Identification of S-phase Cells with PC10 Antibody to Proliferating Cell Nuclear Antigen (PCNA) by Flow Cytometric Analysis

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Introduction
Growth fraction measurement is particularly valuable for characterization of tumors. Monoclonal antibodies (MAb) against several nuclear antigens of proliferating cells, e.g., Ki-67 antigen (10) and DNA polymerase-α (28), are now used to estimate the growth fraction. PCNA (proliferating cell nuclear antigen), an auxiliary protein of DNA polymerase-α, which is closely associated with DNA synthesis and repair (3,25), is also regarded as a marker for the growth fraction as assessed with MAb (1,11,16,17,23,24,29). PCNA is expressed in nuclei of proliferating cells (4–7,11,15,18,20,27,32–36) and has been advocated for assessment of biological behavioral measurement in many fields of research (1,2,16,24,29). Nevertheless, it has been commonly reported that PCNA has been detected in S-phase cells when an autoantibody in sera from patients with SLE is used (19,21,22,26). The discrepancy in PCNA reactivity can be explained by differences between epitopes that the auto- and monoclonal antibodies recognize (15). Moreover, the relationship between PCNA expression and the cell cycle remains controversial (4–7,11,15,16,18–23,27,29,32–36).

PCNA detection in S-phase cells with a cytogram that showed a horseshoe-like pattern with a peak level at mid-S-phase. Flow cytometric dual parameter analysis of PCNA/BrdU was carried out in HeLa cells to confirm detection of PCNA in S-phase cells with acetone/methanol fixation. The population of cells stained for both parameters, i.e., S-phase cells, was obviously discriminated from that of the non-S-phase cell in PCNA/BrdU bivariate cytograms. These results strongly suggest that PCNA used with acetone/methanol fixation would be equal to BrdU as an S-phase marker. (J Histochem Cytochem 42:1177–1182, 1994)

KEY WORDS: PCNA; BrdU; Cell cycle; Flow cytometry; HeLa cells.

We estimated the expression of proliferating cell nuclear antigen (PCNA) in HeLa S3 cells by flow cytometry with monoclonal antibody (MAb) PC10. HeLa cells were fixed with six different fixation procedures: 15-min and 30-min acetone, 15-min acetone followed by 15-min methanol (acetone/methanol), 30-min methanol, 15-min methanol followed by 15-min acetone (methanol/acetone), and a mixture of acetone and methanol. The fixed cells were applied to MAb PC10 against PCNA and then treated with FITC. With five fixation procedures except for acetone/methanol, PCNA was expressed in almost all cells with similar shapes and different FITC intensity levels on PCNA/DNA bivariate cytograms, whereas acetone/methanol fixation allowed expression and the cell cycle remains controversial (4–7,11,15,16,18–23,27,29,32–36).

MAb PC10 to PCNA, which can be applied to formalin-fixed, paraffin-embedded sections, is most useful for PCNA staining of histological materials (1,2,8,11,16,23,29,35). It is important in surgical pathology to characterize the relationship between PC10 reactivity and the cell cycle. The aim of this study was to develop a novel method for detection of PCNA using MAb PC10 for investigation of HeLa S3 cells fixed with acetone and/or methanol (the two fixatives ordinarily employed). We employed flow cytometric (FCM) analysis, which effectively identifies the distribution of cells during the cell cycle (19–22,36), to define the relationship between PC10 reactivity in HeLa cells fixed with different procedures and the cell cycle.

Materials and Methods
Fixation Procedures. HeLa S3 cells (Japanese Cancer Research Resources Bank) incubated in Dulbecco’s modified Eagle’s medium (Nissui; Tokyo, Japan) were used in this study. After the cells were harvested with 0.25% trypsin–EDTA solution (Difco; Detroit, MI) they were fixed in 100% acetone alone or 100% methanol alone, or in combinations of the two fixatives at −20°C in suspension. The procedures used were: (a) acetone for

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with FITC-conjugated PClO were observed by fluorescence microscopy before measurement by flow cytometry.

**Flow Cytometric PCNA/DNA Analysis.** The fixed cells were treated with normal goat serum (diluted 1:4) (Dako; Glostrup, Denmark) for 30 min at room temperature (RT) and then incubated with FITC-conjugated PClO (diluted 1:20 with PBS; Dako) for 60 min at RT, and were kept in the dark until FCM analysis. Cells incubated with FITC-conjugated goat anti-mouse IgG (diluted 1:20 with PBS) (Tago; Burlingame, CA) for 60 min at RT were prepared as negative control cells in each case. After each step the cells were washed three times with PBS. The cells were re-suspended in 2 ml PBS, and 50 μg/ml propidium iodide (PI) was applied to assay DNA content, followed by 1 mg/ml RNASe (ribonuclease A, Type 1-A, from bovine pancreas) (Sigma; St Louis, MO). FCM analysis was performed with a FACScan flow cytometer (Becton-Dickinson; Mountain View, CA). Green fluorescence emanating from FITC was collected by fluorescence detector 1 (FL 1) using a 510-nm detector level. Red fluorescence emanating from PI was collected by fluorescence detector 2 (FL 2), using a 460-480-nm detector level to set the GI population at 300 channel region of DNA content. Debris was excluded by a gating system. Data were stored in a list mode. All experiments were carried out independently at least 10 times. Statistical analysis of values acquired from the FCM analysis was performed with Student's t-test. Staining patterns of PCNA-positive cells in the acetone/methanol procedure were also estimated in this study. Cells stained with FITC-conjugated PClO were observed by fluorescence microscopy before measurement by flow cytometry.

**PCNA/BrdU Dual Parameter Analysis.** HeLa S3 cells previously incubated with 10 μM BrdU labeling reagent (diluted 1:1000) (Amersham, Poole, UK) for 60 min at 37°C were used. The cells were fixed with acetone/methanol and then blocked with 10% human IgG for 30 min at RT to avoid nonspecific reaction. In the first step, cells were incubated with anti-BrdU mouse MAb containing nuclease (diluted 1:50 with distilled/deionized water; cell proliferation kit, Amersham) for 30 min at RT. The nuclease for DNA denaturation was supplied in freeze-dried 1×-buffered saline (TBS) containing bovine serum albumin (BSA), magnesium chloride, and a stabilizer. This antibody has the advantage over previously used antibody in that it denatures DNA structure without procedures that damage other antigens, e.g., strong acid treatment and heating. The cells were then applied to FITC-conjugated goat anti-mouse IgG (Tago) for 30 min at RT. In the second step, cells were stained with RPE-conjugated PClO (diluted 1:50; Dako) for 60 min at RT. The control prepared for dual parameter analysis consisted of cells that were treated with anti-BrdU MAb followed by FITC-conjugated goat anti-mouse IgG and with RPE-conjugated goat anti-mouse IgG (Southern Biotechnology Associates; Birmingham, AL) without PClO. Furthermore, cells incubated with BrdU reagent in serum-free medium for 6 or 12 hr were analyzed by the same methods as above. After being applied to 1 mg/ml RNAse, the cells were analyzed by flow cytometry. FITC for BrdU and red fluorescence from RPE for PCNA were collected by FL 1 using a 490-nm detector level and FL 2 using a 515-nm detector level.

![Figure 1. Bivariate cytograms of DNA content vs PCNA distribution in HeLa cells fixed by the following procedures: (A) 15-min acetone, (B) 30-min acetone, (C) acetone/methanol, (D) 30-min methanol, (E) methanol/acetone, and (F) 30-min mixture. A population of the control cells shows a lower intensity level than FITC-positive cells in each procedure.](image-url)
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Results

PCNA/DNA Bivariate Cytogram

Cells fixed by each procedure were shown in PCNA/DNA bivariate cytograms created with a flow cytometer. Almost all cells, except for those subjected to acetone/methanol fixation, showed positive staining for PCNA, and the PCNA/DNA bivariate cytograms had similar shapes but different maximal levels of FITC intensity (Figures 1A-1F). The FITC intensity of these cells varied widely in each phase of the cell cycle, the level of intensity gradually increasing with progression of the cell cycle. Acetonelmethanol-fixed cells, in contrast, showed a horseshoe-like pattern with a peak level at mid-S-phase, consistent with the pattern of negative control cells in GI and G2/M phases (Figure 1C). This shape was similar to the pattern acquired after BrdU staining of pulse-labeled cells (not shown).

Estimations of PCNA-positive Cells

Calculated from histogram of FITC intensity vs cell number (not shown) for each procedure, the percentages of PCNA-positive cells in the sample, which did not contain control cells, were 91.4 ± 3.1% for 15-min acetone, 90.5 ± 3.6% for 30-min acetone, 33.2 ± 3.2% for acetone/methanol, 90.6 ± 2.8% for 30-min methanol, 91.5 ± 3.2% for methanol/aceton, and 88.4 ± 4.3% for the mixture. The percentage for acetonelmethanol was lower than for the other procedures; there were no significant differences in percentage among the five procedures, except for acetonelmethanol. Figure 2 shows PC10-positive cells fixed with acetone/methanol or 30-min methanol. A large majority of cells fixed with 30-min methanol were stained with PC10 (Figure 2A), whereas a minority of cells fixed with acetone/methanol were stained (Figure 2B). Furthermore, the positive cells after the acetone/methanol fixation procedure could be roughly classified into three types: small nuclei with a small number of dots (Figure 3A), large nuclei with a large number of dots (Figure 3B), and large nuclei with a small number of dots (Figure 3C).

In PCNA/DNA bivariate cytograms (Figure 1), the half value of FITC intensity extent for each phase was measured at G1, mid-S, and G2/M phase regions, which were determined by DNA content. The averages of half values were then calculated a total of 10 times. Table 1 shows the average FITC intensity of each phase in HeLa cells fixed by the different procedures. Figure 4 indicates relative FITC intensity values in each phase when FITC intensity is 1.0 in G1 cells of the control, ignoring the standard deviations in Table 1. The procedures, except for acetone/methanol fixation, in descending order of FITC intensity were: 30-min methanol, methanol/aceton, mixture, 30-min acetone, and 15-min acetone (Figure 4). There were no significant differences of FITC intensity averages in any phases between 30-min methanol and methanol/aceton or between 30-min acetone and 15-min acetone.

PCNA/BrdU Dual Parameter Analysis

Cells that were fixed with acetone/methanol and were doubly stained for PCNA and BrdU with two kinds of fluorescence were analyzed by flow cytometry. As shown in Figures 5A and 5C, cells were divided into three compartments. Cells showing high fluorescence intensity were the positive cells for each parameter. Consequently, other cells showing low intensity might represent unstained cells and debris. In Figure 5B, cells showing higher intensity for two parameters and clearly discriminated from cells showing lower intensity might be doubly stained cells with both parameters. Interestingly, the

Table 1. FITC intensity values (mean ± SD; channel number) of each phase in HeLa cells fixed by different procedures

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
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<tbody>
<tr>
<td>Acetone 15 min</td>
<td>150 ± 40</td>
<td>168 ± 51</td>
<td>213 ± 50</td>
</tr>
<tr>
<td>Acetone 30 min</td>
<td>139 ± 34</td>
<td>146 ± 38</td>
<td>188 ± 42</td>
</tr>
<tr>
<td>Acetone/methanol</td>
<td>65 ± 16</td>
<td>268 ± 60</td>
<td>107 ± 20</td>
</tr>
<tr>
<td>Methanol 30 min</td>
<td>480 ± 61</td>
<td>587 ± 78</td>
<td>715 ± 113</td>
</tr>
<tr>
<td>Methanol/aceton</td>
<td>434 ± 91</td>
<td>524 ± 78</td>
<td>625 ± 122</td>
</tr>
<tr>
<td>Mixture</td>
<td>252 ± 58</td>
<td>282 ± 56</td>
<td>344 ± 65</td>
</tr>
<tr>
<td>Control</td>
<td>58 ± 15</td>
<td>69 ± 17</td>
<td>72 ± 21</td>
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fluorescence intensity of PCNA increased linearly with that of BrdU in doubly stained cells. The control cells showed a high intensity level for FITC and a low level for RPE, in a linear fashion (Figure 6).

Calculated from histograms for two parameters, obtained from 10 experiments, the positive cells as a proportion of total cell number were 30.4 ± 2.7% for PCNA and 31.3 ± 1.7% for BrdU; the difference was not significant. Furthermore, they were correlated in a linear fashion, \( Y = 7.65 + 0.79X \) \((r = 0.736)\), as shown in Figure 7A. In cells incubated with serum-free medium for 6 or 12 hr, the proportions were 19.8 ± 2.4% for PCNA and 20.0 ± 2.2% for BrdU, or 12.8 ± 5.3% for PCNA and 13.3 ± 4.6% for BrdU, respectively. Moreover, they also showed a linear correlation, as indicated in Figures 7B and 7C.

**Discussion**

The cell kinetics of tumors can be characterized by determining the relationship between cell proliferation activity and the cell cycle. The measurement of S-phase in the cell cycle provides much information about cell kinetics (8,12,33). If PCNA is detected only in S-phase cells when MAb antibodies are used, the antigen would meet with greater acceptability. PCNA consists of two different subpopulations. One is closely associated with DNA replication and the other is loosely bound to nuclear structures and is not so associated (4). Consequently, various procedures have been used in attempts to detect the replicon complex population of PCNA with MAb (20,36). Landberg and Roos (20) detected only the replicon complex population, i.e., a limited population in the S-phase, with PC10 antibody by a detergent extraction/fixation method: Triton X-100 followed by methanol fixation. In our preliminary study of PCNA/BrdU double staining using MAb 19A2 in HeLa cell smears fixed with acetone/methanol, PCNA was detected in the S-phase cells (Beppu and Ishida, unpublished observations). However, the judgment of immunostaining positivity differs with the visual interpretation of the observers. In the present study we performed FCM analysis to estimate PCNA detection with MAb PC10 in HeLa cells fixed by various procedures, including acetone/methanol fixation.

In FCM analysis, positive PCNA was detected in almost all cells (approximately 88–91%), except those subjected to acetone/methanol fixation, and bivariate PCNA/DNA cyotograms had similar shapes (Figure 1). Furthermore, Figure 2A showed that a majority of cells fixed with 30-min methanol were stained with FITC-conjugated PC10. These findings were consistent with several studies that have reported PCNA in the nuclei of proliferating cells fixed with either acetone or methanol (14,36). By using acetone/methanol fixation, we found that PCNA positivity was limited to the
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Figure 7. Correlations between percentages of identified S-phase cells for PCNA and BrdU, calculated from histograms in PCNA/BrdU dual parameter analysis. Cells were incubated in (A) normal medium or in serum free medium for (B) 6 hr and (C) 12 hr before FCM analysis.

S-phase, showing a peak level of fluorescence intensity in mid-S-phase, in contrast to the other fixation procedures (Figure 1C). This shape was similar to that of BrdU (30), indicating that cells showing high FITC intensity were in mid-S-phase and those showing the weak intensity were in either early or late S-phase. In contrast to methanol-fixed cells, as shown in Figure 2A, a minority of cells fixed with acetone/methanol were stained with FITC-conjugated PC10 (Figure 2B). We also estimated the staining patterns with FITC-conjugated PC10 in acetone/methanol-fixed cells. As a result, the positive cells were roughly classified into three types (Figures 3A–3C). When these were compared with Figure 1C and some reports (4,6,33), the cells stained with lower intensity in small nuclei (Figure 3A), with higher intensity in large nuclei (Figure 3B), and with lower intensity in large nuclei (Figure 3C) might belong to early S-, mid-S-, and later S-phase, respectively.

To test these concepts, we carried out a flow cytometric dual parameter analysis of PCNA/BrdU in HeLa cells fixed with acetone/methanol. The percentage of PCNA-positive cells obtained from a histogram, as shown in Figure 5A, was consistent with the BrdU value, the difference not being significant. These values were in good agreement with previous estimates of the S-phase in HeLa cells (36). Furthermore, the longer the period of incubation in serum-free medium, the more these percentages decreased with linear correlations (Figures 7B and 7C). These findings indicated that DNA synthesis was inhibited under unfavorable conditions.

Because BrdU is specific for S-phase cells, doubly stained cells discriminated clearly from the other cells in Figure 5B would be S-phase cells. Furthermore, doubly stained cells were linearly described according to increasing fluorescence intensities of both parameters in a bivariate cytogram. These findings support our hypothesis that PCNA is a good S-phase marker but may also be useful for differentiating the three segments in S-phase by use of our fixation procedure. In our preliminary study of PCNA/BrdU double staining with MAb 19A2 to PCNA in HeLa cell smears fixed with acetone/methanol, PCNA staining intensity seemed to parallel that of BrdU in doubly stained cells under the light microscope (Beppu and Ishida, unpublished observations).

We also estimated the differences in FITC intensity levels for each fixation procedure used in this study. There was no significant difference of average FITC intensity value in all phases between 30-min methanol and methanol/acetone. These findings suggest that cells previously fixed with methanol were not affected by later fixation with acetone for PCNA staining. The 15- and 30-min acetone-fixed cells showed lower FITC intensity for PCNA than those with the other procedures, except for acetone/methanol, there being no significant difference between the two procedures. In particular, the average value for the mixture of acetone and methanol (Procedure f) was in between that for acetone fixation and methanol alone or methanol/acetone fixation (Figure 4). Consequently, acetone fixation might not be suitable for PCNA staining. Moreover, acetone inhibits methanol fixation to some degree, excluding the methanol fixation when previously treated. With acetone/methanol fixation we detected PCNA in S-phase cells via FCM analysis, although we are still unable to explain the mechanisms responsible for this phenomenon. Since when acetone fixation (Procedures a and b) was used PCNA was positive in almost all cells identified as proliferating, it appears that the function of acetone in acetone/methanol fixation would not be the same as that of Triton X-100 in the detergent extraction/fixation method (20). When the values for average FITC intensity (shown in Table 1) were corrected by subtracting the control values from the values for each procedure, the S-phase intensity value for acetone/methanol fixation was approximately 38% of that for methanol fixation. This percentage value might be associated with the ratio of chromatin associated to total PCNA, this being 20–35% (4,27,36). It was a little larger than the latter ratio, probably owing to differences in the culture cells used. We speculate that when the acetone/methanol fixation method is used, only the replicon complex subpopulation of PCNA is detected in the S-phase cells, due to some mechanisms different from the one operating in the detergent extraction/fixation method (20). Therefore, it appeared that the two different subpopulations of PCNA were discriminated by the acetone/methanol fixation. Possible explanations for our findings are (a) that the epitope of the subpopulation that is loosely bound to DNA might be modified by some means during the process of continuous fixation with acetone and methanol, and (b) that this subpopulation might be extracted from the nuclei during methanol fixation by previous fixation with acetone.

Although the mechanism(s) of differential staining is unknown, the information acquired from PCNA detection using this fixa-
duction procedure may be equivalent to the cell kinetics provided by BrdU staining. Finally, much more work with our fixation procedure in specimens is indicated and anticipated, as the cell line data from this study do not directly reflect the surgical pathology.

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