Chromatin-bound PCNA as S-phase marker in mononuclear blood cells of patients with acute lymphoblastic leukaemia or multiple myeloma

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Abstract

Objectives: Proliferating cell nuclear antigen (PCNA) has often been used as a marker to aid assessment of tumour growth fraction. This paper addresses the question of whether it can be used as an S-phase marker, when the non-chromatin-bound form of the protein is removed by pepsin treatment.

Materials and methods: Cytofluorometric measurements were carried out after immunofluorescence staining of PCNA and counterstaining of DNA. S-phase fraction was determined with the help of windows on PCNA versus DNA scattergrams, or mathematically from DNA histograms.

Results: S-phase fractions obtained using the two methods correlated well, but did not always agree, exact discrepancies depending on the mathematical model used for histogram analysis.

Conclusions: Determination of S-phase fractions with the help of PCNA immunofluorescence staining is possible, and probably more reliable than calculation of S-fractions from DNA histograms. It thus offers an alternative to assays involving BrdU labelling *in vivo*.

Introduction

Proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerase δ , which is involved in DNA replication and repair (1,2). PCNA is co-localized with characteristic patterns of DNA replication sites of early, middle and late

S-phase (3), disassembling from such sites whenever the replication process is finished and targeting newly active sites, which makes it an effective marker of the spatio-temporal dynamics of DNA replication (4). It is a homotrimeric protein with ring structure, which encircles DNA, and seems to function as a sliding platform to which different types of catalytic and regulatory proteins can bind (5,6). The protein is present throughout the cell cycle, but it seems to bind to chromatin only after phosphorylation at Tyr-211, which depends on tyrosine kinase activity of the EGF receptor (7).

Quiescent cells do not express PCNA (8,9). Antibodies against it have therefore been employed in a number of studies to determine growth fraction in tumours (10–12). Early on however, it was noticed that the results did not always agree with those obtained using other proliferation-specific antibodies, such as Ki-67 (13). In such studies, the PCNA-positive fraction was always higher and less variable than the Ki-67-labelled fraction, which seemed to indicate that upon transition into quiescence, cells do not degrade their PCNA very rapidly; therefore, its concentration in the cell does not accurately reflect current growth state (14). Detectability of the antigen, moreover, depended on quite a number of other factors, such as fixation procedure and clone of the antibody used (15,16). Most importantly, when cells were treated with detergents such as Triton X-100, up to 90% of PCNA could be extracted. What remained was the chromatin-bound fraction of the antigen, and thus it has been suggested that under these conditions, PCNA was a marker of S-phase cells rather than proliferating cells in general (17–19).

Working with human tumour cells *in vitro*, we have previously developed an alternative method of using PCNA as an S-phase marker (13). Instead of treating whole cells with detergent to extract non-chromatin-bound fraction of PCNA, we isolated cell nuclei with the help of pepsin treatment, digesting cytoplasm and allowing free

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PCNA to be washed away. Our scattergrams at the time showed excellent separation of cells in S-phase from those in G₁- or G₂-phases, similar to results usually obtained using the BrdU labelling technique (20). In fact, percentage of PCNA-positive cells at different cell culture ages agreed very closely with the BrdU labelling index (14). Now, we have tested this method on mononuclear blood cells of patients with acute lymphoblastic leukaemia (ALL) or multiple myeloma (MM), which cannot – at least not routinely – be labelled with BrdU *in vivo*. The ‘classical’ method for determining S-phase fraction in such cases is mathematical analysis of DNA histograms, and this also was employed in this study, in order to compare results of the two methods and assess their relative merits.

There are numbers of earlier studies in which S-phase fraction has been determined from DNA histograms and results have been correlated with clinical data. For instance, low S-phase fraction has been found to be related to significantly better prognosis in ALL (21), chronic lymphocytic leukaemia (22), acute myeloid leukaemia (23) and chronic myeloid leukaemia (24,25) as well as in MM (26,27). The objective of the study described here was to evaluate two-parameter flow cytometry with the help of PCNA immunostaining as a possible alternative.

Materials and methods

Patients

All patients with ALL included in this study were diagnosed at the Department of Paediatric Oncology of the Paediatric Centre, University Hospital Essen. Peripheral blood samples were obtained from 20 patients, and in addition, bone marrow samples from 10 of these patients. Patient age ranged from 0.1 to 16.5 years (median 5 years). As there remain some questions concerning paediatric and adult forms of ALL representing the same disease process, the limited age range of this group should be emphasized.

All patients with MM included here, presented themselves at the Department of Orthopaedics of the Kasturba Medical College, Manipal. Samples of sternal bone marrow were obtained from 20 patients. Patient age ranged from 30 to 62.5 years (median 52 years).

All samples were obtained before the start of treatment, after obtaining informed consent from the patient and/or a responsible relative. Diagnosis was confirmed cytologically and immunologically. Overall, patients in this study are representative of the larger groups to which they belong [ALL in Essen (28), MM in Manipal (29)].

Immunofluorescence staining

Mononuclear cells were obtained by density gradient centrifugation (Lymphodex, density 1.077–1.080 g/ml) of heparinized peripheral blood or bone marrow samples. Cells were washed twice in Tris buffer and were fixed in 96% ethanol.

For two-parameter flow cytometry of PCNA and DNA, the cell suspension was centrifuged, nuclei were isolated in pepsin solution (0.5% in 0.55N HCl, pH 1.8, 10 min, 37 °C), washed twice with PBS–Tween (0.05%) and incubated with anti-human PCNA (PC10, Dako, Glostrup, Denmark; 1:10 in PBS–Tween, 30 min, 4 °C in the dark). After washing twice in PBS–Tween (0.05%)–BSA (1%), they were incubated with FITC-conjugated goat anti-mouse IgG (DuPont, Wilmington, Delaware, USA; 1:100 in PBS–Tween–BSA, 30 min, 4 °C in the dark). Finally, nuclei were washed again in PBS–Tween, resuspended in PBS and stained with propidium iodide (2.5×10^{-5} M in 0.1 M Tris, 0.1 M NaCl, pH 7.5). Cytofluorometric measurements were carried out using a FAC-Scan instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Green (515–545 nm) and red (>650 nm) fluorescence after blue (488 nm) excitation was determined from 5000 to 10 000 cells. Cell cycle fractions were quantified with the help of windows on the scattergrams. Placing of cut-off lines between PCNA-positive and -negative cells was checked with the help of an appropriate irrelevant antibody or using only secondary antibody (both these controls leading to the same results).

DNA histogram analysis

For one-parameter DNA histogram analysis, frequency distribution of red fluorescence values was analysed with the so-called RFIT (rectangle fit) algorithm of CellFit software (Becton Dickinson). In this program, the S-phase fraction is calculated from a rectangle extending between the G₁- and G₂M-peaks at the height of average mid-S-phase. An alternative would have been the SOBR (sum of broadened rectangles) algorithm, which includes background correction, where an exponential function is fitted to portions of the histogram below G₁ and above G₂M, and subtracted from all channels. The program then assigns Gaussian distributions to G₁- and G₂M-peaks and three (or more) broadened rectangles to the S-phase region. The sum of these functions is fitted to the actual histogram by a least squares procedure. We would have preferred to use this algorithm, because we consider it more reliable and have used it in an earlier study (30), but unfortunately and for unknown reasons it did not work with about a third of our histograms. For the remaining two-thirds, however, results were very closely

correlated with those obtained with RFIT [assuming linear relationship between the two, we applied the Pearson method and obtained SOBR S-phase (%) = $2.3 + 0.94$ RFIT S-phase (%), $r^2 = 0.883$, $n = 32$].

Statistical analysis

Correlations between PCNA-positive fractions and S-phase fractions from DNA histograms were assessed in parallel with both Pearson and Spearman tests. Calculation of Pearson's product-moment correlation coefficient presumes a linear relationship between the variables, whereas Spearman's rank correlation coefficient is a non-parametric alternative. Both tests were performed on three subsets of patients (ALL – peripheral blood, ALL – bone marrow, MM – bone marrow) as well as with the combined set of all patients. Calculations were carried out with the help of spreadsheets offered by the 'Handbook of Biological Statistics' which are available online (Pearson: <http://udel.edu/~mcdonald/statregression.html>; Spearman: <http://udel.edu/~mcdonald/statspearman.html>).

Results

Flow cytometer scattergrams of mononucleate blood cells after PCNA immunofluorescence staining and staining of DNA, were similar to those of cultured human tumour cells processed in the same way, of which we have published earlier (14). Examples are shown in Fig. 1. Separation

Figure 1. Representative scattergrams of PCNA-labelled cells from ALL patients (upper panel: aneuploid tumour, lower panel: diploid tumour).

tion between PCNA-positive and -negative cells was fairly unambiguous, even though average FITC fluorescence of the former was just 2.3 ± 0.4 (SD) times higher than the latter. With BrdU labelling, much better separation can usually be achieved (14,20). Comparison of results obtained by three different observers showed, however, that individual differences in setting of windows on scattergrams had only marginal influence on PCNA-positive fraction calculated (data not shown in this study).

For ALL, S-phase fraction from DNA histograms was between 0.0% and 11.9% (median 3.1%) and PCNA-positive fraction between 2.1% and 18.9% (median 6.6%). Both S-phase fraction from DNA histograms and PCNA-positive fraction were on average twice as high in bone marrow as in peripheral blood (median of S-phase fraction 4.5 versus 2.6, median of PCNA positive fraction 10.4 versus 5.3 respectively). For MM, S-phase fraction from DNA histograms was between 0.6% and 46.0% (median 14.0%) and PCNA-positive fraction between 2.5% and 26.7% (median 11.6%).

The impression created by these figures, namely that in ALL, histogram analysis lead to lower values than by evaluation of PCNA immunostaining, whereas the opposite was the case in MM, is somewhat misleading. There is a tendency for PCNA-positive fractions to be higher than S-phase fractions from DNA histograms in cases of low cell proliferation, but lower in cases of high cell proliferation.

This is seen when the two parameters are plotted against each other (Fig. 2). PCNA-positive fractions are closely correlated to S-phase fractions from DNA histograms, but the linear regression line for all data combined has intercept of 5.9 and slope of considerably less than unity, 0.4. Similar results were obtained for all three subsets of patients (ALL – peripheral blood, ALL – bone marrow, MM – bone marrow; see Table 1). Both Pearson

Figure 2. Correlation between PCNA-positive fractions and S-phase fractions from DNA histograms for the three subsets of patients (ALL – peripheral blood, ALL – bone marrow, MM – bone marrow).

Table 1. Results of Pearson's linear regression and Spearman's rank correlation for different subsets of patients and the combined set

	Pearson's linear regression				Spearman's rank correlation	
	Intercept	Slope	R ²	P-value	ρ	P-value
ALL – peripheral blood	4.95	0.525	0.183	0.060	0.324	0.163
ALL – bone marrow	4.90	0.740	0.704	0.002	0.657	0.039
MM – bone marrow	6.73	0.362	0.593	<0.001	0.832	<0.001
Combined data	5.95	0.401	0.604	<0.001	0.686	<0.001

and Spearman tests showed statistical significance in the combined data set as well as in ALL and MM bone marrow subsets, but not in the ALL – peripheral blood subset. This may be because in the latter, 80% of all S-phase fractions from DNA histograms were below 5%, where RFIT analysis becomes unreliable due to influence of background events.

Discrepancies between two- and one-parameter approaches are most probably a result of peculiarities of DNA histogram analysis. As indicated in the Materials and methods section, certain choices have to be made concerning the mathematical model used for approximation of S-phase distribution, which overlaps with G₁- and G₂M-peaks. In an earlier study in which we compared results of CellFit analysis (in this case: S-phase approximated by a sum of several broadened rectangles) with those obtained from the same histograms with a different algorithm (correction for background events, S-phase approximated by a single broadened trapezium), there was good correlation between the two, but the latter method yielded 75% smaller values on average. That result was a finding in colorectal carcinomas, where S-phase fractions are typically much larger than in leukaemias, we found the same differences in the subset of ALL samples (peripheral blood and bone marrow combined) from the present study; approximation of S-phase by a single broadened trapezium yielded 67% lower values than the sum of broadened rectangles algorithm.

In conclusion, fraction of cells in S-phase among mononuclear cells from patients with ALL or MM, can be determined flow cytometrically by PCNA immunostaining of cell nuclei after pepsin digestion. Results obtained correlate well with those from DNA histogram analysis, but not in a 1:1 relationship. Depending on the mathematical model used to calculate S-phase fraction from DNA histograms, lower or higher estimates are obtained. Determination of S-phase fractions with the help of PCNA, therefore, seems to yield more reliable results. It

thus offers an alternative to assays involving BrdU labelling, whose application *in vivo* is viewed only with some reservation.

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