Hamilton Regional Cancer Centre, 699 Concession Street, Hamilton, Ontario, L8V 5C2 Canada.

Table 1 *Changes in G_1 and G_2-M phase accumulation with corresponding changes in whole-cell ATP in the presence of increasing doses of oligomycin*

Cells were treated for 24 h after which time, whole-cell ATP was measured using the luciferin/luciferase bioluminescence assay (19). Cell cycle distributions were achieved on a Coulter Epics Profile flow cytometer using standard DNA staining techniques (20). All values are expressed as % control; data represent $n = 3$. ATP data shown above represent one typical experiment.

	% control		
Dose (μg/ml)	ATP	G_1	G_2-M
0.05	88	121 ± 5.9[a]	106 ± 5.8
0.625	83	124 ± 4.2[a]	128 ± 9.4
1.25	85	126 ± 2.5[a]	127 ± 10.3[a]
2.5	62	119 ± 3.9[a]	152 ± 9.2[a]
5.0	59	119 ± 2.8[a]	159 ± 10.1[a]

[a] Significant at $P < 0.05$.

Results

Effect of Oligomycin on Energetic Checkpoints. Using doses of oligomycin ranging from 0.05 μg/ml up to 5 μg/ml, the viability of control samples was approximately 94%, while viability of the treated samples was >90%. These small changes in viability were not sufficient to explain the observed decrease in whole-cell ATP or the observed changes in the cell cycle distribution. Significant reductions in ATP were achieved with doses of oligomycin that did not significantly reduce population viability. At the smallest oligomycin dose that could elicit a detectable change in cellular ATP (at 0.05 μg/ml, ATP is reduced by 12%), a significant increase in the proportion of cells in G_1 (from 56 to 68%, *i.e.*, up to 121% control) was observed (Table 1). There were no further increases in G_1 at higher doses. At higher doses of oligomycin, as ATP continued to decrease, the proportion of cells in the G_2-M phase of the cycle increased (from 10 to 16%, *i.e.*, up to 160% control; Table 1). The most substantial drop in ATP occurs between the doses of 1.25 and 5 μg/ml, where ATP is reduced from ≈80 to ≈60%, and this change correlates with the increase in the G_2-M population of 1.6-fold. The effects of oligomycin were reversible as the cell cycle profiles were similar to those of control samples 36 h after changing the media.

Effect of Rhodamine-123 on Energetic Checkpoints. The initial increase in the G_1 population is not surpassed at higher doses nor does a longer incubation time induce a dose-responsive G_1 accumulation. Longer incubation time does, however, induce further accumulation at each dose (*i.e.*, G_1 accumulation is time and not dose dependent). Increases in dose do, however, elicit an increase in the G_2-M fraction of the cell cycle, which also increases with time. Viability was not significantly affected up to and including 2.5 μM rhodamine-123. The whole-cell ATP levels showed a dose-dependent decrease with increasing rhodamine-123 levels (Table 2).

Effect of Dequalinium Chloride on Energetic Checkpoints. The cell cycle changes observed with dequalinium chloride were similar to those induced by both oligomycin and rhodamine-123. The viability of the cells in doses up to 1 μM was not different from control. Whole-cell ATP levels were observed to decrease with increasing doses of dequalinium chloride (Table 3).

Discussion

Our data indicate that progress through G_1 to S is exquisitely sensitive to small decreases in whole-cell ATP, as evidenced by the fact that the smallest detectable decrease in whole-cell ATP was sufficient to cause a G_1 accumulation that was not surpassed at higher drug concentrations (*i.e.*, lower ATP levels; Fig. 1*a*). Progress through G_2-M also appears to be sensitive to changes in ATP, although accumulation continues to increase as ATP levels decrease (Fig. 1*b*). Significant G_2-M accumulation is not observed until ATP is reduced by 30–40%, whereas G_1 accumulation is evident at a 10% reduction in whole-cell ATP.

The phenomenon of cell cycle redistribution observed in this study lends itself to several possible lines of interpretation: (*a*) only a selective portion of cells at a vulnerable stage in their cycle are susceptible to death induced by these agents, and their loss is the source of both the change in the cell cycle proportions and the fall in ATP; (*b*) the agent used to reduce ATP content is interfering directly with one or several of the molecular cell cycle events, and the decrease in ATP is merely a reflection of the arrested population; and (*c*) the agent is altering the homeostatic balance within the cell (through induction of ionic changes, alterations in pH or redox status for example), and this signals the cell cycle controls to initiate arrest due to an environment of suboptimal conditions for proliferation. The results presented here, however, are unlikely to conform with these explanations for a variety of reasons. In any study of cell cycle redistribution, it is important that a significant proportion of the

Table 2 *Changes in G_1 and G_2-M phase accumulation with corresponding changes in whole cell ATP in the presence of increasing doses of rhodamine-123.*

Cells were treated for either 24 or 48 h prior to cell cycle and DNA analysis. At 48 h, 10 μM rhodamine-123 induced significant cell death.

	% control					
	ATP		G_1		G_2-M	
Dose	24 h	48 h	24 h	48 h	24 h	48 h
2.5 μM	69	56	114 ± 4.2[a]	150 ± 13.4[a]	143 ± 9.4[a]	169 ± 27.5
5.0 μM	67	55	118 ± 3.9[a]	143 ± 9.0[a]	145 ± 13.4[a]	175 ± 14.1[a]
10 μM	56		117 ± 4.1[a]		168 ± 39	

[a] Significant at $P < 0.05$.

Table 3 *Changes in G_1 and G_2-M phase accumulation with corresponding changes in whole-cell ATP in the presence of increasing doses of dequalinium chloride.*

Cells were treated for either 24 or 48 h prior to cell cycle and DNA analysis. At 48 h, 2.0 μM dequalinium chloride caused population viability to fall to ~80% control.

	% control					
	ATP		G_1		G_2-M	
Dose	24 h	48 h	24 h	48 h	24 h	48 h
0.5 μM	83	81	111 ± 0.6[a]	108 ± 0.6[a]	110 ± 3.6	114 ± 5.9
1.0 μM	79	61	116 ± 1.2[a]	124 ± 4.2[a]	121 ± 7.6[a]	148 ± 7.9[a]
2.0 μM	78	58	118 ± 3.2[a]	143 ± 5.7[a]	123 ± 6.7[a]	169 ± 1.35[a]

[a] Significant at $P < 0.05$.

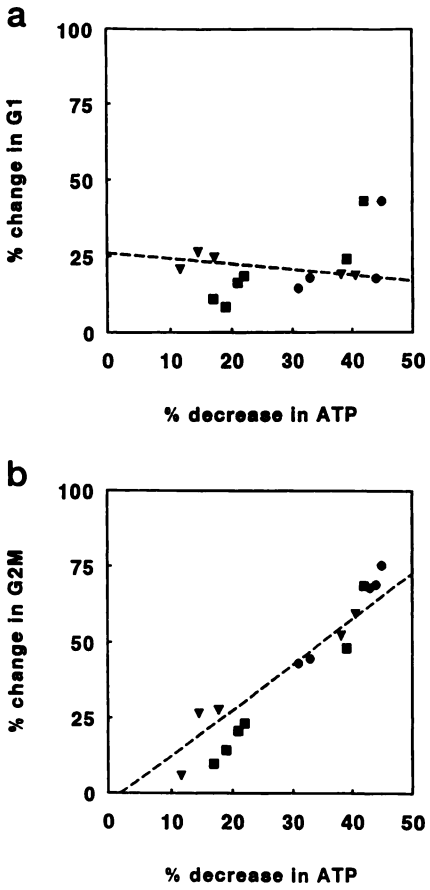

Fig. 1. *a*, changes in the proportion of cells in G_1 following incubation with various agents with respect to control G_1. Decreases in ATP of up to 45% (regardless of agent) induce moderate G_1 accumulation (slope, −0.185; r = 0.743). *b*, changes in the proportion of cells in G_2-M following incubation with various agents with respect to control G_2-M. Decreases in ATP cause a dose-dependent increase in the proportion of cells in G_2-M (slope, +1.5; r = 0.956). ▼, oligomycin; ●, rhodamine-123 (*Rh 123*); ■, dequalinium chloride (*deca*).

population is not lost due to toxicity. For this reason, doses that did not induce substantial cell loss were deliberately chosen.

To confirm that the decrease in ATP was not solely a reflection of a redistribution of the population (*i.e.*, if G_1 cells have less ATP than G_2 cells and the cycle shows increases in G_1, the population would have a different total ATP level), the ATP content of homogeneous G_1, S, and G_2-M populations achieved through elutriation centrifugation was measured. We verified that the redistribution alone was not sufficient to account for the observed decreases in whole-cell ATP.

Furthermore, similar cell cycle effects were observed, regardless of the agent used or the time of incubation. Irrespective of any other potential effect of each agent within the cell, each drug independently induced a decrease in ATP content that resulted in a predictable alteration in the cell cycle profile of these cells.

Our interpretation of these results includes the existence of an energy-sensitive cell cycle checkpoint at both the G_1-S and G_2-M transitions in HL-60 cells. This checkpoint would, in theory, assess the cell's availability of ATP and its capacity to generate ATP throughout the cycle. For example, following a moderate decrease in ATP (≈10%), the ATP status of a cell that had passed the G_1-S transition when the ATP was decreased would again be assessed at the G_2-M border. Because this checkpoint appears to have a lower ATP threshold, these cells would complete the cycle and return to G_0-G_1, where they would be prevented from initiating another cell cycle by the high ATP threshold that exists at that checkpoint. As ATP levels are further decreased, the cells no longer meet the energetic requirement to pass the G_2 checkpoint; therefore, the population exhibits G_2 accumulation at higher doses of ATP inhibitors when the minimum requirement for ATP cannot be met.

It has been proposed (21) that depletion of ATP, independent of mitochondrial membrane depolarization, is alone sufficient to induce lethal cell injury. Using rat hepatocytes, it was shown that ATP levels must fall by at least 15% before viability is affected, and further reductions in ATP could induce the onset of cell death. Our data on ATP reduction and viability are in agreement with these findings.

It has been observed that nutrient deprivation will induce cell cycle arrest at the G_1-S border due to decreased macromolecular synthesis necessary for the remaining cell cycle phases (22). Consistent with our results, Van den Bogert *et al.* (15, 23) used doxycycline to selectively block mitochondrial protein synthesis and showed proliferation arrest in the G_1 phase of the cell cycle and that the arrest is accompanied by an increasing shortage of ATP. The same group also demonstrated that following doxycycline treatment, mammalian cells were no longer able to proliferate if the activity of enzymes involved in oxidative phosphorylation was reduced to 50% and suggested that the lack of ATP-generating capacity was the primary cause of arrest (24). Proliferation continued only if culture conditions allowed for an enhanced rate of glycolysis that could compensate for the loss of mitochondrial ATP production. These studies however did not report any observed G_2 accumulation. The absence of the G_2 phenomenon may be a result of the manner in which ATP synthesis was affected (*i.e.*, indirectly through mitochondrial protein synthesis inhibitors), or it may be attributed to different cellular characteristics of the cell lines involved. The possibility exists that the ATP levels were not reduced sufficiently to induce G_2 arrest or simply that the cell cycles were not monitored at that time. Nonetheless, our data are consistent with these previously reported results, suggesting that energetic checkpoints exist which must be satisfied if a cell is to continue through its proliferative cycle.

Because similar cell cycle effects were seen with reduced ATP regardless of the agent used to achieve the reduction (Fig. 1), it is likely that the observed cell cycle phenomena are related to the change in the energy status of the cell. The practical implications of these findings include the potential use of mitochondrially directed agents in combination with chemotherapy regimens where a synchronous population with a reduced energy-generating capacity is desirable prior to administration of the antineoplastic agent. At the basic cell biology level, the existence of energetic checkpoints at the G_1-S and the G_2-M borders presents a field of inquiry that has experienced only limited investigation. It will now be possible to complement the existing information on the machinery of the cell cycle engine with a consideration of the fuel required to make the engine run. These data also reinforce the idea that mitochondria are sensitive targets for cancer chemotherapy (25). Other energetic processes, such as glycolysis and the creatine kinase pathway, may also be important targets for the induction of cell cycle arrest.

To establish a solid molecular link between the energetic status of the cell and the cell cycle machinery, we are currently examining the effects of decreased ATP content on the levels of cyclins, the activity of the cyclin/cdk complexes, and the inhibitors that control the G_1-to-S transition of the cell cycle.

In conclusion, it is evident that progression through the cell cycle of HL-60 cells is sensitive to changes in mitochondrially derived ATP. Energetic checkpoints exist at both the G_1-S and the G_2-M borders that prevent the cell from continuing through its proliferation cycle when the energetic status of the cell is not sufficient to allow competent cell division.

References

1. Pardee, A. B. G1 events and regulation of cell proliferation. Science (Washington DC), *246:* 603–608, 1989.
2. Sherr, C. J. Mammalian G1 Cyclins. Cell, *73:* 1059–1065, 1993.
3. Pagano, M., and Draetta, G. Cyclin A, cell cycle and oncogenesis. Prog. Growth Factor Res., *3:* 267–277, 1991.
4. Solomon, M. J. Activation of the various cyclin/cdc2 protein kinases. Curr. Opin. Cell Biol., *5:* 180–186, 1993.
5. Hunter, T., and Pines, J. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell, *79:* 573–582, 1994.
6. Sherr, C. J. G_1 phase progression: cycling on cue. Cell, *79:* 551–555, 1994.
7. Goodrich, D. W., Wang, N. P., Qian, Y., Lee, E. Y. H. P., and Lee, W. The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. Cell, *67:* 293–302, 1991.
8. Helin, K., and Harlow, E. The retinoblastoma protein as a transcriptional repressor. Trends Cell Biol., *3:* 43–46, 1993.
9. Frebourg, T., and Friend, S. H. The importance of *p53* gene alterations on human cancer: is there more than circumstantial evidence? J. Natl. Cancer Inst., *85:* 1554–1556, 1993.
10. Rotter, V., Foord, O., and Navot, N. In search of the functions of normal p53 protein. Trends Cell Biol., *3:* 46–49, 1993.
11. Levine, A. J., Momand, J., and Finlay, C. A. The *p53* tumor suppressor gene. Nature (Lond.), *351:* 453–456, 1991.
12. Hartwell, L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell, *71:* 543–546, 1992.
13. O'Connor, P., Ferris, D. K., Pagano, M., Draetta, G., Pines, J., Hunter, T., Longo, D. L., and Kohn, K. G_2 delay induced by nitrogen mustard in human cells affects cyclin a/cdk2 and cyclin B1/cdk2-kinase complexes differently. J. Biol. Chem., *268:* 8298–8308, 1993.
14. Nurse, P. Universal control mechanism regulating onset of M-phase. Nature (Lond.), *344:* 503–508, 1990.
15. Van den Bogert, C., Van Kernebeek, G., De Leij, L., Kroon, A. M. Inhibition of mitochondrial protein synthesis leads to proliferation arrest in the G_1 phase of the cell cycle. Cancer Lett., *32:* 41–51, 1986.
16. Kroll, W., and Schneider, F. Cell cycle kinetics and metabolism of Ehrlich ascites tumor cells in the presence of chloramphenicol as inhibitor of mitochondrial protein synthesis. Z. Naturforsch., *39:* 126–135, 1984.
17. Chen, L. B. Mitochondrial membrane potential in living cells. Annu. Rev. Cell Biol., *4:* 155–181, 1988.
18. Weiss, M. J., Wong, J. R., Ha, C. S., Bleday, R., Salem, R. R., Steele, G. D., and Chen, L. B. Dequalinium, a topical antimicrobial agent, displays anticarcinoma activity based on selective mitochondrial accumulation. Proc. Natl. Acad. Sci. USA, *84:* 5444–5448, 1987.
19. Garewal, H. S., Ahmann, F. R., Schifman, R. B., and Celniker, A. ATP assay: ability to distinguish cytostatic from cytocidal anticancer drug effects. J. Natl. Cancer Inst., *77:* 1039–1045, 1986.
20. Vindelov, L. L. Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. Virchows Arch. B. Cell Pathol., *24:* 227–242, 1977.
21. Nieminen, A., Saylor, A. K., Herman, B., and LeMasters, J. J. ATP depletion rather than mitochondrial depolarization mediates hepatocyte killing after metabolic inhibition. Am. J. Physiol., *267*: C67–C74, 1994.
22. Hartwell, L., Culotti, J., Pringle, J. R., and Reed, J. Genetic control of the cell division cycle in yeast. Science (Washington DC), *183:* 46–51, 1974.
23. Van den Bogert, C., Muus, P., Haanen, C., Pennings, A., Melis, T. E., and Kroon, A. M. Mitochondrial biogenesis and mitochondrial activity during the progression of the cell cycle of human leukemic cells. Exp. Cell Res., *178:* 143–153, 1988.
24. Van den Bogert, C., Spelbrink, J. N., and Dekker, H. L. Relationship between culture conditions and the dependency on mitochondrial function of mammalian cell proliferation. J. Cell. Physiol., *152:* 632–638, 1992.
25. Singh, G., and Moorehead, R. Mitochondria as a target for cancer chemotherapy. Int. J. Oncol., *1:* 825–829, 1992.