Different Manner of DNA Synthesis in Polyploidizations of Meth-A and B16F10 Cell Lines

Kohzaburo Fujikawa-Yamamoto1,*, Zhi-ping Zong1, Manabu Murakami1, and Shizuo Odashima2

1Division of Basic Science, Research Institute of Medical Science and 2Department of Pathology, Kanazawa Medical University, Uchinada, Ishikawa 920-02, Japan

Key words: DNA synthesis/polyploidization/Meth-A cells/B16F10 cells

ABSTRACT. Polyploidization of Meth-A and B16-F10 cells by demecolcine was examined using flow cytometry (FCM). In the presence of demecolcine, both cell lines were polyploidized to more than 16c DNA content. A marked difference was observed in the durations of S phase of polyploidy. The S-phase duration of Meth-A cells was doubly increased with ploidy, but that of B16F10 cells remained constant. When the rate of DNA synthesis in the polyploidizing cells was examined through the BrdU-uptake experiments, it was confirmed that the level of DNA-synthesis rate was constant in Meth-A cells but increased in B16F10 cells. The cellular content of c-Myc protein in polyploidized cells was also examined using anti-c-Myc monoclonal antibody. The c-Myc level of Meth-A cells was constant regardless of the ploidy but that of B16F10 cells increased with ploidy. Thus, the c-Myc content seems to be related to the duration of S phase in polyploidy.

A relationship between DNA synthesis rate and DNA content has been reported for Chinese hamster cells by Graves and McMillan (6). They concluded that the duration of S phase in mammalian cells is almost constant regardless of the DNA content. This was supported by the finding of the increasing BrdU uptake in polyploid CHO cells (13).

Constant rate of cell-cycle progression in polyploidizing cells has been demonstrated in human lymphocytic leukemic MOLT-4 cells by Gong et al. (5) and in V79 cells by us (3), suggesting that intracellular contents of enzymes and precursors relating to DNA synthesis increased with the ploidy increase. However, the duration of each phase of polyploidized cells has not been examined. If the duration of S phase is almost constant, how does the durations of G1 and G2 phase increase in polyploidy?

To confirm whether or not the durations of G1 and G2 phases elongate with the ploidy increase, we examined cell cycle parameters of polyploidizing Meth-A and B16F10 cells. Methylcholanthrene-derived murine abdominal dropsy tumor cells (Meth-A cells) in culture always contain large cells which are self-induced polyploid Meth-A cells. Meth-A cells were polyploidized by demecolcine, K-252a and staurosporine (4). B16F10 cells, selected as a control, were polyploidized by demecolcine and K-252a but not by staurosporine (4). Here, we demonstrated an exception that the rate of DNA synthesis is constant in Meth-A cells, independent of the ploidy.

MATERIALS AND METHODS

Cells. Murine abdominal dropsy tumor cells (Meth-A cells) were maintained in a humidified atmosphere of 5% CO2 at 37°C as suspension culture in a Leibovitz’s L15:Ham’s F10 mixture (7:3) supplemented with 10% fetal bovine serum, streptomycin (100 mg/ml) and penicillin (50 units/ml). B16F10 cells, a highly metastatic subline of mouse B16 melanoma cells, were maintained under the same conditions as above except that they were cultured in monolayers. Both cell lines were cultured at low density.

Synchronization of cell lines. Exponentially growing Meth-A and B16F10 cells were plated in Petri dishes (100 mm diameter, NUNC) at a density of about $2 \times 10^5$ and $1 \times 10^4$ cells/dish, respectively, with the medium being changed 24 h after seeding. Twelve hours thereafter, the cells were exposed to hydroxyurea (HU) at a final concentration of 0.6 mM. Five hours thereafter, the Meth-A cells were washed twice by centrifugation and the B16F10 cells were rinsed twice with medium. Two hours after HU release, the cells were exposed to hydroxyurea (HU) at a final concentration of 0.6 mM. Five hours thereafter, the Meth-A cells were washed twice by centrifugation and the B16F10 cells were rinsed twice with medium. Two hours after HU release, the cells were exposed to hydroxyurea as follows.

Polyploidization of synchronized cell lines. The synchronized cells were exposed to hydroxyurea at a final concentration of 0.1 µg/ml. At various times, as shown in Fig. 2, Meth-A and B16F10 cells were fixed with 20% ethanol/PBS (divalent cation-free phosphate buffered saline) and stored at 4°C. B16F10 cells were trypsinized (0.17% trypsin and 30 mM
EDTA) before the ethanol fixation.

The fixed cells were incubated with 0.25% RNase (Type II-A, Sigma Chemical Co.) for 3 h at 4°C. Immediately prior to measurements, the cells were stained with PI solution (propidium iodide, 7.5 x 10⁻³ M) and examined for red fluorescence by flow cytometry (FCM). Under these staining conditions, the signal due to residual double-stranded RNA is negligible and the relative intensity of red fluorescence corresponds to the DNA content (8).

**DNA synthesis in polyploid cells.** Exponentially growing Meth-A and B16F10 cells were plated in Petri dishes (60 mm diameter, NUNC) at a density of about 2 x 10⁵ and 1 x 10⁶ cells/dish, respectively, with the medium being changed 24 h after seeding. Twenty-four hours thereafter, the cells were exposed to demecolcine at a final concentration of 0.1 µg/ml. At various times, the cells were exposed to 5-bromodeoxyuridine (BrdU, Wako Pure Chemical Industries, Ltd.) at a final concentration of 10 mM for 30 min. The cells were washed with PBS⁻¹, fixed with 20% ethanol and stored at 4°C.

The fixed cells were incubated with 0.25% RNase for 3 h at 4°C, after which they were treated with 2N HCl for 45 min at 25°C, and then neutralized with 0.1 M Na₂B₄O₇ (pH 8.5). A 1 x 10⁶ cell suspension in 100 µl PBS⁻¹ containing 2% BSA (bovine serum albumin), 0.5% Tween 20 and 5 µg anti-BrdU monoclonal antibody (Becton Dickinson) was then incubated for 12 h at 4°C. After these cells had been washed 3 times with washing solution (PBS⁻¹ containing 0.5% Tween 20 and 2% BSA), they were stained with FITC-conjugated rabbit anti-mouse IgG (E.Y. Labs. Inc.) for 3 h at room temperature (RT). The cells were stained with PI solution and examined by FCM for green and red fluorescences, which correlated to c-Myc protein and DNA content, respectively.

**Measurements of c-Myc protein in polyploid cells.** Exponentially growing Meth-A and B16F10 cells were plated in Petri dishes (60 mm diameter, NUNC) at a density of about 2 x 10⁵ and 1 x 10⁶ cells/dish, respectively, with the medium being changed 24 h after seeding. Twenty-four hours thereafter, the cells were exposed to demecolcine at a final concentration of 0.1 µg/ml. At various times, the cells were harvested, fixed with 20% ethanol, and then treated with RNase as described above.

A 1 x 10⁶ cell suspension in 100 µl PBS⁻¹ containing 0.5% Tween 20, 2% BSA and 5 µl of anti-c-Myc monoclonal antibody (Cambridge Res. Biochem. Ltd.) was incubated for 30 min at RT. The cells were washed 3 times with washing solution, and then stained with FITC-conjugated rabbit anti-mouse IgG (E.Y. Labs. Inc.) for 30 min at RT. The cells were stained with PI solution and examined by FCM for green and red fluorescences, which correlated to c-Myc protein and DNA content, respectively.

**Flow Cytometry (FCM).** Fluorescence from individual cells was measured with a Cytofluorograf system 50H (Ortho Instruments) and a Facsort (Becton Dickinson Immunocytometry Systems). The fluorescence of individual cells irradiated with a focused laser light at a wavelength of 488 nm was detected by photomultiplier tubes. Relative intensities of green and red fluorescence were measured, and DNA histograms were obtained. FCM measurements were performed as soon as possible after sample preparation.

**RESULTS**

To examine the cell cycle parameters of polyploidizing Meth-A and B16F10 cells, the cells were partially synchronized by HU to exclude G₂/M phase cells and exposed to demecolcine. At various times, the DNA distribution of cell population was obtained by FCM. Figure 1 shows representative DNA fluorescence histogram of Meth-A and B16F10 cells after the addition of demecolcine. In Fig. 1, 16c and 32c DNA peaks appeared on the histograms at 96 (C in Fig. 1) and 112 h (F in Fig. 1) after the drug addition to B16F10 and Meth-A cells, respectively, suggesting that both cell lines were polyploidized by demecolcine.

In Fig. 1, histograms C and F contain more than three peaks, indicating that not all of the cells in the population were polyploidized. A large fraction of the shoulder population appeared in the histogram of the Meth-A cell population (F in Fig. 1). They belong to the cell debris of dead cells and due to the lack of medium change.

To quantify the cell-cycle response after drug exposure, the DNA contents of the main peaks (most frequent peaks) and the sub-peaks (those other than the main peaks) were plotted against time after demecolcine addition (Fig. 2). In Fig. 2, the solid lines were drawn by considering the peaks of the most progressive sub-populations that had the maximum DNA content at the time. In drawing the S phase, the shoulders in the histograms were taken into account. Panel B in Fig. 2 represents the results of two independent experiments. Figure 2 reveals that the DNA content of Meth-A and B16F10 cells increased step by step from 2c to above 16c, implying progressive polyploidization.

The cell cycle parameters of the polyploidizing cells are listed in Table I. The cell cycle time (defined as G₂/M phase of lower ploidy plus G₁/S phase of higher ploidy) gradually increased with a ratio of about 1.5 in both cell lines. In the Meth-A cell population, the length of the S phase increased at 4.2, 8.3, 14.2 and 29.6 h with the ploidy increase but was a constant 6 h in the B16F10 cells. These findings suggest that the S-phase duration is not always constant in polyploidization.

To examine the rate of DNA synthesis in the polyploidized Meth-A and B16F10 cells, BrdU uptake in these polyploidized cells was measured by FCM using anti-BrdU monoclonal antibody (Fig. 3). Figure 3 shows the BrdU/DNA cytograms and the DNA histograms (the insert figure) of polyploidizing Meth-A and
B16F10 cells. The relative content of BrdU incorporated in cells was estimated in the mid S phases, and the results are listed in Table I. The BrdU uptake relating to DNA synthesis rate was increased with the ploidy increase in both cell lines, but the manner of increase was essentially different. The rate increased doubly as 1.0, 4.0 and 8.4 in B16F10 cells but was almost constant as 1.0, 1.2 and 1.8 in Meth-A cells.

To explain the difference in the DNA synthesis rate between Meth-A and B16F10 cells, we measured the cellular content of c-Myc protein, which might be related to the DNA synthesis, using an anti-c-Myc monoclonal antibody (Fig. 4). Figure 4 shows the c-Myc/DNA cyto-grams of Meth-A and B16F10 cells. The relative content of c-Myc in cells was estimated in the mid S phases, and the findings are listed in Table I. The c-Myc content was increased with ploidy increase in both cell lines, but the manner of increase, as well as that of the BrdU uptake was essentially different. The c-Myc content increased doubled as 1.0, 2.8 and 4.8 in B16F10 cells but was almost constant as 1.0, 1.2 and 1.4 in Meth-A cells. We concluded that the constant level in c-Myc content is responsible for the elongation of the S-phase duration and the constant rate of DNA synthesis in the polyploidizing process of Meth-A cells.

**DISCUSSION**

Polyploid cells are observed in various organs, particularly in the aged or partial hepatectomized liver, but the mechanism of polyploidization is unclear (9, 10,
Table 1. The cell cycle parameters in polyploidizing B16F10 and Meth-A cells.

<table>
<thead>
<tr>
<th>Phase DNA level</th>
<th>S (2c-4c)</th>
<th>G2+M+G1 4c</th>
<th>S (4c–8c)</th>
<th>G2+M+G1 8c</th>
<th>S (8c–16c)</th>
<th>G2+M+G1 16c</th>
<th>S (16c–32c)</th>
<th>G2+M+G1 32c</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B16F10 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase Durations (h)</td>
<td></td>
<td>6.0</td>
<td>18.0</td>
<td>6.0</td>
<td>30.0</td>
<td>6.0</td>
<td>&gt;48.0</td>
<td></td>
</tr>
<tr>
<td>Cycle time (h)</td>
<td></td>
<td>24.0 →</td>
<td>36.0 →</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Synthesis ratio*</td>
<td>1.0</td>
<td>4.0</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(relative width)</td>
<td>(0.4–1.4)</td>
<td>(2.4–5.6)</td>
<td>(4.6–12.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Myc content ratio*</td>
<td>1.0</td>
<td>2.8</td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(relative width)</td>
<td>(0.6–1.6)</td>
<td>(2.0–3.6)</td>
<td>(3.4–6.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Meth-A cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase Durations (h)</td>
<td></td>
<td>4.2</td>
<td>13.0</td>
<td>8.3</td>
<td>19.4</td>
<td>14.2</td>
<td>18.8</td>
<td>29.6</td>
</tr>
<tr>
<td>Cycle time (h)</td>
<td></td>
<td>21.3 →</td>
<td>33.6 →</td>
<td>48.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Synthesis ratio*</td>
<td>1.0</td>
<td>1.2</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(relative width)</td>
<td>(0.5–1.7)</td>
<td>(0.5–2.5)</td>
<td>(0.8–2.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Myc content ratio*</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(relative width)</td>
<td>(0.6–1.4)</td>
<td>(0.7–1.5)</td>
<td>(0.7–2.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* values at the mid-S phase.

Fig. 3. Representative BrdU/DNA cytograms of B16F10 (A and B) and Meth-A (C and D) cells, and the DNA histograms (insert figures). Exponentially growing B16F10 and Meth-A cells were exposed to demecolcine of 0.1 µg/ml. At various times, the cells were exposed to BrdU for 30 min, and stained with anti-BrdU antibody and PI. BrdU/DNA cytograms and the histograms were then obtained. In each cytogram, the ordinate and the abscissa represent the BrdU and DNA content, respectively. Note that the scale of BrdU content differs between B16F10 cells (A and B) and Meth-A cells (C and D). The insert figures represent the DNA histograms for the cytograms. Panels A and C are of the control, and panels B and D are the histograms 48 hours after demecolcine addition.
DNA Synthesis in Polyploidization

Fig. 4. Representative c-Myc/DNA cytograms of B16F10 (A and B) and Meth-A (C and D) cells. Exponentially growing B16F10 and Meth-A cells were exposed to demecolcine of 0.1 μg/ml. At various times, the cells were stained with anti-c-Myc antibody and PI, and c-Myc/DNA cytograms were obtained. In each cytogram, the ordinate and the abscissa represent the c-Myc and DNA contents, respectively. The insert figures represent the negative control in which cells were stained with PBS instead of the first antibody. Dotted bands represent the base lines. Panels A and C are of the control, and panels B and D are the cytograms 40 and 30 h after the drug addition, respectively.

It has been reported that the duration of S phase in mammalian cells is almost constant regardless of the ploidy (6), suggesting that certain substances, such as enzymes and DNA precursors, are responsible for the rate-limiting step of DNA synthesis increase with the DNA level. Although many studies have noted the mechanism of polyploidization of cultured cells (1, 2, 7, 15, 18-21), increasing duration of the S phase in the process of polyploidization has not been reported.

We showed an exception that the duration of S phase increased with the ploidy increase in Meth-A cells. The prolongation in S phase is in harmony with the results that the rate of DNA synthesis, as well as c-Myc content, was almost constant in the level of magnitude. It should be mentioned that the length of G2/M + G1 phase markedly increased with ploidy increase in B16F10 cells, but it did not increase in Meth-A cells. If the increase of G2/M + G1 phase primarily depends on that of G1 phase, the elongation of G1 phase might be used to accumulate the substances for the subsequent DNA synthesis in B16F10 cells. It seems that Meth-A cells enter the polyploid S phase without accumulating the substances required in DNA synthesis.

B16F10 cells responded per the hypothesis that duration of S phase in mammalian cells is almost constant regardless of the ploidy (6). These acceptable results with B16F10 cells may confirm the validity of our findings with Meth-A cells. Many studies have shown polyploidization of cells by drugs, such as mitotic poisons (11, 12, 16), topoisomerase inhibitors (22) and kinase modulators (17), and different responses among cell species in the polyploidization were also noted (12, 16). Though we presently did not demonstrate the difference in cellular substance between Meth-A and B16F10 cells, except for cellular c-Myc content, further findings on the number of replicon, the elongation speed in DNA replication and the content of DNA polymerase may reveal the mechanism of polyploidization in Meth-A cells.

It should be emphasized that this study of the cellular response to a specific drug refers only to intracellular variations in Meth-A and B16F10 cells cultured under defined proliferating conditions.

Acknowledgments. This study was supported in part by a grant from the Project Research Fund of Kanazawa Medical University (P96-2, 1996).

REFERENCES


(Received for publication, April 9, 1997 and in revised form, June 23, 1997)