Laser scanning cytometry (LSC) allows detailed analysis of the cell cycle in PI stained human fibroblasts (TIG-7)

M. Kawasaki, K. Sasaki, T. Satoh*, A. Kurose†, T. Kamada, T. Furuya, T. Murakami and T. Todoroki‡

Department of Pathology, Yamaguchi University School of Medicine, Ube, *Chromosome Research Center, Olympus Optical Co., Hachioji, †Department of Pathology, Iwate Medical University, Morioka, and ‡Department of Surgery, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan

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Abstract. We have demonstrated a method for the *in situ* determination of the cell cycle phases of TIG-7 fibroblasts using a laser scanning cytometer (LSC) which has not only a function equivalent to flow cytometry (FCM) but also has a capability unique in itself. LSC allows a more detailed analysis of the cell cycle in cells stained with propidium iodide (PI) than FCM. With LSC it is possible to discriminate between mitotic cells and G₂ cells, between post-mitotic cells and G₁ cells, and between quiescent cells and cycling cells in a PI fluorescence peak (chromatin condensation) vs. fluorescence value (DNA content) cytogram for cells stained with PI. These were amply confirmed by experiments using colcemid and adriamycin. We were able to identify at least six cell subpopulations for PI stained cells using LSC; namely G₁, S, G₂, M, postmitotic and quiescent cell populations. LSC analysis facilitates the monitoring of effects of drugs on the cell cycle.

An analysis of the cell cycle is frequently necessary in both biological research fields and clinical medicine. Cell cycle analysis is usually made by DNA measurement for cells stained with a DNA specific dye such as propidium iodide (PI). Flow cytometry (FCM) permits a rapid measurement of cellular DNA content, and at present this is a representative method of cell cycle analysis. However, it provides limited information concerning the cell cycle, namely the percentages of G₁, S and G₂M of a cell population. When we want to know further information concerning the cell cycle, additional steps, which are usually cumbersome and laborious, are required for FCM. Another disadvantage in FCM is that it cannot give morphological data for an individual cell of interest. In contrast, varying systems for image cytometry (ICM) give morphological information coupled with cell kinetic data, although the analytical speed of ICM is much slower than FCM. ICM as well as FCM has rapidly been improved with great advances in computer and optical technology. Laser scanning cytometry (LSC), a newly developed cytometric technology (Kamentsky & Kamentsky 1991), which is applicable to cells fixed on a microscope slide, offers cell kinetic information equivalent to that of FCM (Martin-Reay *et al.* 1994, Clatch *et al.* 1996, Kakino *et al.* 1996, Li *et al.* 1996, Sasaki *et al.* 1996, Kamada *et al.* 1997). Since functionally LSC has the capability of both FCM and ICM, it has several advantages over FCM. Recently Luther and Kamentsky

Correspondence: Dr K. Sasaki, Department of Pathology, Yamaguchi University School of Medicine, Ube 755, Japan.

reported that LSC allowed discrimination of mitotic cells from those in other phases in PI stained preparations (Luther & Kamensky 1996). In this brief report, we show that LSC easily identified six different populations of the cell cycle in PI stained cells, namely G₁, S, G₂, M, post-mitotic and quiescent cell populations. LSC is convenient for monitoring the effects of drugs including anticancer agents on the cell cycle, and it will be a potentially powerful instrument for cell cycle analysis.

MATERIALS AND METHODS

Cell line

A cell line derived from fetal lung fibroblasts, TIG-7, was cultured in Dulbecco's modified minimum essential Eagle's medium (DMEM, Nissui, Tokyo) supplemented with 10% FBS in an incubator containing 5% CO₂ at 37°C. The experiments were performed during the exponentially growing condition of the cells, unless otherwise mentioned. The cells were grown on a glass slide and fixed in 70% ethanol at room temperature. The cells were rinsed in a phosphate-buffered saline (PBS) containing RNase (1 mg/ml, Sigma St Louis, MO) and then stained by propidium iodide (PI, Sigma) staining solution at a concentration of 25 µg/ml in PBS. For the study of quiescent cells, cells were cultured at confluence, which virtually removed the population of S phase cells.

Drugs

Colcemid (2 µg/ml) and adriamycin (ADM, 1 µg/ml) were used to accumulate cells at metaphase and G₂ phase, respectively. The cells were incubated with colcemid for 3 h, followed by culturing in normal medium without the drug. The slides were fixed in 70% ethanol after 0, 10, 20, 30, 40, 60, 120 and 180 min. Alternatively, the cells were cultured in the medium containing ADM for 18 h, and then transferred to an ADM free medium. The slides, as well as the experiments using colcemid, were fixed in 70% ethanol after incubation for 0, 10, 20, 30, 40, 60, 120 and 180 min.

Laser scanning cytometry (LSC)

We used a laser scanning cytometer LSC 101 (Olympus, Tokyo) for measuring nuclear DNA. Three parameters were employed for expression of nuclear characteristics; i.e. nuclear area, PI fluorescence peak and PI fluorescence value. We showed two-parameter cytograms of the possible permutations of these three parameters. The PI fluorescence peak which indicates the highest pixel value within the segmenting contour of the nucleus corresponds to the state of chromatin condensation, and the value is the sum of all the pixel values within the nucleus (Kamensky & Kamensky 1991, Luther & Kamensky 1996). Usually >5000 cells were counted, and data of each cell were recorded in list mode.

Since LSC measurements are microscopic slide based, location of each cell on the slide is automatically recorded together with cellular data. It is possible to view cellular morphology coupled with cellular data in the interesting cells. In this experiment, morphological features were examined in cells stained with PI, but a detailed examination of cellular morphology was made after restaining with Giemsa stain.

RESULTS

The PI fluorescence value, which is the sum of all of the pixel values within the nucleus, corresponds to the DNA content of the nucleus, and consequently it generates a DNA

histogram. The coefficient of variation of the DNA histograms was usually around 2.8% for TIG-7 (Figure 1d). The fluorescence peak, which indicates the highest pixel value of PI fluorescence within the nucleus, varied greatly even among cells with the same DNA content, especially in G_1 and G_2M regions (Figure 1c). A PI fluorescence peak vs. PI fluorescence value cytogram allowed us to identify two regions different from G_1 , S and G_2 phases in an exponentially growing cell population (Figure 2). One was a region including cells with the high PI fluorescence peak and 2C DNA content, and the other consisted of cells manifesting a high PI fluorescence peak and a 4C DNA content. The visual examination of cellular

Figure 1. A representative display of LSC DNA measurement of exponentially growing TIG-7 cells stained with propidium iodide. Two-parameter cytograms of the possible permutations of nuclear area, PI fluorescence peak and PI fluorescence value, and a DNA histogram. PI fluorescence area corresponds to nuclear area. PI fluorescence peak indicates the highest pixel value within the segmenting contour of the nucleus, and the PI fluorescence value is the sum of all the pixel values within the nucleus. PI fluorescence (nuclear) area vs. PI fluorescence peak (A), PI fluorescence (nuclear) area vs. PI fluorescence value (B), PI fluorescence peak vs. PI fluorescence value (C) and DNA histogram (D).

morphology revealed postmitotic cells in the former region (Figures 2a–c) and mitotic cells in the latter region (Figures 2l & m).

Treatment of cells with colcemid, which accumulated metaphase cells (Figure 3), disclosed a small but distinct cluster of dots with a high PI fluorescence peak in the tetraploid region (Figure 4). Cells in metaphase showed the highest PI fluorescence peak in the cell population. The population of mitotic cells was apparently separated from a G_2 cell population, based on the level of PI fluorescence peak. The level of the PI fluorescence peak was 2.5 times higher in M cells than in G_2 cells in which the PI fluorescence peak was 1.2 times that in G_1 cells. The PI fluorescence peak of prophase nuclei was lower than that in metaphase cells, but higher than that in G_2 cells. The nuclei arrested in metaphase were smaller than those in other regions in a cytogram of PI fluorescence value *vs.* area (Figure 4). Thereby, the population of metaphase cells was easily identified in both cytograms of value *vs.* area and of value *vs.* peak. Postmitotic cells with the same DNA content as G_1 cells virtually disappeared from the cytogram (Figure 4). This experiment using colcemid also settled the borderline between postmitotic cells and G_1 cells. Cells arrested in metaphase were released from the colcemid treatment, then moved to the latter part of the mitotic process and subsequently postmitotic cells appeared again within 30 min after release from the drug treatment.

On the basis of the level of the PI fluorescence peak, mitotic cells were segregated from G_2 cells, as mentioned above. This was amply clarified by an experiment using ADM which accumulated cells in the G_2 phase and consequently depleted mitotic cells. As a result, the border between the regions for G_2 and M cells in the cytogram was distinctly defined in the experiment using ADM (Figure 5). Dots reappeared in the region of the mitotic phase immediately after cells arrested at the G_2 phase by ADM were transferred to the normal medium. The cells migrated toward the higher level of peak on a cytogram, and subsequently cells reappeared in the post-mitotic cell region 40 min after release from the G_2 phase (Figures 5c & d).

As the number of cells increased in the culture, additional populations of cells with a low fluorescence peak appeared in a cytogram of value *vs.* peak (Figure 6). Eventually, a small but distinct cluster of cells was formed at confluent culture conditions where the clusters of mitotic and postmitotic cells were virtually absent and S phase cells were scarcely found (Figure 6). The cell cluster was separable from G_1 and G_2 cells. The newly appeared clusters under such a culture condition were considered quiescent cells.

The results in this study are supported by the microscopic observation that chromatin condensation rapidly increases during the prophase, and that it reaches a maximum level at metaphase. The fluorescence peak in telophase and anaphase cells decreased as compared to that in metaphase. Furthermore, the cell cycle progression was able to be visualized even during the mitotic phase by monitoring the changes in the fluorescence peak and its value (Figure 2).

DISCUSSION

The conventional univariate analysis of the cell cycle measuring nuclear DNA content provides information on the percentages of $G_1/0$, S and G_2 M cells in a population, but it cannot discriminate between cells with the same DNA content such as G_2 and M cells. This limitation may be circumvented partly by measurement of the right angle light scatter (Epstein, Watson & Smith 1988, Zucker *et al.* 1988). Immunocytochemical detection of a cell cycle phase specific constituent of cells may permit the identification of particular cells in a

Figure 2. A two-parameter (PI fluorescence value vs. PI fluorescence peak) cytogram shows discrimination of mitotic cells from G_2 cells and of post-mitotic cells from G_1 cells. Furthermore, subclassification of mitotic cells is possible based on the value and the peak for cells stained with only propidium iodide. At least six phases of the cell cycle can be distinguished on a cytogram. This is confirmed by the visual examination of each dot. (a–c) Post-mitotic cells; (d) early G_1 cells; (e) G_1 cells; (f, i) quiescent cells; (g, h) S cells; (j) G_2 cells; (k) late G_2 cells; (l–n) mitotic cells. Giemsa stain, $\times 400$ (original magnification).

Figure 3. The treatment with colcemid (2 µg/ml) accumulates cells at metaphase. The mitotic index (the number of mitotic cells/the number of total cells) increases with the incubation time.

cell population (Gong, Traganos & Darzynkiewicz 1993, Sasaki, Kurose & Ishida 1993, Traganos *et al.* 1994, Pellicciari *et al.* 1996). However, the immunocytochemical staining is usually cumbersome and time consuming. In contrast, nuclear DNA staining with PI is very simple and rapid. It is convenient because PI stained cells give detailed information of the cell cycle. Fortunately, it was reported that LSC allows discrimination between G_2 and mitotic cells in cells stained with PI (Luther & Kamensky 1996). As indicated in this paper, LSC makes it possible to know the cell cycle position of each cell, based on the level of the PI fluorescence peak. This is very difficult, infact almost impossible, for FCM analysis.

Figure 4. In cells treated with colcemid (2 µg/ml) for 3 h, a cluster of cells with high PI fluorescence peak and with small area in the tetraploid region indicated by a rectangular window is distinct in cytograms (a) and (b), respectively. Direct morphological observation reveals that these were mitotic cells. Tetraploid cells without the window are G_2 cells in both cytogram (a, b). PI fluorescence value vs. PI fluorescence peak (a), and PI fluorescence value vs. PI fluorescence area (b).

The treatment with ADM perturbs the cell cycle progression from G₂ phase to M phase, and consequently dots with high PI fluorescence peak disappeared from the cytogram, indicating no entry of cells in the mitotic phase. As a matter of course, this was accompanied by depletion of the dots corresponding to post-mitotic cells. Cells incubated with ADM were able to enter the mitotic phase a short time after release from ADM. This indicates that the treatment with ADM accumulates the cells immediately before the mitotic phase. Once the cells enter the mitotic phase, nuclear chromatin rapidly condenses as indicated by the PI fluorescence peak level. This was clarified by experiments using colcemid. At metaphase, chromatin condensation reached its maximum level which was 2.5 times more than that in G₂ cells. After metaphase, the PI fluorescence peak levels in anaphase lowered slightly, and then

Figure 5. Cells are accumulated at the G₂ phase by incubation with adriamycin (1 µg/ml) for 18 h. Virtually no cells are detected in the window which indicates the region of mitotic cells in cytograms (a) and (b). Cells appear in the box of cytograms (c) and (d) as early as 40 min after that cells are transferred to normal medium. These cells are mitotic cells.

Figure 6. Three representative cytograms. Only two populations are seen in cells under the confluent condition (a). Although such populations are not seen in an exponentially growing cell population (c), they become clear with the increase in culture density (b).

daughter chromosomes began to separate. Daughter cells just leaving the mitotic phase show a much higher peak level than the cells in the G_1 phase. During the progression through the early G_1 phase, the chromatin progressively decondensed to the same level as G_1 cells. The elevation of the PI fluorescence peak was slight from G_1 to G_2 . The combination of the PI fluorescence value (DNA content) and the PI fluorescence peak (chromatin condensation) allows determination of the cell cycle position of each cell. The PI fluorescence peak representing chromatin condensation is a parameter given exclusively by LSC. In exponentially growing cells stained with only PI, at least five different populations in the cell cycle are able to be discriminated by LSC analysis, namely G_1 , S, G_2 , M and post-mitotic cells.

Under confluent condition, normal fibroblasts are in a quiescent state. Generally, it is not easy to discriminate quiescent cells from cycling ones in a cell population. Non-cycling cells can be distinguished from cycling counterparts by a fascinating method using acridine orange (Darzynkiewicz 1990). Since the optimal condition of the method is very strict, the development of a simple method for discrimination between non-cycling and cycling cells is necessary for a detailed analysis of the cell cycle. Chromatin condensation/decondensation is considered to reflect DNA structure and function. Since the cells under confluent condition exhibited a low PI fluorescence peak, they were clearly separated from G_1 and G_2 populations based on the level of the PI fluorescence peak. This indicates that quiescent cells show low chromatin condensation and that chromatin condensation is different between growing and confluent conditions. However, this is inconsistent with findings from peripheral blood lymphocytes, which are regarded as having highly condensed chromatin. It is likely that the quiescent condition of cultured cells in this experiment may be different from that in peripheral blood lymphocytes. Although further investigation is necessary to answer this question, it is true that we were able to identify at least six different cell populations in the cell cycle.

In conclusion, LSC analysis allows us to identify at least six populations of the cell cycle; namely mitotic, post-mitotic, quiescent, G_1 , S and G_2 cells for PI stained samples without a combination of cell cycle specific markers (Figure 2). The fact that visual information

coupled with the position of the cell cycle is always provided for the cells of interest by LSC facilitates the monitoring drug effects on cells.

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