Flow Cytometric Localization within the Cell Cycle and Isolation of Viable Cells following Exposure to Cytotoxic Agents

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ABSTRACT

Viable cell sorting, based on flow cytometric analysis of DNA content and cell volume, was used to evaluate the cycle position and survival potential of Adriamycin (AdR)-treated or 1-β-D-arabinofuranosylcytosine (ara-C)-treated CHO cells. Drug-treated cells initially stained with the vital, DNA-specific fluorochrome, Hoechst 33342, were analyzed for DNA content and volume, and sorting “windows” were established for subsequent sorting of duplicate unstained cell samples based only on cell volume. Another portion of the cell sample was fixed in ethanol, and stained with three fluorochromes for correlated flow cytometric analysis of DNA, RNA, and protein. Similarities in the viable cell volume distributions and the protein content distributions of the ethanol-fixed samples provided a means for indirectly determining the DNA and RNA contents of the sorted cells. Three regions (S, L, and I) were selected in the cell volume distributions corresponding to the range of near normal cell size (S), larger than normal cell size (L), and the extremely large cells (I). Adriamycin-treated or ara-C-treated cells sorted from the S region had survival values, respectively, 46 times and 7 times greater than the abnormally large cells in region L. Cells from the S region also respectively survived 14-fold (AdR-treated) and 7-fold (ara-C-treated) greater than the cells sorted from the I regions. RNA content levels for cells within the L region were three times and two times greater, respectively, than the AdR-treated and ara-C-treated subpopulations in the S regions. Survival of subpopulations of G2-arrested, AdR-treated cells (I and L regions) was better correlated with relative abnormality in cell size than with position in the cell cycle. In addition to providing further support for the validity of the “balanced growth hypothesis,” the results of this study suggest that two-parameter DNA content and cell volume measurements would be extremely useful for providing general guidelines for judging the effectiveness of therapy, especially in clinical diagnoses where cell sorting is impractical or impossible. From these analyses the frequency and cycle position of cells resistant to therapy can be estimated. Such information would be particularly useful for rapidly detecting drug-resistant cells and designing of subsequent therapeutic regimens.

INTRODUCTION

Evaluation of the effectiveness of cancer therapeutic agents is often based on information obtained from cytokinetic and tumor cell survival studies performed during or following the initial standard treatment regimen. Primary treatment generally perturbs cell cycle progression and cell proliferation, thereby inducing an abnormal redistribution of cells throughout the cell cycle (1–4). DNA content, cell cycle analysis by FCM2 (5) is a valuable and rapid method for detecting and quantitating the percentage of cells in the various phases of the cell cycle following therapy. The FCM technique is particularly advantageous for studies on therapeutic agents since the method provides “total cell cycle frequency accountability”; that is, the cycle position of all cells in the population can be ascertained irrespective of their rate of cycle progression or their capability for incorporating tritiated thymidine (6). However, FCM-cell cycle analyses cannot distinguish (a) intact cells that have irreversibly lost their reproductive capacity from (b) cells merely experiencing a transitory drug-induced cycle delay and that still retain colony formation capacity. From a clinical standpoint, information on the fraction of surviving cells and their position in the cell cycle would be extremely valuable in designing subsequent therapeutic regimens.

Attempts to overcome some of these analytical limitations have led to the development of FCM techniques that allow for correlated analysis of DNA and other cellular constituents such as RNA (7) or RNA and protein (8). Using the latter approach we previously analyzed RNA/protein and RNA/DNA ratios to detect and quantitate CHO cells in various degrees of “unbalanced growth” at different positions in the cell cycle 15 h after treatment with AdR (9). Drug-treated cells arrested in the G2 + M phase of the cell cycle (i.e., 85%) had RNA/protein and RNA/DNA ratio values elevated 31 and 44%, respectively, above the untreated control G2 + M cells. Few cells remained in S phase (<5%), but cells arrested in G1 (≈10%) had ratio values nearly identical to that of G1 control cells. Population survival (cloning efficiency) of drug-treated cells was 12%. Based on the theory that cells with grossly perturbed macro-molecular ratios (cells in unbalanced growth) are the most likely to be rendered nonviable (10–13), the subpopulation of cells in G2 + M phase in our AdR-treated cultures would be expected to have low survival values, while cells in G1 would have a much higher survival probability. DNA content, cell cycle phase sorting by FCM, and survival studies were not possible in our previous studies (9), since cells must be ethanol fixed for the correlated DNA, RNA, and protein content assay.

In the present study, viable cell sorting, based on DNA content and cell volume, was used to evaluate survival potential of Adriamycin-treated or ara-C-treated CHO cells of normal or abnormal size residing within distinct regions of the cell cycle. The results show an extremely good proportionality between cell volume of viable cells and the protein content of fixed cells that were stained for DNA, RNA, and protein contents. Extrapolation of the cell volume and protein content data permitted viable cell sorting and survival assays to examine indirectly the correlation between the degree of unbalanced growth, as reflected by abnormality of the protein and RNA contents of fixed and stained cells, with cell survival. Viable control and drug-treated cell populations stained for 1 h with the vital, DNA-specific dye, Hoechst 33342, provided FCM-generated bivariate DNA and cell volume profiles that served to accurately set “windows” in the volume distribution that corresponded to cell cycle position as well as cell size. Colony-formation assays performed on unstained, viable cells sorted from the preselected windows in the volume distribution of control and drug-treated populations showed that bivariate DNA content and cell size data provided important information for evaluating drug efficacy with regard to relative fraction of cell survivors in distinct regions of the cell cycle.

MATERIALS AND METHODS

Cell Culture. Suspension cultures of CHO cells were maintained in exponential growth phase in Ham’s F-10 medium containing 15% newborn calf serum, streptomycin, and penicillin (8).
Drug Treatment. AdR (Adria Laboratories, Columbus, OH) was added for a single 2-h interval at a final concentration of 6 or 10 μg/ml to exponentially growing CHO cells. Cells were then centrifuged, washed briefly, and cultured in drug-free medium.

ara-C (Upjohn Laboratory, Kalamazoo, MI) was added to a final concentration of 10 or 50 μg/ml to exponentially growing CHO cells. Cells were continuously exposed to the drug for 24 h.

DNA-specific Staining of Viable CHO Cells with Hoechst 33342. Following AdR treatment (2 h) and subsequent culture in drug-free medium for 14 h, HO 33342 (5 μg/ml) was added to suspension cultures of both drug-treated populations and untreated control cells. After 1-h staining (37°C) in culture medium, an aliquot of each cell was harvested and directly analyzed for DNA content and cell volume by flow cytometry (14). The G1 peak position for viable, Hoechst-stained, AdR-treated CHO cells was decreased about 5 to 8% in the DNA content histograms compared to histograms obtained for untreated Hoechst-stained populations. These results illustrate the effects of AdR on cellular Hoechst uptake and/or fluorescence, and they are similar to those cell-staining results previously shown by Preisler (15) for viable, Hoechst-stained, AdR-treated human bone marrow cells. However, these effects produced no distortion in the cell cycle frequency distribution, based on FCM results obtained from AdR-treated cells fixed in ethanol prior to staining either with Hoechst or with mithramycin.

At 23 h after continuous ara-C treatment, the control and drug-treated CHO cells were stained in suspension culture with HO 33342 as described above, and harvested for correlated FCM analysis of DNA content and cell volume.

Cell Sorting and Survival Studies. Duplicate cultures of drug-treated and control cells were maintained in all studies. One culture from each set was viably stained with HO 33342 (i.e., no fixation procedure required), after which the cells were analyzed via FCM with DNA content and cell volume as the parameters of interest, using a FCM system that was described previously (14). On the basis of the cell volume measurements, three sorting windows designated small (S), intermediate (I), and large (L) were arbitrarily selected. Drug-treated cells in the S window possessed primarily G1 and early S phase DNA contents and a cell volume range equivalent to that of the non-drug-treated control culture. Cells from the drug-treated culture located in the I window contained an abundance of cells that were slightly larger than their non-drug-treated counterparts and DNA contents varying from S to G2 + M phases. The cells in the L volume window were at least 1.5 to 2 times larger than the largest control G2 + M cells. Once sorting windows had been established through use of the HO 33342-stained culture, known number of cells from the duplicate culture that had not been treated with HO 33342 were sorted on the basis of volume differences (i.e., the S, I, and L window) directly into tissue culture dishes containing 5 ml of complete F-10 medium. The dishes were incubated 7 days in a 37°C CO2 incubator prior to staining and determination of the number of colonies (50 or more cells).

DNA, RNA, and Protein Staining. Aliquots of viable, unstained cells were collected from both drug-treated and control cultures, at specified times, harvested by centrifugation, fixed in 70% ethanol as previously described (8), and maintained at 4°C for subsequent FCM analysis and correlation of DNA, RNA, and protein contents (9). Prior to analysis, cells were centrifuged and the ethanol fixative was removed. The cell pellet was then resuspended in a dye solution containing 0.5 μg/ml Hoechst 33342, 1.0 μg/ml pyronin Y, and 0.08 μg/ml fluorescein isothiocyanate to stain DNA, RNA, and protein, respectively (8). Thirty min after staining cells were analyzed in a FCM equipped with three lasers as previously described (16). The intensity of the blue, red, and green fluorescence reflecting relative DNA, RNA, and protein content was correlated for each cell and the protein and RNA contents were determined from the green and red fluorescence measurements, respectively (8). Computer generated "gated" analyses were used to determine the RNA content and cell cycle position (DNA content) of drug-treated, viably sorted cells, from the comparisons made between measurements of volume (of the sorted cells) and protein content (of the fixed cells subjected to multiple parameter FCM analysis).

RESULTS

Cell Volume Sorting and Survival of Drug-treated CHO Cells. Computer-generated, bivariate distributions correlating FCM data obtained for simultaneous DNA content and cell volume measurements provided a means for directly comparing the size range of viable HO 33342-stained cells located in various phases of the cell cycle. Comparison of data obtained for the drug-treated and for the control populations allowed direct examination of drug-induced effects on cell volume and cell cycle position of any abnormal cells. Fig. 1 shows bivariate DNA and cell volume data obtained for a control CHO population (Fig. 1A), and for populations treated for 2 h with 6 μg/ml (Fig. 1B), or 10 μg/ml (Fig. 1C) of Adriamycin, then returned to drug-free medium for 15 h prior to analysis. We are aware that the concentration of AdR used in this study was considerably higher than those used in clinical studies and in some investigations with cell types other than CHO. However, survival of exponentially growing CHO cells even at these high levels of AdR was 10–14%, just as we have found in previous studies (see Ref. 9). These results indicate that CHO cells are somewhat resistant to AdR. In this study we were primarily interested in using drug concentrations that were high enough to decrease population survival to about 10% and to demonstrate further that, under such conditions, cells enter a state of unbalanced growth that is observable as early as 15 h following treatment.
It may be noted in Fig. 1 that AdR treatment perturbed cycle progression and in some instances induced abnormal cell growth, as reflected by the dramatic increase in cell size, particularly for cells in the G₂ + M region of the cell cycle. However, drug-treated cells in G₁ phase retained near normal values for cell volume growth when compared to control G₁ cells (CS region, Fig. 1A). Data shown in Fig. 1 were used to establish the sorting regions designated in S, I, and L in the cell volume distribution. Sorting based on volume was then performed within these preselected windows on unstained control and drug-treated populations, and the percentage of survivors in each sort group was calculated. Survival values for the sorted control cells (Fig. 1A, CS and CL region), and unsorted control cells were identical (data not shown), indicating that cell sorting had no effect on cell survival. Furthermore, microscopic examination of cells showed that, at the time of sorting, >97% of the drug-treated populations had intact membranes as based on exclusion of propidium iodide (17). Thus, we were not dealing with a population of grossly damaged cells, but instead with a population of intact cells with reduced reproductive capacity.

It is apparent from Fig. 1 that long-term cell viability was dramatically decreased with increasing abnormality in cell size. Also it is clear that, irrespective of drug concentration, cells in the G₁ region that maintained near normal size, had the highest survival values. As seen in Fig. 1B, survival of G₁ cells (S region) was 46 times greater than for cells in the L region. However a small percentage of these extremely large cells also survived.

Results obtained from similar studies on ara-C-treated CHO populations are shown in Fig. 2. Electronic gain settings used for DNA and cell volume measurements were different from those used in Fig. 1. The correlation of decreasing survival with increased abnormality of cell size is similar to that noted in Fig. 1. Cells in the S sorted region that maintained near normal size during the 24-h ara-C-treatment period had survival values that were 7 times (Fig. 2B) and 4.6 times (Fig. 2C) greater than cells in the L region. Cells sorted from the I region that were, in general, larger than the largest control cells (Fig. 2A, CL) also had significantly diminished survival values. CHO cell populations are somewhat more resistant to ara-C than to AdR, but the survival and cell volume correlations are similar.

Correlated FCM Analysis of Cellular DNA, RNA, and Protein in Adriamycin-treated CHO Cells. While the data obtained with HO 33342-stained cells (Fig. 1, B and C), indicated that AdR increased the proportion of G₂ + M cells in the imbalanced growth state, much more convincing evidence for this phenomenon is provided in AdR-treated cultures that were fixed and subjected to three-parameter FCM analysis (Fig. 3).

Many of these G₂-arrested cells had abnormally high RNA and protein contents compared to the control exponentially growing CHO population (Fig. 3, top). Few drug-treated cells remain in S phase but cells in G₁ phase have mean cellular protein and RNA content ranges 13 and 10% lower than the mean values of control G₁ cells. These results are similar to those obtained in our previous cell staining and analysis studies (9). However, in those earlier studies, cell sorting and viability assays were not performed in conjunction with the FCM, DNA, RNA, and protein analyses which are performed on ethanol-fixed cells. It may be noted that the bivariate DNA versus protein content distributions of the drug-treated population are similar to the bivariate DNA versus cell volume distributions of the viable cell population shown in Fig. 1C, with the exception that the DNA content resolution in the FCM-generated histograms is better for the fixed cells. For example, the cells arrested in the G₂ + M phase with abnormal cell size (Fig. 1C) also appear to possess abnormal protein content (Fig. 3).

Comparison of Viable Cell Volume and Protein Content Distributions. Before indirect comparisons can be made between volume-sorted, viable cells (Fig. 1) and fixed, multiply stained cells (Fig. 3), it first must be shown that the cell volume profile of unfixed cells correlates moderately well with the protein content profiles of fixed cells. Comparisons of the volume and protein content profiles for both control and AdR-treated cultures are presented in Fig. 4. It is immediately apparent that the agreement between these two parameters is sufficiently close to permit indirect comparisons to be made between the size of viable cells and the protein content of the fixed cell populations. The DNA and RNA content distributions of the AdR-treated
Fig. 4. Cell volume distribution and protein content profiles in unfixed and fixed control and AdR-treated cells. Cell volume distribution for viable control cells (A) and protein content distribution for the same cells (duplicate sample) fixed in ethanol, stained for DNA, RNA, and protein (C). Cell volume and protein content distributions for AdR-treated (6 μg/ml for 2 h followed by 15 h in drug-free medium) are shown in B and D, respectively. Regions S, I, and L in the cell volume distribution were designated sorting regions used in Fig. 1B. Regions S, I, and L of the protein content distribution were obtained by indirect extrapolation of B. Computer-generated gated analysis of the S, I, and L regions in the protein content distribution was used to obtain DNA and RNA content ranges for the sorted viable cells.

Fig. 5. DNA and RNA content distributions obtained by extrapolation of protein content in Fig. 4 for cells sorted over the small (S), intermediate (I), and large (L) size range of the AdR-treated (6 μg/ml) CHO cells.

population obtained by computer-generated gated analysis over the S, I, and L protein content ranges shown in Fig. 4 are shown in Fig. 5. These data show that the degree of abnormality in RNA content also correlates with decreased cell survival. These data also clearly show the position in the cell cycle of the metabolically drug-perturbed cells. It should be noted that the cycle position ranges indicated in Fig. 5, A, C, and E, are in very good agreement with the ranges (DNA content) shown in Fig. 1B for viable cells sorted from the S, I, and L regions. The RNA content range of the small CHO subpopulation (Fig. 5B), obtained by gating on the S region in the protein distribution (Fig. 4D), is within the RNA range of untreated control cells. Survival of sorted viable cells (Fig. 4B, S region) was highest. These cells are predominantly in G1 phase (Fig. 5A). Intermediate size cells arrested in G1 + M (Fig. 5C) predominantly had mean RNA content values slightly larger (i.e., 30%) than the mean value for most normal cells in the G1 + M region. These cells had, by extrapolation, a survival value 3 times greater than G1 + M phase-arrested cells within the L region with extremely large cell volume (Fig. 1C) and a mean RNA content value (Fig. 5F) almost 3 times that of normal cells.

Similar data for RNA content were obtained by gated analyses over the protein content distribution (data not shown) for the ara-C-treated (10 μg/ml) population. As with the AdR-treated cells, protein content and cell volume were extremely well correlated for the ara-C-treated populations. Comparison of the survival values (Fig. 2B) and RNA content range of the S, L, and I subpopulations showed a trend similar to that seen for AdR-treated cells in Fig. 5. Notably, survival of ara-C-treated cells decreased (Fig. 2B, S, I, and L) with progressive increase in abnormality in cellular RNA content. Small, normal size G1 and S phase cells with a mean RNA content 10% above control cells had survival values about 4 times and 7 times greater, respectively, than cells with intermediate (mean RNA content 60% above control values) or large RNA contents (mean RNA content 2 times above control values). Survival also decreases as cells progress further across the cell cycle while continuously exposed to ara-C. Cells in G2 + M had lower survival values (Fig. 2B, L region) and higher RNA content, as well as higher protein contents (data not shown) than did the intermediate (I) subpopulation.

DISCUSSION

The ongoing design of chemotherapeutic regimens could be significantly improved if information on cell kinetics and tumor cell survival were readily available following initial treatment. Correlated analyses of these cellular parameters would be ideal for determining both the cycle position and the relative frequency of cells surviving therapy. To date, no techniques have become available for such determinations. Flow cytometric DNA content analysis has provided a rapid method for detecting drug-induced perturbations in cycle progression and the frequency redistribution of cells within various phases of the cell cycle. However, the fraction of surviving cells in each cycle phase are not available from these analyses.

In this study, we have examined the potential of correlated DNA content and cell volume, coupled with cell sorting and subsequent survival studies, for improving analysis of drug-treated populations. Previous studies (10–12) have shown dramatic changes in cell volume following treatment with various agents. Such changes in cell size are often accompanied by unbalanced metabolism in nucleic acid and protein contents as well (10). The term unbalanced growth (as opposed to normal or "balanced" growth) has been used to designate these cellular metabolic perturbations (10–13). An important prediction of the balanced growth hypothesis is that severe alterations in the content of major cellular macromolecules, such as RNA and protein, should lead to cell death, a result consistent with our findings. In the past, evidence for the validity of the balanced growth hypothesis has been provided chiefly by biochemically measured alterations in population contents of DNA, RNA, and protein (i.e., the measured parameters reflected population...
averages) (10). The correlation, in this study, of survival properties with measurements of volume, DNA, RNA, and protein contents greatly strengthens the conclusions reached in earlier biochemical studies since in the present report, the measured parameters reveal the cycle position, the volumes, and the RNA and protein contents of individual cells comprising large populations.

In terms of specific results, data presented on the effects of two different chemotherapeutic agents, AdR and ara-C, clearly show that subpopulations of cells refractory to drug effects maintain near normal cell volume and retain a state of balanced growth as determined by FCM analyses of RNA and protein contents. In contrast, the largest cells in the drug-treated populations existed in a state of gross biochemical imbalance. The abnormality in volume of the large cells was directly related to the degree of imbalance in RNA and protein, indicating that the observed drug-induced increase in cell volume was not merely related to vacuolation or swelling of cells.

Cell survival was more related to the cell size (and increased RNA and protein contents) than position in the cell cycle. AdR treatment (6 µg/ml), for example, caused a dramatic arrest of about 85% of cells in the G2 phase. However, G2-arrested (I) cells, slightly larger than normal size G2 cells, had survival values that were three times greater than that of the extremely large (L) G2 cells. Normal-size, predominantly G1 cells had survival values 46 times greater than the L cells. Similar abnormal size patterns were also shown for ara-C-treated cells but the magnitude of the decreased survival values was not as great. Interestingly, small (S) cells treated with 10 µg/ml ara-C which possessed a relative survival of 77% contained a significantly large fraction of early S phase cells. This is somewhat surprising in view of the fact that ara-C is presumably an S phase-specific drug. Possibly, in this instance, the ara-C is acting in the capacity of an agent (such as hydroxyurea) that accumulates in cells in early S phase without permitting sufficient synthesis of DNA to cause the cells to enter a state of acute ara-C-specific toxicity (18). Whatever the explanation, the large ara-C-treated cells survived to a somewhat greater extent than the large AdR-treated cells; however, it should be noted that the RNA content data indicate that these cells are not as metabolically perturbed as the AdR-treated cells in Fig. 5F. However, the large ara-C-treated cells still had survival values 7 times less than the normal size (S) cells.

Evaluation of the efficacy of chemotherapeutic regimens is a complex and difficult task. From a clinical standpoint, it is most important to determine quickly the effectiveness of treatment and, as well, to provide an estimate of the frequency and the cell cycle position of drug-resistant subpopulations. It is certainly not feasible, in most instances, to perform cell sorting analyses could easily be acquired by needle biopsy. The analysis is rapid, and information could be available within 12 to 24 h. We recognize that it is not possible in every instance to assign levels of abnormality in cell volume with cell survival; however, it is certain that cells which retain near normal size have a high probability for survival and that survival decreases proportionally with increasing abnormality in cell size. In view of the few analytical tools available to the clinician, rapid FCM analysis as proposed should prove a valuable adjunct method for coupling with the established clinical diagnostic approaches.

A major problem to be overcome if the technique described in this report is to be adapted for routine clinical use is the difficulty in obtaining adequately resolved DNA distributions for Hoechst 33342-treated, nonfixed tumor cells. Several recent reports (20, 21) describe protocols for improving DNA resolution in nonfixed cells that are normally refractory to treatment with HO 33342. Inclusion of these procedures in cell preparatory protocols may increase the clinical feasibility of this approach.

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