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Specific Cell Cycle Synchronization with Butyrate and Cell Cycle Analysis by Flow Cytometry for Madin Darby Bovine Kidney (MDBK) Cell Line

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Abstract: We investigated the property of the cell cycle arrest induced by butyrate and compared cell synchronization induced by butyrate, serum deprivation and the combination of serum deprivation and aphidicolin. The site of growth inhibition and cell cycle arrest was studied by BrdU incorporation and flow cytometry analyses. By combining BrdU incorporation and DNA content analysis, not only can the overlapping between different cell populations be eliminated, but also the frequency and nature of individual cells that have synthesized DNA can be determined. Exposure of MDBK cells to 10 mM butyrate caused inhibition of growth and cell cycle arrest in a reversible manner. Evidence is presented that butyrate affected the cell cycle at a specific point immediately after mitosis and at a very early stage of the G1 phase. After release from butyrate arrest, MDBK cells underwent synchronous cycles of DNA synthesis and transited through the S phase. The results showed that most serum deprivation treated cells were arrested at the G1 phase and aphidicolin treated cells were arrested at the early S phase. The effects of the three treatments were reversible. Using butyrate-synchronized cells, we determined that it takes about 8 h for G1 synchronized cells, induced by butyrate, to progress into the S phase and 8 h for the completion of the S phase. One cycle of cell division for MDBK cells is about 20 h.

Key words: Aphidicolin, bovine kidney epithelial cell, brdu incorporation, butyrate, cell cycle synchronization, flow cytometry

INTRODUCTION

It is now well established that microbial fermentation in the gastrointestinal tract contributes to the energy balance of all mammalian species[1,2]. Short-chain fatty acids (acetate, propionate and butyrate) are formed during microbial fermentation of dietary fiber in the gastrointestinal tract of mammalian species and then are directly absorbed at the site of production. Short-chain fatty acids contribute up to 70% of the energy requirements of ruminants[2]. We have reported recently that butyrate can arrest MDBK cells specifically at the early G1 phase[3]. However, the properties of the cell cycle arrest induced by butyrate and cell cycle progression after release have not been characterized in MDBK cells. An optimal cell cycle synchronization protocol is required to study the proliferative biomarkers of cell cycle. Highly synchronized cell populations greatly facilitate cell cycle analysis[4,5]. A number of agents and protocols have been described to block cell cycle progression reversibly. These protocols and agents can be used either to arrest actively dividing cells in chemotherapeutic protocols or to devise synchronizing regimens for cultured cells[6]. Most of these agents have an extensive history as chemotherapeutic agents and their mechanisms of action are well understood. Among the most widely used protocols are utilizing nutritional or serum deprivation to arrest cells in G0/G1, using inhibitors of DNA synthesis to block cell cycle at the early S phase and using nocodazole, a mitotic inhibitor, to synchronize cells at M/G2 phases. However, not all agents that produce cell cycle blocks are suitable for different kinds of block-and-release experiments or different kind of cell types. To obtain cell populations at specific cell cycle stages and to develop a model for studies of cell growth regulation, we examined different cell synchronization protocols and compared the properties of cell cycle arrest by butyrate and protocols such as serum deprivation and aphidicolin. We demonstrated that butyrate blocks the cell cycle at the very early G1 phase and butyrate induced cell cycle block is reversible. Using butyrate-synchronized cells, we also determined that the cell division cycle of MDBK cells is

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about 20 h. The use of flow cytometry allows us to monitor the progression of the cell cycle and to characterize the cell cycle arrest induced by butyrate.

MATERIALS AND METHODS

Cell culture: Madin-Darby bovine kidney epithelial cells (MDBK, American Type Culture Collection, Manassas, VA, Catalog No. CCL-22) were cultured in Eagle's minimal essential medium supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 25 cm^2 flask, where the medium was renewed twice per week. Cell cultures were maintained in a water-jacked incubator with 5% CO_2 at 37°C. Sub-cultivations were performed when cells attained 80 to 90% confluence, according to the product information supplied by American type culture collection. Cells were used for treatment testing at approximately 50% confluence, during the exponential phase of growth.

Cell treatments: Sodium butyrate (Calbiochem, San Diego, CA) was prepared as 1 M stock by dissolving it in ultra pure, deionized water (tissue culture grade, Advanced Biotechnologies Inc., Columbia, MD). The procedure for cell treatment of butyrate was detailed in our previous publication[3]. Adding up to 10 mM sodium butyrate into cell culture medium did not cause measurable pH change. For serum deprivation, cells were seeded in 25 cm^2 culture flasks. The culture medium was discarded and cells were rinsed with fresh-wormed serum free media and then replaced with media containing 0.5% fetal bovine serum and cultured for another 72 h. For aphidicolin treatment, cells were first cultured in serum-depraved medium for 48 h; cells were released into fresh medium with 5% fetal bovine serum. Aphidicolin (Aphidicolin (Calbiochem) dissolved in dimethyl sulfoxide (DMSO) at 1 mg mL^{-1}) was added to a final concentration of 10 μg mL^{-1} for another 24 h. Duplicated flasks of cells were used for each of treatment.

5-Bromo-2'-deoxyuridine (BrdU) incorporation labeling and flow cytometric analysis of cells: Cells were labeled with BrdU using BrdU Flow kits (BD Pharmingen, San Diego, CA). To pulse label the cells, 10 il of BrdU solution (1 mM BrdU in PBS) was carefully added directly to each mL of culture media and cultured for 40 min. To immunofluorescent stain cells, cells were collected with trypsinization, fixed and permeabilized with BD Cytofix/Cytoperm buffer supplied with the kits. Cells were then treated with DNase to expose incorporated BrdU (30 μg of Dnase to each sample, incubated at 37°C for 1 h). After washing with 1 ml of 1XBD Perm/wash buffer, cells were resuspended with 50 μL of BD Perm/Wash buffer containing diluted fluorescent (Fluorescent Isothiocyanate, FITC) anti-BrdU antibody and incubated for 20 min at room temperature. After washing with BD Perm/Wash buffer, cells were resuspended in 20 il of the 7-AAD solution (supplied with kits) to stain the cellular DNA content.

Flow cytometry analysis is based on measurements of increased DNA content in proliferating cells going through cell cycle phases and immuno-staining on the newly synthesized DNA containing BrdU, which reflects the cell cycle progression status. Cellular DNA content increases from the original amount of two copies (2C) in the G1 phase to twice that amount to four copies (4C) in M/G2 phases with intermediate DNA content in S phase cells. The amount of DNA was measured by the amount of dye taken up by the cells and indirectly by the DNA content. The detailed procedures were reported in our previous publication[3]. Cell DNA content and BrdU labeling were analyzed using flow cytometry (FC500, Beckman Coulter Inc., Palatine, IL) and collected data were analyzed using Cytomics RXP (Beckman Coulter Inc.). At least 10,000 cells per sample were analyzed.

RESULTS

Synchrony/release of MDBK cells and flow cytometric analysis using DNA content and BrdU incorporation analyses: Asynchronously growing MDBK cells were treated with different protocols and collected for flow cytometric analysis of DNA content. Following butyrate treatment for 24 h, in comparison with the profile of normal growing cells, both G1 (the first peak of the profiles) and M/G2 populations (the small and second peak) of the survival cells were increased and S phase cells (cells falling in-between the first and second peaks) were decreased Fig. 1B suggesting three possibilities. One, cells were possibly arrested at G0/G1; two, cells were arrested at G1/S boundary; and three, DNA replication was blocked at an early stage of the S phase by the butyrate treatment. The results in Fig. 1 also show that serum deprivation, aphidicolin and butyrate treated cells have similar cell population profiles. The flow cytometry

Table 1: Flow cytometry analyses of cell cycle arrest induced with different protocols*

	% G1/G0 (Mean±SEM)	% S (Mean±SEM)	%M/G2 (Mean±SEM)
Exponential growing cells	42.4±1.5	48.8±1.8	8.9±0.4
100% confluence	66.4±4.6**	28.4±3.0**	5.2±2.9
Starvation (72 h)	72.5±1.0**	20.3±1.8**	7.3±0.6
Aphidicolin	80.4±3.4**	16.1±1.9**	3.6±3.5
10 mM BT	81.0±2.9**	8.9±4.1**	10.1±2.4

*: data from 4 separate experiments, **:$p<0.01$, compared to exponential growing cells

Fig. 1: Synchronization of Madin-Darby bovine kidney (MDBK) epithelial cells. One set of representative histogram plots of flow cytometry analysis (DNA content) of MDBK cells: A) Flow cytometric analysis of exponential growing cells. G1/G0: cells in G1/G0 cell cycle phases; S: cells in S cell cycle phase; M/G2: cells in M/G2 cell cycle phase; 2C and 4C: two and four copies of DNA content respectively. B) Histogram plot of flow cytometry analysis of MDBK cells treated with 10 mM sodium butyrate. C) Histogram plot of flow cytometry analysis of MDBK cells treated with serum deprivation for 72 h. D) Histogram plot of flow cytometry analysis of MDBK cells treated with serum deprivation for 48 h and aphidicolin (10 μg mL^{-1}) for 24 h

analyses from four experiments were quantified and the results are summarized in Table 1. However, since the mechanism of cell cycle arrest induced by serum deprivation and aphidicolin are different[7], indifference in profile of the DNA content analysis may indicate that the resolution afforded by flow cytometry based on DNA content is not sufficient to adequately subdivide the late G1/early S phase of the cell cycle. Therefore, we utilized double staining flow cytometric analysis that combined the immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and DNA content stained by DNA marker. BrdU is a TdR analogue and is incorporated into cellular DNA through the same pathway. DNA synthesis can be visualized *in situ* by direct immunofluorescence using an antibody against BrdU[8]. Using this technique, we can not only analyze cell cycle synchrony with high resolution, but also monitor the cell cycle progression after cells are released from the treatments. Figure 2 shows one representative results from the normal growing cell population (control). Fluorescent signal generated by FITC was acquired in a

Fig. 2: Double staining flow cytometric analysis. For flow cytometric analysis, exponential growing cells were first pulse labeled with BrdU for 30 min. Collected cells were first stained with diluted fluorescent (Fluorescent isothiocyanate, FITC) anti-BrdU antibody and then stained with DNA marker (7-ADD). The fluorescent signal generated by FITC was acquired in a logarithmic mode and fluorescent signal from the DNA-content marker 7-ADD was normally acquired in the linear signal amplification mode. A: Cells were separated into three clusters by double staining analysis. a) G1/G0 cells, with 2C DNA content and without any DNA synthesis activity; b) S phase cells, with DNA content between 2C and 4C (two and four copies of DNA content respectively) and high BrdU incorporation (DNA synthesis) activity c) G2/M cells, with 4C DNA content and also without DNA synthesis activity; and. B: Flow cytometric analysis of cell DNA content for the same cell preparation

logarithmic mode and fluorescent signal from the DNA-content marker 7-ADD was acquired in the linear signal amplification mode. Using double color staining (immunofluorescent staining of the BrdU incorporation and fluorescents staining of total DNA), cells can be separated into three major clusters. The first is a cell population with two copies of DNA and without any

DNA synthesis activity, e.g., the cells in G1/G0 phases (cluster a). The second is the cell population (G2/M phases) that has four copies of DNA and without any synthesis activity (cluster c). The last is a cell population (cluster b) has the DNA content between cluster a and c, but has a strong shift of immunofluorescent staining of newly synthesized DNA. Those cells are in the S phase. In comparison with the profiles from the DNA content analysis, the immunofluorescent staining of incorporated BrdU and flow cytometric analysis provides a high-resolution technique to eliminate the overlap between different cell populations and to determine the frequency and nature of individual cells that have synthesized DNA.

Synchrony/release MDBK cells from butyrate treatment: Asynchronously growing MDBK cells were incubated 24 h in the presence of 10 mM butyrate; cells were washed with fresh medium to eliminate the dead cells and then replaced in fresh medium with 5% fetal bovine serum. Cells were pulse-labeled with BrdU at indicated time points and collected for flow cytometric analysis. Figure 2A and 2B are the flow cytometric data representing the 1 h and 6 h time points after release from butyrate. Average flow cytometric cell cycle distribution from 4 experiments showed an arrest of cells in the G1 phase with the majority of cells in G0/G1 (76±2.9%), while 8.9±1 and 15±2.4% of the cells were in S and G2/M phases, respectively. However, during the first 8 h post-release, G1 synchronized cells progressed very little into the succeeding stage. Only 18.5±3.7% of the cells at 6 h post-release and 38.1±5.5% of the cells at 8 h post-release progressed into S phase Fig. 3, A, B and C. Those data indicated that butyrate blocks the cell cycle at very early G1 phase and may induce some of the cells into G0.

Fig. 3: Cell cycle progression after release from butyrate. After treated with 10 mM, butyrate cells were released into fresh medium containing 5% fetal bovine serum. At the indicated time point, cells were pulse labeled with BrdU. Collected cells were first stained with diluted fluorescent (Fluorescent isothiocyanate, FITC) anti-BrdU antibody and then stained with DNA marker (7-ADD). A: A representative flow cytometric analysis of the cells 1 h after released from butyrate. B: A representative flow cytometric analysis of cells 6 h after released from butyrate. C: Cell cycle progression during the first 8 h after released from butyrate. Data are from three individual experiments and presented as Mean±SEM

Synchrony/release MDBK cells from serum deprivation.: To confirm that the blocking site of the cell cycle by butyrate was indeed at the very early G1 stage, we also monitored the cell synchronization and the cell cycle progression in MDBK cells induced by serum deprivation and aphidicolin. Asynchronously growing MDBK cells were incubated for 72 h in medium containing only 0.5% fetal bovine serum, which was subsequently washed and replaced with fresh medium with 5% fetal bovine serum. At the indicated time point, cells were pulse labeled with BrdU for 40 min and collected for flow cytometric analysis. Figure 3 A and B are the representative flow cytometric data for 1 h and 6 h postrelease from serum deprivation. The results in Fig. 3 show that most serum deprivation treated cells were arrested in G1 phase. Average flow cytometric cell cycle distributions showed that the

Fig. 4: Cell cycle progression after release from serum deprivation. After incubated in serum-depraved medium for 72 h, cells were released into fresh medium containing 5% fetal bovine serum. At the indicated time point, cells were pulse labeled with BrdU. Collected cells were first stained with diluted fluorescent (Fluorescent isothiocyanate, FITC) anti-BrdU antibody and then stained with DNA marker (7-ADD). A: A representative flow cytometric analysis of cells 1 h after released from butyrate. B: A representative flow cytometric analysis of the cells 6 h after released from butyrate. C: Cell cycle progression during the first 8 h after released from butyrate. Data from three individual experiments and presented as Mean±SEM

Fig. 5: Cell cycle progression after release from aphidicolin treatment. Cells were first incubated in serum-depraved medium for 48 h and replaced with fresh medium with 5% fetal bovine serum and 10 ig/ml aphidicolin for another 24 h. Cells were released into fresh medium containing 5% fetal bovine serum. At the indicated time point, cells were pulse labeled with BrdU. Collected cells were first stained with diluted fluorescent (Fluorescent isothiocyanate, FITC) anti-BrdU antibody and then stained with DNA marker (7-ADD). A: A representative flow cytometric analysis of the cells 1 h after released from butyrate. B: A representative flow cytometric analysis of cells 6 h after released from butyrate. C: Cell cycle progression during the first 8 h after released from butyrate. Data from three individual experiments and presented as Mean±SEM

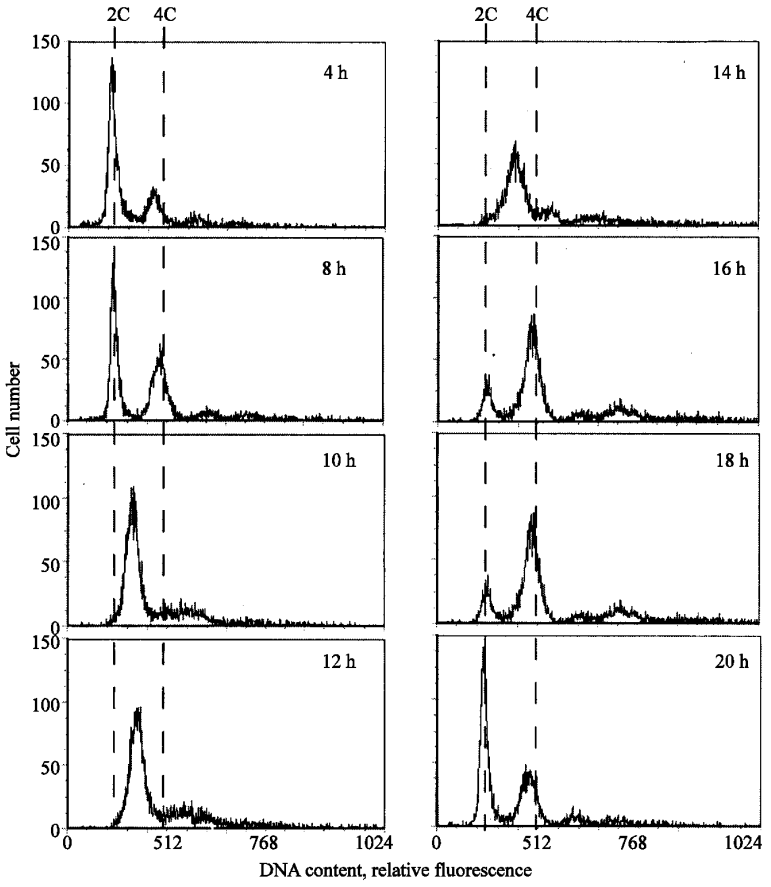

Fig. 6: Cell cycle determination using butyrate synchronized MDBK cells. After released from the butyrate treatment, cells were collected at indicated times post-release and processed for cycle analysis by flow cytometry. 2C and 4C: two and four copies of DNA content, respectively

majority of cells were in G0/G1 (78.5±4.1 %), while 6.5±0.5 and 14.5±5.1% of the cells were in S and G2/M phase, respectively. During 6 h post-release, G1 synchronized cells progressed into the succeeding stages. The cell cycle distribution was 60.2±5.0, 31.4±5.9 and 8.4±1.0% in G0/G1, S and G2/M phase, respectively Fig. 3C. At 8 h post-release, about 47% of the cells had reached the S phase. Monitoring cell cycle progression for 8 h after released from serum deprivation showed that the synchronized G1 cells remained in the G1 phase for at least 6 h with an increase in the S phase and decrease in the G1 phase between 4 and 6 h. These results confirmed that serum deprivation blocks cells at an early stage in G1 and prevents their cell cycle progression[9]. By comparison, cells treated with butyrate take a little longer than the cells treated with serum deprivation to progress into the S phase; therefore, the blocking site of the cell cycle induced by butyrate should be at the very early stage of the G1 phase.

Synchrony/release MDBK cells from serum deprivation and aphidicolin: For aphidicolin treatment, cells were first cultured in serum-depraved medium for 48 h; cells were then released into fresh medium with 5% fetal bovine serum. Aphidicolin was added to a final concentration of 10 μg mL^{-1} and cells were incubated for another 24 h. The aphidicolin was removed by replacement with fresh medium. The cells were collected at the indicated time points after pulse-labeled with BrdU for flow cytometric analysis of DNA content and BrdU incorporation. The effects of aphidicolin were also reversible. DNA content analysis indicated that aphidicolin treated cells were blocked at the G1 phase (80.4±2.0%) immediately after release. However, using combined DNA content and BrdU incorporation analysis gave higher-resolution results. Our data showed that aphidicolin treated cells were arrested at the early S phase. BrdU incorporation (DNA synthesis) started almost immediately after the cells were released from treatment Fig. 1, A and B, 1 h and 6 h

post-release, respectively. At 1 h post-release, 52.9±7.4% of the cell population incorporated BrdU. DNA synthesis peaked at 2 h post-release (74.8±4.9%) and leveled off thereafter. Completion of the S phase takes about 8 h Fig. 5C.

One cycle of cell division for MDBK cells takes about 20 h. Using butyrate induced synchronized cells, we determined the length of cell division cycle of MDBK cells Fig. 6. After released from the butyrate treatment, cells were collected at various times post-release and processed for cycle analysis by flow cytometry. Monitoring cell cycle progression for 24 h after of removal of butyrate showed that synchronized G1 cells underwent synchronous cycles of DNA synthesis and transited through the S phase. Synchronized G1 cells remained in the G1 phase for at least 8 h with a weak increase in the S phase and a weak decrease in the G1 phase between 6 and 8 h. At 10 h post-release, almost all cells progressed into the S phase. Between 16 to 18 h post-release, the majority of the cells were in G2/M phase. At 20 h post-release, the G1 become dominant population of the cells again. These results demonstrated that butyrate blocks cells at an early stage in G1 and prevents their cell cycle progression. Once released from butyrate, the cells' cycle block was reversed and cells progressed in synchrony into next cell cycle phases. It took about 8 h for completion of the S phase and about 20 h for one cycle of cell division in MDBK cells.

DISCUSSION

This study was undertaken to examine the cell cycle characteristics of the Madin Darby Bovine Kidney (MDBK) cell line after culture in the presence of 10 mM butyrate. This study demonstrated that the Madin Darby Bovine Kidney (MDBK) cells, a commonly used cell line in veterinary research, can be effectively synchronized using butyrate. For the precise determination of the phase where MDBK cells are arrested by butyrate, we used some marker points. For this, we used the serum deprivation and aphidicolin treatment. Among the most common experimental approaches to cell cycle study is to arrest cell growth using incubation in low-serum medium or cell cycle inhibitor such as aphidicolin. It is generally accepted that serum deprivation produces cells arrest at a point in the G1 phase[10]. Aphidicolin is a DNA synthesis inhibitor that blocks cell cycle at the very early S phase (not at the G1/S boundary) by inhibiting the activity of DNA polymerase α complex in eukaryotic cells[9,11]. Our results verify that serum deprivation and aphidicolin block are efficient means of synchronizing MDBK cells in G0/G1 and the early S phase, respectively. With these time points for comparison, we have also clearly shown that the major population of butyrate-treated cells is arrested at the very early G1 phase, possible immediately after mitosis and some cells may be in G0.

During the course of this study, we also experimented with nocodazole. Nocodazole, an inhibitor of microtubule formation, has been extensively used for cell synchronization for many different cell types[12,13]. Nocodazole-treated cells gradually progress into mitosis but without cell division. After a short treatment of 3 to 4 h, accumulated M phase cells can be collected by shake-off. This was also used as a whole-culture synchronization method[13]. However, it works poorly on the MDBK cells in our laboratory (unpublished observation). After treated with nocodazole (0.5 ng/ml) for 4 h, there were no accumulation of M phase cells. 24 h treatment of nocodazole (0.5 ng mL^{-1}), however, induced a massive (more than 80%) death of cultured cells (data not show). By BrdU incorporation analysis, we also found that surviving cells were blocked for DNA synthesis even though DNA content analysis indicated that many cells were in the S phase.

Flow cytometry has emerged as a major new technology that is now viewed as an essential component of any research laboratory in animal science[14]. Using double staining flow cytometric analysis that combined the immunofluorescent staining of newly incorporated BrdU and DNA content stained by DNA marker enabled us to analyze the cell cycle synchrony with high resolution and to monitor the cell cycle progression after cells were released from the treatments.

CONCLUSION

The present study characterized the cell cycle arrest induced by butyrate. In comparison with common experimental approaches, such as serum deprivation and aphidicolin, our data show that butyrate can effectively synchronize MDBK cells at early G1/G0 phases. In comparison to 72 h serum deprivation for cell synchronization, 24 h treatment of butyrate induces similar synchrony of cell populations. Using butyrate synchronized cells, we were able to determine the length of the cell cycle for MDBK cells at about 20 h. Because short-chain fatty acids are common nutrients, understanding their important biological functions additional to a simple energy supply certainly will help us to understand critical control points in cell life cycle function that could lead to improvements in the efficient production of food animals. In addition, MDBK as an

established bovine cell line with inducible apoptosis and cell cycle regulatory events certainly is ideal and invaluable tool for functional genomic studies on homeostasis in animals and butyrate can be an invaluable tool to study cell cycle regulatory events.

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