Determination of the Optimal Conditions for Flow Cytometric Analysis by Simultaneous Staining with Bromodeoxyuridine (BrdU) and Propidium Iodide (PI) in Isolated Nuclei of BG-1 Human Ovarian Cancer Cells

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ABSTRACT. This study was undertaken to determine the optimal conditions for flow cytometric analysis of isolated nuclei by simulutaneous staining with bromodeoxyuridine (BrdU) and propidium iodide (PI) particularly in BG-1 human ovarian cancer cells. The following results were obtained: (1) The optimal concentration of BrdU to label the isolated nuclei of the BG-1 cells was 20 μM. (2) The optimal concentrations and times for HCl treatment were 2 N for 20 min, 2.5 N for 15 min, and 2.5 N for 20 min.

Key words: bromodeoxyuridine (BrdU) and propidium iodide (PI) analysis — flow cytometry — nuclear isolation — ovarian cancer

Abnormal multiplication of cells is the most prominent characteristic of tumors. Therefore, analysis of cell kinetics is important in cancer research. Analysis of cell kinetics is usually performed with a flow cytometer (FCM) for each phase of the cell cycle using DNA stained with propidium iodide (PI) based on theoretical calculations.[1,2] However, there are some problems with these calculations, such as differentiation of the quiescent cells in the S-phase which do not synthesize DNA.[3] Therefore, the true cell kinetics of cells cannot be defined by a method based on theoretical calculations. Bromodeoxyuridine (BrdU) is an analogue of thymidine constructing DNA and can be taken into the cells synthesizing DNA. In 1982, Gratzner *et al* developed the anti-BrdU monoclonal antibody (MAb), making it easy to identify cells in the S-phase.[4] Dolbeare *et al* defined the two-dimensional relationship between the DNA synthesis and the total amount of DNA in the S-phase by using BrdU and the anti-BrdU MAb with a FCM.[5] This technique has been widely used, but experiments have been performed only with whole cells. Virtually no studies of the optimal conditions for the preparation of nuclei for simultaneous staining with BrdU and PI have been done. However, using the BrdU incorporation assay, it is possible to investigate the relationship between cell cycle perturbation and various nuclear properties. Therefore, it is important to determine the optimal conditions for BrdU incorporation for isolated nuclei. In this study, we attempted to clarify the optimal conditions for simultaneous staining of isolated nuclei of BG-1 human ovarian cancer cells with BrdU and PI.

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MATRIALS AND METHODS

Cells : BG-1 cells, derived from a human ovarian carcinoma,[6,7] were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 0.05% L-glutamine, 1% nonessential amino acids, 100 U/ml penicilline G, and 100 μg/ml streptomycin sulfate, and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in 95% air. Then they were plated at 1×10^6 cells/75-cm^2 flask and incubated.

BrdU incorporation : Exponentially growing BG-1 cells (Day 3) were treated with BrdU diluted with phosphate buffered saline (PBS), and incubated for 30 min at 37°C in a humidified atmosphere containing 5% CO_2 in 95% air. The cells were washed with McCoy's 5A medium to remove the effect of BrdU.

Nuclear isolation : BG-1 cells were trypsinized with 0.1% trypsin and 0.01% EDTA and resuspended. The nuclei of the BG-1 cells were isolated by a previously described method.[8] BG-1 cells 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 Triton-X solution (1% Triton X-100, 0.08 M NaCl, 0.02 M EDTA) for 10 min to remove cytoplasm and centrifugation at 2000 rpm for 5 min, the cells were washed with 10 ml of 0.88% saline.

Denaturing of the DNA : The isolated nuclei of BG-1 cells were treated with 1 ml HCl at room temperature to denature DNA. The concentration and time for treatment are described below. After centrifugation at 1500 rpm for 5 min, the HCl was 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.

Staining : The nuclei treated with HCl were stained with 100 μg/ml propidium iodide (PI) for DNA analysis and with FITC-conjugated anti-BrdU MAb diluted 100 times for BrdU analysis. Then they were incubated overnight at 0°C for FCM analysis.

Flow Cytometry : Flow cytometric analysis was performed on a FACStar (Becton Dickinson) with an argon laser using a 488-nm excitation beam at 15 mW. Four parameters ; *i.e.*, forward scatter, side scatter, PI (red fluorescence, 610 nm), and FITC (green fluorescence, 525 nm), were analyzed. The optimal condition for BrdU-PI staining was defined as clear observation of an inverted U pattern on the two-dimensional flow cytometric analysis.

Optimal concentration and time of HCl treatment for DNA denaturization : To determine the optimal condition for denaturing DNA with HCl, we tested nine combinations of concentrations at 1.5, 2, or 2.5 N and at exposure times of 10, 15, or 20 min. BG-1 cells labeled for 30 min with 20 μM BrdU were treated with each combination of HCl.

Optimal concentration of BrdU : To determine the optimal concentration of

BrdU, exponentially growing BG-1 cells, originally plated in a 75-cm^2 tissue culture flask at 1×10^6, were labeled for 30 min with BrdU at 2.5, 5, 10, or 20 μM on Day 3. The nuclei of the cells in these flasks were treated with 2 N $\times$ 20 min HCl based on results for the determination of the optimal concentration for DNA denaturization.

Growth curve of BG-1 cells with or without BrdU incorporation: BG-1 cells were plated at 1×10^6 in a 75-cm^2 tissue culture flask. The cells either untreated or treated with 20 μM BrdU on Day 3. The number of cells in the flasks were scored on Days 3, 5, and 7.

RESULTS

1) Optimal concentration and time for HCl treatment.

The effects of staining with BrdU under each condition of the concentration and time for the HCl treatment are shown in Fig 1. All the contours and histograms showed a similar inverted U pattern. The intensity of BrdU became higher as a function of the concentration and time of HCl. The isolated nuclei of BG-1 cells labeled with BrdU were best stained with HCl either at 2 N $\times$ 20 min, 2.5 N $\times$ 15 min or 2.5 N $\times$ 20 min.

2) Optimal concentration of BrdU.

The two-dimensional contour patterns and three-dimensional histograms of the nuclei stained with PI and BrdU at 2.5, 5, 10, or 20 μM are shown in Fig 2. Although the BG-1 nuclei were even stained at an extremely low BrdU concentration, the best concentration of BrdU for the two-dimensional analysis using PI and FITC was 20 μM.

3) Effect of BrdU on the growth of BG-1 cells.

The growth curves of the BG-1 cells with or without BrdU incorporation at 20 μM are shown in Fig 3. There was no significant difference. Therefore, it was concluded that BrdU treatment at 20 μM did not affect the cell proliferation of the BG-1 cells.

DISCUSSION

Flow cytometric analysis using the simultaneous staining with PI and BrdU with anti-BrdU MAb is a useful method for analysis of cell kinetics of cancer cells. When simultaneous staining with BrdU and DNA is performed, treatment with single-strand DNA for BrdU staining and double-chained DNA for PI staining is required to obtain the reproducible and quantitative results. It is necessary to determine the optimal condition for denaturing DNA to obtain single-strand DNA. Although HCl, formamide, heat and their various combinations are known to denature DNA, HCl is the most flexible as it can be used for a variety of cells. Previous studies have reported concentrations of HCl ranging from 2 N to 4 N, and treatment times ranging from 20 min to 30 min. However, these conditions were almost always for whole cells. Similar results have been reported by Jinno *et al* in a study in which treatment of nuclei with 2 N HCl for 20 min at room temperature was optimal.[11] We found that the isolated nuclei of BG-1 cells were poorly stained if the

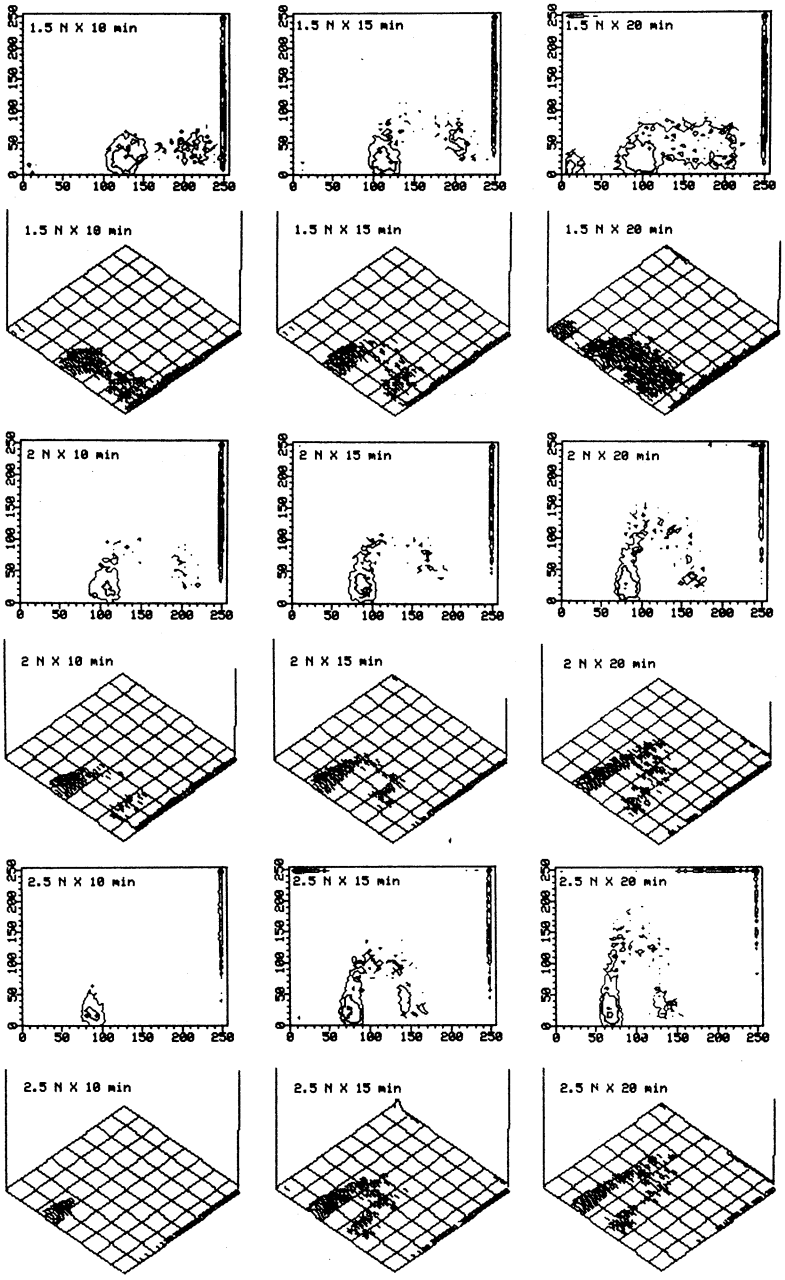

Fig 1. BrdU/DNA distributions measured for the isolated nuclei of BG-1 cells labeled with 20 μM BrdU
The concentrations and times for HCl treatment were 1.5 N × 10 min, 1.5 N × 15 min, 1.5 N × 20 min, 2.0 N × 10 min, 2.0 N × 15 min, 2.0 N × 20 min, 2.5 N × 10 min, 2.5 N × 15 min or 2.5 N × 20 min. The upper row shows a two-dimensional contour with the DNA contents on the X axis, and the BrdU content on the Y axis. The lower row shows a three-dimensional histogram as isometric plots with the DNA contents on the X axis, the BrdU contents on the Y axis, and the relative cell number on the Z axis.

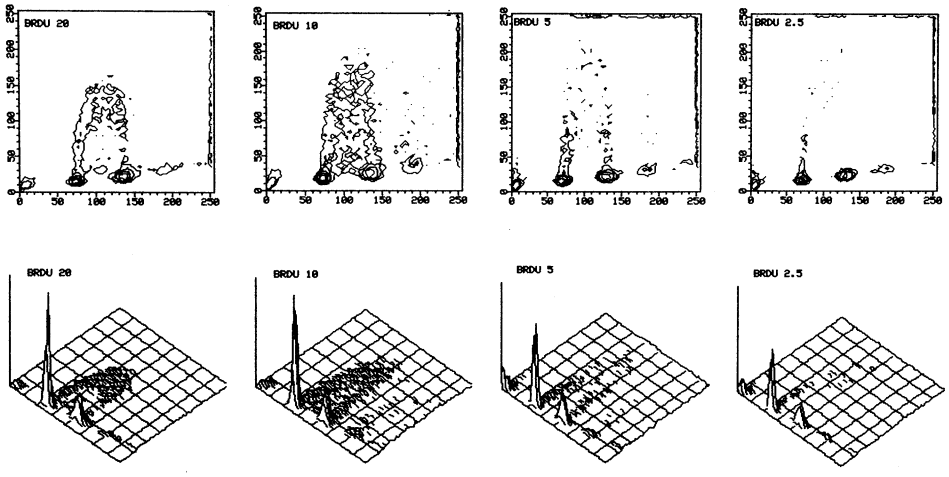

Fig 2. BrdU/DNA distributions measured for the isolated nuclei of BG-1 cells labeled with BrdU of concentrations of 2.5 μM, 5 μM, 10 μM and 20 μM
The upper row shows a two-dimensional contour with the DNA contents on the X axis, and the BrdU contents on the Y axis. The lower row shows a three-dimensional histogram as isometric plots with the DNA contents on the X axis, the BrdU contents on the Y axis, and the relative cell number on the Z axis.

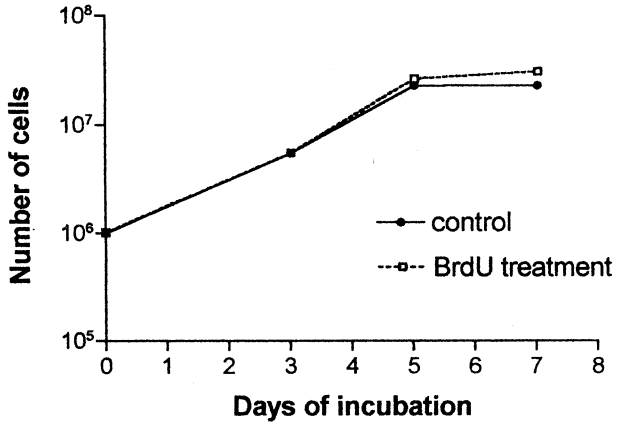

Fig 3. Growth curve of BG-1 cells with or without 20 μM BrdU
Data are shown with the days of incubation on the X axis and the number of cells on the Y axis.

concentration of HCl was too low or if the treatment time was too short, but were well stained at 2 N $\times$ 20 min, 2.5 N $\times$ 15 min, and 2.5 N $\times$ 20 min. Treatment with HCl at a low concentration or a short treatment time was found to be inadequate because DNA was not sufficiently denatured.

BrdU is often used as a radio-sensitizer and is known to suppress cell profilation at high dosages. Goz reported that long-time exposure to a haloprymidine such as BrdU at a high concentration of over 50 μM increased cell mutaion.[9] Therefore, the concentration of BrdU in studies of cell kinetics

has to be within a range in which it does not affect cell proliferation, but yet is sufficient to label the cells. Although Dolbeare *et al* originally treated cells with 10 μM BrdU,[5] many different concentrations, from 10 μM to 500 μM, are now used to label cells with BrdU. Shimabukuro reported that concentrations of BrdU at 1.25 μM, 2.5 μM, 5 μM, 10 μM, 20 μM for 30 min did not cause significant differences in green fluorescence intensities produced by FITC-labeled anti-BrdU MAb.[10] We also found that isolated nuclei were well stained by BrdU at 2.5 μM, 5 μM, 10 μM, 20 μM for 30 min, but the best concentration was 20 μM. Shimabukuro *et al* also reported that the colony efficiency of exponentially growing MBT-2 cells treated with BrdU at 20 μM for two hours did not differ from that in untreated cells.[9] We found that there were no significant differences in the growth on Days 5 and 7 with or without treatment with 20 μM BrdU for 30 min. Therefore, we concluded that the proliferation of BG-1 cells was not influenced by treatment with 20 μM $\times$ 30 min BrdU.

In conclusion, we suggest that the optimal concentration of BrdU to label the isolated nuclei of BG-1 cells is 20 μM for 30 min and the cell growth of the BG-1 cells was not affected by this treatment. The optimal concentration and time for HCl treatment is 2 N $\times$ 20 min, 2.5 N $\times$ 15 min, and 2.5 N $\times$ 20 min. This information should be valuable for future experiments using BrdU-PI double staining in the isolated nuclei of cells under normal or abnormal growth conditions.

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