Two-cell-divisions-BrdU-pulse-chase Analysis in Human Ovarian Cancer Cells at Two Different Growth Statuses

Hirofumi KOIKE

Department of Obstetrics & Gynecology,
Kawasaki Medical School, Kurashiki 701-0192, Japan

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ABSTRACT. To analyze cell cycle progression dynamically and in detail, a new method was proposed using a modified bromodeoxyuridine (BrdU)-pulse-chase method with a flow cytometer. The method was used to analyze cell cycle progression of two cell divisions in BG-1 human ovarian cells at two different cell growth statuses, initially plated at 1×10^6 or 3×10^6. In this study, cells pulse-labeled with BrdU were divided into six compartments in the S phase; i.e., two segments for BrdU content correspondent to half of the highest channel number and three segments for DNA content correspondent to the G_1, S and G_2/M phases. This division is based on the theory that BrdU content is reduced to half after one cell division. The labeled-cell progression was observed from compartment to compartment. Then this method was compared with the analysis of DNA histograms and conventional BrdU-pulse-chase methods. Detailed cell progression could be identified with the present method more dynamically than with DNA histograms or conventional BrdU-pulse-chase methods, and two different growth statuses could also be distinguished. Therefore, this two-cell-divisions-BrdU-pulse-chase method may facilitate the analysis of complicated alterations of cell cycle progression.

Key words: two-cell-divisions — BrdU-pulse-chase study — flow cytometry — nuclear isolation — overian cancer

The analysis of cell cycle progression has been performed by a flow cytometer (FCM), and the proportions of the G_1, S and G_2/M phases in the cell cycle have been theoretically calculated by mathematical methods with computer simulations proposed by several authors.[1-9] These approaches have been mainly involved the use of DNA histograms. Such DNA histograms have been previously reported to be very useful, but they exhibit frequent inaccuracies due to the high coefficient of variation and small subpopulations in asynchronously growing cell populations.[5,10,11] There is also a possibility that the dynamic progression of the cell cycle, especially in the S phase, cannot be exactly reflected by these conventional methods.[9,10,12] The BrdU labeling index (LI) technique is one method for specifically detecting cells in the S phase.[11,13,14] Higashikubo et al extended this BrdU-LI technique to more dynamic analysis of cell cycle progression in a BrdU-pulse-chase study and demonstrated its usefulness.[15,16] With their method, however, observation was done through only one cell division in the cell cycle progression.

BG-1 human ovarian cells are known to be sensitive to various anti-cancer agents.[17,20] For this reason, we felt it would be worthwhile to investigate

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differences in cytotoxic mechanisms particularly in relation to cell cycle perturbation. Therefore, we decided to carry out dynamic analysis of BG-1 cells using various anti-cancer agents. To the best of our knowledge, this study presents the first data regarding the behavior of untreated BG-1 cells in a BrdU-pulse-chase study.

In this study, we attempted to extend the BrdU-pulse-chase study through two cell divisions using human ovarian cancer cells in two different growth situations with the FCM to determine if better dynamic analysis can be achieved.

MATERIALS AND METHODS

Cells

BG-1 human ovarian carcinoma cells[17,18] were cultured in McCoy's Medium 5A supplemented with 10% fetal bovine serum, 0.05% L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Exponentially growing BG-1 cells were plated at 1×10^6 (group A) or 3×10^6 (group B) cells/75-cm^2 tissue culture flask with 15 ml medium, and then were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in 95% air.

BrdU incorporation assay

On day 3, the cells were pulse-labeled for 30 min with 20 μM BrdU, as previously described.[19] On days 3, 4, 5, 6, and 7, they were removed from the flasks with 0.1% trypsin and 0.01% EDTA and the cell numbers were scored for growth curves. Then the BG-1 cell nuclei were isolated using a previously described method.[20] Briefly, they were washed with 10 ml spinner salts (5.4 mM KCl, 0.4 mM $MgSO_4$, 0.12 M NaCl, 26 mM $NaHCO_3$, 10 mM NaH_2PO_4, 5.5 mM D-glucose), and collected by centrifugation at 2000 rpm for 5 min three times. After resuspension in 10 ml TritonX solution (1% Triton X-100, 0.08 M NaCl, 0.02 M EDTA) for 10 min to remove the cytoplasm and centrifugation at 2000 rpm for 5 min, the pellets were washed with 10 ml of 0.88% NaCl. The isolated nuclei were treated with 1 ml of 2 N HCl for 20 min at room temperature to denature DNA.[19] After centrifugation at 1500 rpm for 5 min, they were neutralized by 1 ml of 0.1 M $Na_2B_4O_7$ for 5 min at room temperature, and centrifuged at 1500 rpm for 3 min. The nuclei were stained with 100 μg/ml propidium iodide (PI) for DNA analysis and with 1% fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody for BrdU analysis. Then, they were incubated overnight at an ice-cold temperature for FCM analysis.

Flow cytometry

Analysis was performed on a FACSort (Becton Dickinson) with a 488 nm excitation beam. Two parameters, PI fluorescence (red, 610 nm) and FITC fluorescence (green, 525 nm), were measured. The dynamic progression in the cell cycle was analyzed by a BrdU-pulse-chase method as follows. The BrdU/DNA distributions of the cells were displayed on two-dimensional (2D) dot-plots and contour-plots. The scales were linear for DNA content (X axis) and logarithmic for BrdU content (Y axis). The BrdU-labeled portion in the

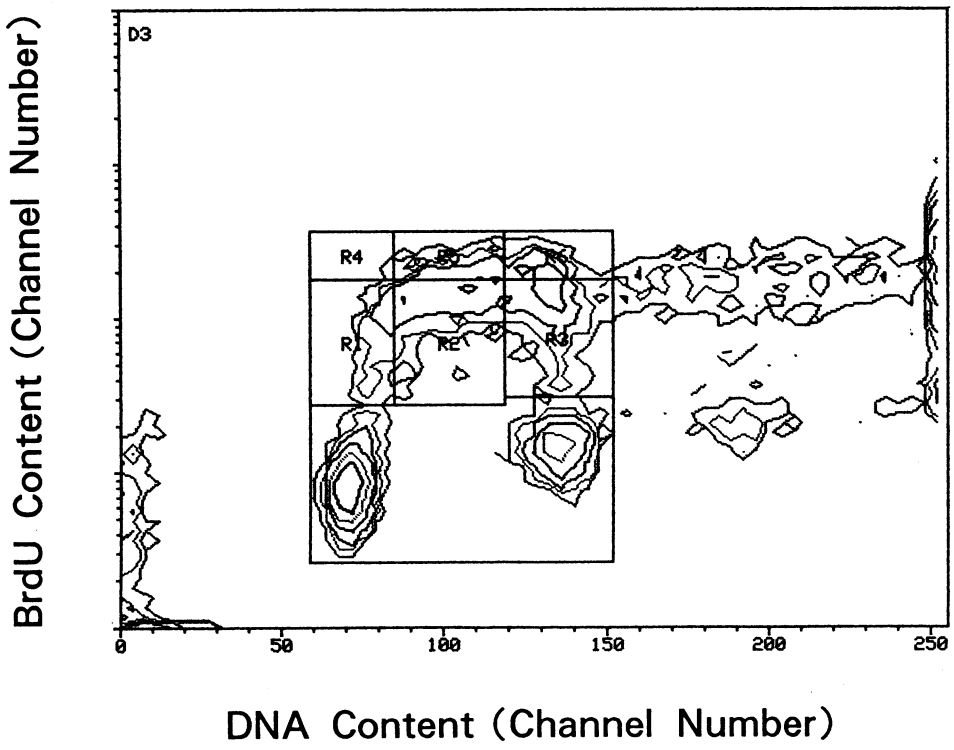

Fig 1. Division of S phase into six compartments. R1 and R4 represent the early S phase, R2 and R5 show the middle S phase, and R3 and R6 indicate the late S phase. The total cell cycle population includes these compartments and the compartment below them, which represents the G_1 and G_2/M phases. This contour-plot is shown with the DNA contents on the X axis (linear scale) and the BrdU contents on the Y axis (logarithmic scale).

S phase was divided into six compartments (R1 - R6) (Fig 1). Since BrdU content is theoretically reduced to half after one cell division,[12,21-24], we divided the BrdU-labeled portion into two segments by dividing it at the channel number corresponding to half of the highest channel number for BrdU content. The compartments of R4, R5, and R6 reflected higher BrdU contents than those of R1, R2, and R3. To measure DNA content, the BrdU-labeled portion was divided into three segments corresponding to the G_1 phase (R1 and R4), S phase (R2 and R5), and G_2/M phase (R3 and R6). With this method, we assumed that the cells pulse-labeled with BrdU would pass the compartments in order of R4, R5, R6, R1, R2, and R3, and then enter into unlabeled levels. For example, the cells in the R5 compartment on day 3 passed through the R6 compartment and entered into the R1 compartment after one cell division, and then shifted into unlabeled portions following the next cell division. Then the percentages of compartments in the whole cell cycle populations were calculated ([Number of cells in compartments]/[Number of whole cell cycle populations]). The percentages of cells in the G_1, S, and G_2/M phases were also analyzed using CellFIT, a cell cycle analysis program.

RESULTS

Cell Growth

Fig 2 shows the growth curves of BG-1 cells. When the BG-1 cells were plated at 3×10^6 (group B), they grew exponentially until day 5, and reached a plateau. On the other hand, when the BG-1 cells were plated at 1×10^6 (group A), they continuously grew until day 7, although a slight slowdown in the cell growth was observed from day 5.

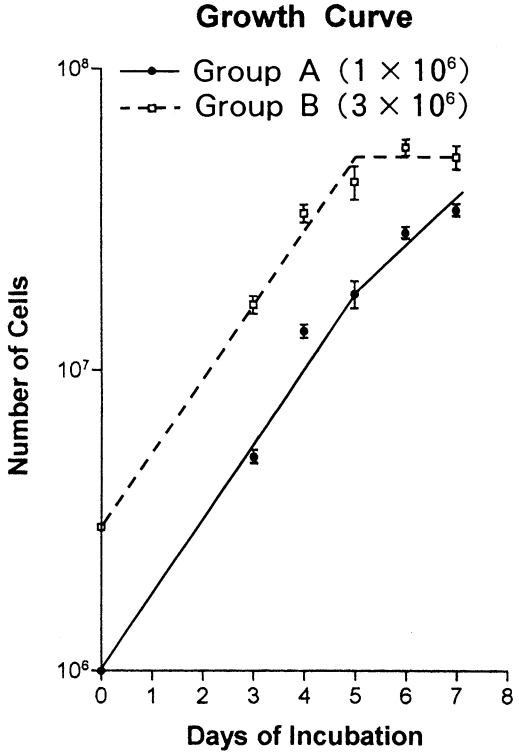

Fig 2. Growth curve of BG-1 cells labeled with BrdU, which were plated at 1×10^6/flask (group A) and 3×10^6/flask (group B). Data are shown with the days of incubation on the X axis and the number of cells on the Y axis.

BrdU-pulse-chase study

Fig 3 shows changes in BrdU/DNA 2D dot-plots and DNA histograms for BG-1 cells from day 3 to day 7. Typical inverted U patterns for the S phase were observed for the day 3 cells at two different growth statuses. Then the patterns changed as a function of incubation time. When the changes in the DNA histograms of groups A and B were compared, no distinguishable differences were observed. Differences in the changes in the patterns of the cells in these two groups on the 2D dot-plots for the BrdU-labeled cells were difficult to recognize visually (Fig 3). Significant differences were observed only in the cells which were not labeled with BrdU.

Fig 4 summarizes the percentage of BG-1 cells in the G_1, S and G_2/M phases calculated by CellFIT (top row), compartments of R1+R4, R2+R5 and

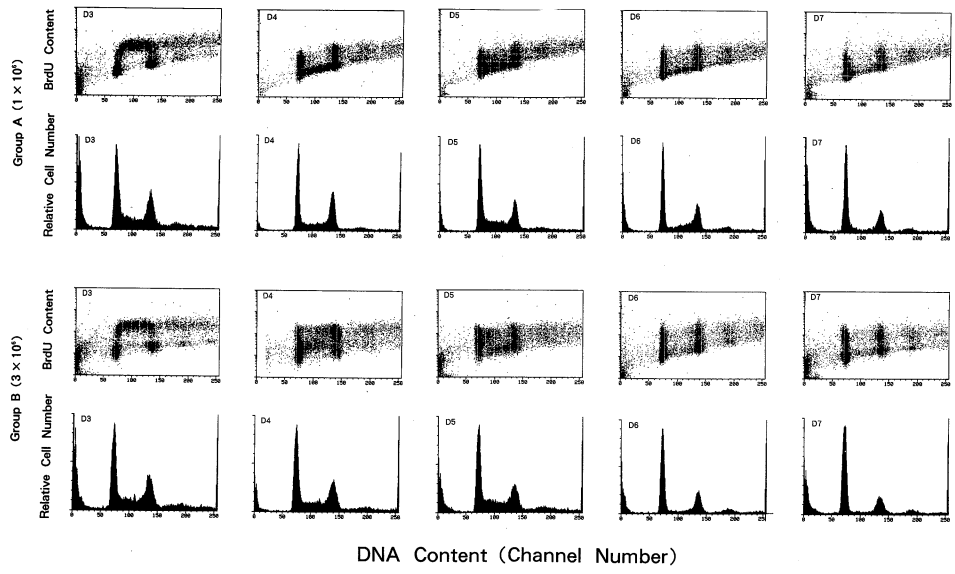

Fig 3. Changes in patterns in 2D dot-plots for BrdU/DNA distributions and DNA histograms. The upper two rows indicate the changes in BG-1 cells plated at 1×10^6/flask and the lower two rows represent those plated at 3×10^6. The BrdU/DNA distributions were analyzed with gates described in the method section.

R3+R6 as a conventional BrdU-pulse-chase model (second row), and compartments of R1 through R6 determined by gating (bottom two rows), as shown in Fig 1.

The changes in cells in the G_1, S and G_2/M phases (Fig 4, top row) demonstrated accumulation of cells in the G_1 phase as a function of days in incubation. The accumulation was observed earlier in the cells of group B than in those of group A. This observation well correlated with the delay in the achievement of a plateau in the growth curve of the group A cells (Fig 2). The increase in the percentage of cells in the G_1 phase reflected the decrease in the S phase after day 5 in both groups. There were no significant changes observed in the G_2/M phase.

On the other hand, the conventional BrdU-pulse-chase method showed an early accumulation of the group A cells in the R1+R4 compartment followed by a subsequent decrease in the percentages on days 5, 6, and 7. The group B cells showed a plateau formation that correlated with the growth curve (Fig 2) in the R1+R4 compartment on days 5 through 7. The percentages of the group A cells in the R2+R5 compartment decreased on day 4, but a transient increase was observed on day 5. The transient increase was probably due to the evidence of some of the cells in the R1+R4 compartment into the R2+R5 compartment. However, the percentages of cells plated in group B continuously decreased from day 3 through day 7 because there was no influx of cells from the R1+R4 compartment to the R2+R5 compartment. In spite of these significant changes in the R1+R4 and R2+R5 compartments, there were no remarkable changes in the percentages of cells in the R3+R6 compartment except for a remarkable decrease in the percentages of cells in groups A and B from day 4 to day 5. This observation indicated that the

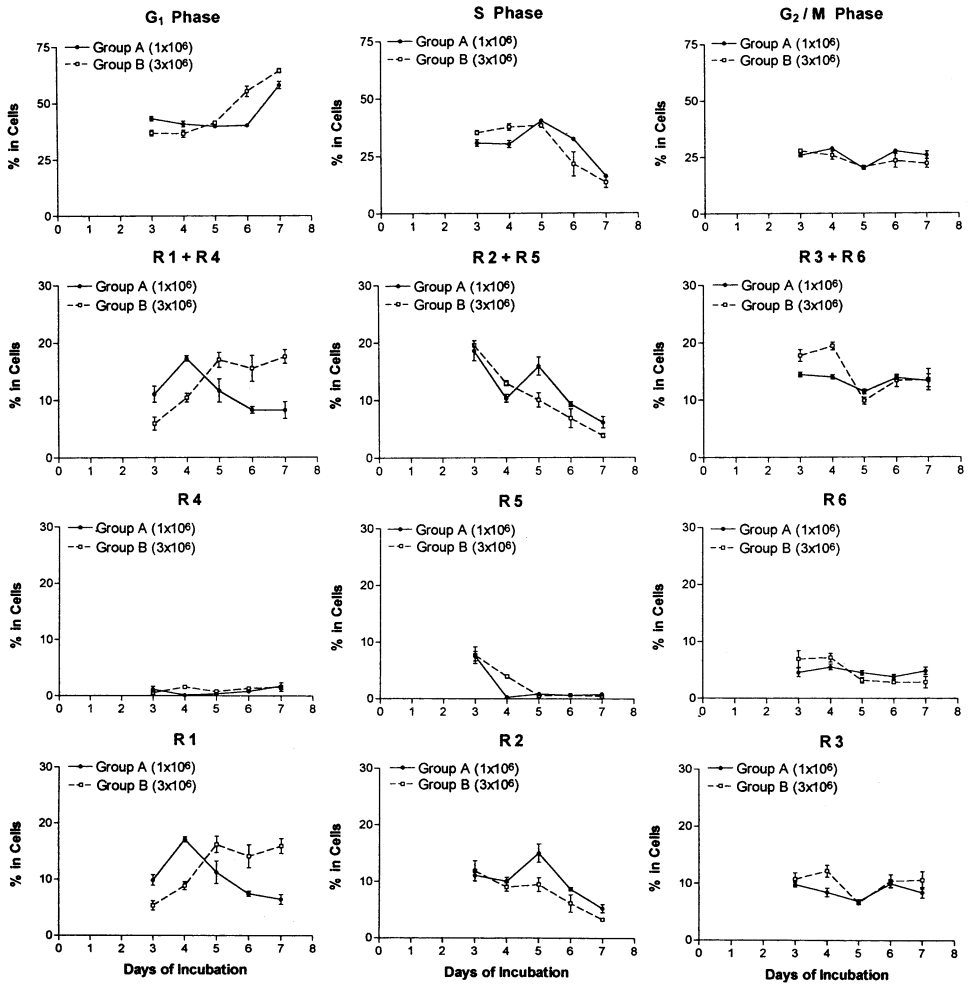

Fig 4. Comparison of DNA histograms (top row), conventional BrdU-pulse-chase (second row), and our methods (bottom two rows). The early S phase (R1 and R4) of this study corresponded to the G_1 phase in DNA histograms, the middle S phase (R2 and R5) to the S phase, and the late S phase to the G_2/M phase. Data are shown with the days of incubation on the X axis and the percentage in cells on the Y axis. Error bars represent the standard error of means of at least three independent experiments.

transit of BG-1 cells through the G_2/M phase is too quick to reflect the changes in the S phase.

The increase of cells in the R1+R4 compartment cannot be explained if we use this conventional BrdU-pulse-chase analysis because the R1+R4 compartment is the first compartment of BrdU-labeled cells. In other words, there is no source for the cell increase in this compartment. However, by subdividing these three compartments (R1+R4, R2+R5, and R3+R6) into six compartments (R1−R6), the answer became obvious. There was a decrease in the percentages of cells in the R5 compartment in both groups A and B. The degree of decrease differed between the groups, but the difference was well reflected by the increase in the percentages of cells in the R1 compartment.

There were, again, no remarkable changes in the R6 compartment in spite of significant changes in the R5 and R1 compartments. Therefore, we can assume that the cells in the R5 compartment rapidly passed through the R6 compartment and reached the R1 compartment. Since the cells labeled with BrdU in the R5 compartment passing through the R6 compartment can be assumed to be in the G_2/M phase, the absence of significant changes in the R6 compartment also supports the assumption that the BG-1 cells pass through the G_2/M phase quickly.

DISCUSSION

The analysis of the cell kinetics of normal and neoplastic cells has been greatly developed by a FCM, using several measurement methods, such as DNA histogram patterns, the proliferating index, S phase duration, DNA aneuploidy, or the DNA index.

However, the most difficult problem in cell cycle analysis using DNA histograms is how to accurately estimate the S phase. Two methods have mainly been proposed. One of these involves simplifying the S phase to a trapezoid or rectangle or polynomials.[1,2,4,5] The other involves changing the S phase to a convolution of multiple subdivided distributions, such as the constrained sum of Gaussians or the convoluted multirectangle model.[3,6-9] Although the former methods simply require the percentage of the S phase fraction, they cannot accurately distinguish the actual S phase fraction from regions overlapping with the G_1 or G_2/M phase.[6,7,10,25] Although the latter methods have more flexibility and applicability for asynchronous or perturbed cell populations than the former models,[26] they involve the use of several complicated mathematical computer programs.[3,6-9]

A double staining technique with BrdU/DNA developed by Gratzner[27] and Dolbeare *et al*[22] enabled us to estimate the actual S phase more precisely, Yanagisawa *et al* demonstrated the feasibility of a pulse-chase method with bivariate BrdU/DNA distributions,[12] and Higashikubo *et al* elucidated dynamic alterations in heat and X-rays in cell cycle progression using a BrdU-pulse-chase method,[15,16] but observations with these methods were done in only one cell cycle division. In addition, most of the conventional BrdU-pulse-chase methods have been demonstrated within 24 hours.[12,15,16,23,28-33] We assumed that there might be some factors of cell cycle analysis which could not be identified by DNA histograms or conventional BrdU-pulse-chase analysis.

As shown in Fig 4, the DNA histogram method showed only accumulations of cells in the G_1 phase on day 7 for group A (cells plated at 1×10^6) and from day 6 to 7 for group B (cells plated at 3×10^6). The conventional BrdU-pulse-chase method used in this study provided different information demonstrating cell accumulation in group A on day 4 and plateau formation in group B from day 5 to 7 in the R1+R4 compartment, and this accumulation corresponded to the growth curve. In addition to these results, the conventional BrdU-pulse-chase method demonstrated cell transitions from the R1+R4 compartment to the R2+R5 compartment. However, this method could not identify the source of cells accumulated in the R1+R4 compartment. As shown with our method, by dividing the conventional BrdU-pulse-chase

method into two segments for BrdU content so that we can analyze until two cell divisions, we were able to distinguish that these cell accumulations in the R1 compartment for both groups corresponded to a decrease of cells in the R5 compartment.

Although with the conventional BrdU-pulse-chase method early transition of BG-1 cells through the G_2/M phase has been assumed, our method could identify this phenomenon more clearly.

Therefore, our two-cell-divisions-BrdU-pulse-chase analysis could identify detailed cell progression more dynamically than DNA histograms or the conventional BrdU-pulse-chase method, and it was also able to distinguish different cell growth statuses. Furthermore, it seems that the cell traverse of entry into a compartment or exit from that compartment can be directly determined by measuring the changes in the fraction of cells and slopes forward or backward in the respective compartments.

In this study, we have described the possibilities of two-cell-divisions-BrdU-pulse-chase analysis for more dynamic and detailed analysis of cell kinetics. Although the cells labeled with BrdU in this study were chased every 24 hours, with shorter intervals for chasing, such as 8, 12, and 16 hours, more precise cell kinetic analysis may be possible. In addition, this method can be extended to a complicated interpretation of the cell cycle perturbation caused by various cytotoxic agents.

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