Distinct Dynamics of Mitotic Transition in B-Cell Lymphoma and Reactive B-Cell Lymphoproliferations Determined by H3S10 Phosphohistone Immunolabeling

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Keywords

Cell proliferation · Mitosis · Chromatin · Modification · Lymphoma · Cell cycle

Abstract

Objectives: Clonal selection in the follicular germinal centers in lymphatic tissues is accompanied by an intense proliferation of polyclonal B cells in a precisely regulated fashion. In contrast, B-cell neoplasias proliferate autonomously due to endogenous stimuli. The cell kinetic activity is obvious at many levels including progressive chromatin modification and elevated mitotic rates. We asked if there are differences in the kinetics of histone H3S10 phosphorylation required for mitotic entry between highly proliferating B cells of reactive germinal centers and in B-cell lymphomas with different proliferative capacity. **Material and Methods:** Phospho-H3 histone (pH3S10)-specific immunohistochemistry was applied to cultivated cell, reactive and selected indolent and aggressive lymphoma samples (diffuse large B-cell lymphoma, Burkitt lymphoma, lymphoblastic lymphoma, follicular lymphoma and small lymphocytic lymphoma). Microscopic quantification of the “dot-type” (representing late G₂ phase) and “mitotic” immunolabeling patterns per field of view was performed and compared with classical cell proliferation

markers. **Results:** In addition to the dense homogeneous chromatin labeling highlighting mitotic figures, we stated a selective dot-type nuclear labeling representing ongoing chromatin condensation in premitotic G₂ phase cells. While cell proliferation and mitotic counts correlated in general with histology, statistical analysis indicated an accumulation of G₂ phase pH3S10 pattern in the reactive germinal centers in contrast to lymphomas. The dot-type G₂ staining pattern was surprisingly overrepresented ($1,321.7 \pm 356.5/10$ HPF) in the reactive germinal centers compared to aggressive lymphomas (101.3 ± 33.1) ($p < 0.005$). The relative G₂/M value was significantly higher (4.6 ± 0.6) in reactive germinal center B cells than in any lymphoma entity evaluated (0.7 ± 0.2 in Burkitt lymphoma, 0.9 ± 0.4 in grade 3b follicular lymphoma, 1.3 ± 1.1 in diffuse large B-cell lymphoma, 1.5 ± 0.6 in lymphoblastic lymphoma, and 0.9 ± 0.2 in small lymphocytic lymphoma). **Conclusions:** pH3S10 immunohistochemistry enabled the presentation of significant differences in the cell cycle kinetics between reactive and neoplastic B-cell lymphoproliferations. Accumulation of G₂ phase B cells in reactive folliculi directs to physiological G₂/M checkpoint blockade. In contrast, accelerated G₂/M transition in lymphomas is potentially associated with impaired genomic repair and cell death mechanisms.

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Introduction

The activation of mature B lymphocytes following antigen experience proceeds to a massive induction of cell proliferation and to the formation of the follicular germinal centers in secondary lymphatic organs [1]. This physiological expansion of the B lineage is strictly regulated under the control of antigen presentation and follicular T helper cell interactions [2]. In contrast, cell proliferation in transformed neoplastic B cells (lymphomas) is induced by acquired genetic/epigenetic stimuli, which result in continuous intracellular growth signaling [3, 4]. Some of the gene mutations or translocations occurring in lymphoma are directly associated with intense cell growth (e.g. *c-myc*) while others do not directly influence cell proliferation (e.g. *bcl-2* in follicular lymphoma).

Progression through the cell cycle and the cell division process is precisely associated with changes of the chromatin structure. The condensation and decondensation grade of chromatin is principally determined by dynamic epigenetic marks, such as the reversible phosphorylation, acetylation, and methylation of histone proteins [5, 6].

Histone H3 is a major core histone protein playing a crucial role in chromatin arrangement and chromosome formation [7–9]. Phosphorylation at serine 10 of H3 by the kinase Aurora B results in immediate condensation of the chromatin in the late G₂ phase of the cell cycle [10]. By the end of the mitotic prophase the H3 phosphorylation must be completed in order to enable the segregation of chromosomes before entering metaphase [11]. Phospho-H3 was reported to be representative for mitotic activity and, thus, an excellent marker to highlight dividing cells [12]. Mitotic counts determined following phospho-H3 serine 10 (S10; pH3S10) immunohistochemistry (IHC) has been experimentally and clinically applied to measure cell proliferation in different types of cancer [13–19]. In a recent paper we could establish an excellent relation between H3S10-based mitotic counts and histological grade in follicular lymphoma [20].

The dynamic increase and cell cycle dependence of pH3S10 labeling was elegantly demonstrated in cell culture experiments [21]. Premitotic cells characteristically show isolated pH3S10-positive intranuclear dots, the amount of which varies from single to multiple minute foci transforming to larger confluent spots packed in single cell nuclei [22, 23]. In our earlier studies using pH3S10 IHC the dot-type pH3S10 labeling could be clearly identified and differentiated from mitotic figures in histological conditions. In our hypothesis this IHC staining pattern represents late G₂ phase figures with ongoing H3 phos-

phorylation terminating in complete chromosome condensation, followed by the disruption of the nuclear envelope during the transition to mitosis. Thus, based on the characteristic appearance of the G₂-specific “dot-type” pattern, premitotic cells can be identified and counted in histological/cytological preparations allowing a deeper insight into the process occurring prior to cell division in different pathological conditions.

In the present study we evaluated the occurrence of the obviously different IHC staining patterns of pH3S10 in different B-cell lymphoproliferations. Interestingly, we found significant differences in cultivated cells, as well as in reactive and neoplastic proliferations regarding the frequency of both premitotic/G₂ and M phase type H3 immunolabeling.

Materials and Methods

Cell Culture Conditions (Raji, K562)

The Raji lymphoma cell line and K562 myeloid leukemia cell line were cultivated according to a general standard protocol using RPMI-1640 supplemented with 10% fetal calf serum at 37°C in a CO₂ incubator. Cells were grown for 24 h after splitting to reach logarithmic growth phase before preparation for flow cytometric cell cycle analysis.

Cell Cycle Determination by Flow Cytometry

Cells were washed twice with HEPES buffer, then incubated, at a concentration of 10⁶ cells/mL for 30 min with 5 µg/mL Hoechst 33342 (Sigma-Aldrich, Darmstadt, Germany) at 37°C. Cells were then kept at 4°C and sorted using a FACSaria III (Becton-Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Hoechst 33342 was excited with a 375-nm laser, and Hoechst Blue fluorescence was detected through a 450/20-nm bandpass filter. G₁, S, and G₂/M phases were gated for sorting. Samples of sorted cells were run through the system to validate purity, which was above 99.9%. Sorted cells were collected at a minimum cell number of 10⁴, and cytopsins were fixed for immunocytochemistry in 1% formaldehyde/PBS after drying at room temperature. Experiments were performed in triplicate.

Selection of Reactive Hyperplasia and Lymphoma Cases

Altogether 18 lymph node samples with signs of unspecific follicular hyperplasia and 89 neoplastic B-lymphoid disorders were selected for detailed analysis. All lymphoma samples were diagnosed according to the recent WHO lymphoma classification [24]. The following B-cell lymphomas were analyzed: 24 follicular lymphomas, 10 small lymphocytic lymphomas, 44 diffuse large B-cell lymphomas (DLBCL), 6 Burkitt lymphomas, 5 lymphoblastic lymphomas. Follicular lymphomas were categorized as grade 1–3 by counting intrafollicular centroblasts as described [20]. Germinal center and postgerminal center subtypes of DLBCL were determined following CD10, *bcl-6*, and MUM-1 immunostainings using the algorithm proposed by Hans et al. [25].

Immunohistochemistry (Phospho-H3-Specific Antibody)

For IHC, slides were subjected to Dako EnVisionFLEX Peroxidase-Blocking Reagent (DM821, Dako, Glostrup, Denmark) following deparaffination and rehydration. Antigen retrieval was performed in an epitope retrieval solution (Leica RE-7113) at pH6 using a pressure cooker. This step was followed by washing with distilled water and EnVision Flex Wash Buffer (Tris-buffered saline solution containing Tween 20, pH 7.6 $\pm$ 0.1). Samples were incubated with anti-histone H3 (phospho-S10) (ab25873, Abcam) primary polyclonal antibody at a dilution of 1:4,000 or with anti-Ki-67 protein antibody (clone Mib-1, Dako, Glostrup, Denmark) at a dilution of 1:100 for 1 h at 37°C in a humid chamber. After washing antibody binding was visualized by the Dako EnVision FLEX/HRP (DM822) and FLEX DAB3 Chromogen detection system (DM827, Dako). Slides were covered following counterstaining with Gill hematoxylin solution, washing in tap water and dehydration in ascending ethanol series. The intensity and distribution of pH3S10 expression were assessed by light microscopy (DM2500 microscope, Leica, Wetzlar, Germany) equipped with a DFC 420 digital camera and the Leica Application Suite V3 software.

Cell culture-derived cytospin preparations were fixed in 1% paraformaldehyde/PBS for 10 min followed by permeabilization in ice-cold acetone for 10 min after washing in distilled water. Immunocytochemistry to demonstrate pH3S10 was identical with the process described above.

Data Acquisition and Analysis

Phospho-H3 and Mib-1-related immunopositivity was determined in representative parts of each section. In cases with follicular hyperplasia individual germinal centers were selected and evaluated at $\times 400$ magnification in the microscope. Immunopositive events representing premitotic (dot-type) and mitotic pH3S10 staining pattern were counted for each field of view (Leica N Plan 40/0.65) for all changes; altogether at least 10 high power fields (HPF) were evaluated as described [20]. Premitotic G₂ cells were defined as cells with nuclei presenting at least 1 sharply occurring immunopositive dot. Nuclei packed with signals were considered as premitotic if pH3S10 spots were still distinguishable and the nuclear membrane appeared to be intact. Cells with dense pH3S10 staining and condensed chromatin material (pro-/metaphase) or cells in division (ana-/telophase) were considered as mitotic. The relative (mean) areal frequency of immunopositivity (number of positive events/number of fields counted) was calculated.

The premitotic/mitotic fraction was calculated to present the relative mass of premitotic G₂ cells compared to the true cell division capacity determined by the mitotic rate.

Statistical analyses were performed using the GraphPad Prism 6.04 Trial software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Cell Cycle Analysis and Identification of G₂ and M Phase-Related pH3S10 Labeling Pattern

Immunocytochemistry with the anti-phospho-H3 antibody in the cell lines Raji and K562 both represented a labeling spectrum from the single intranuclear dot in the

Table 1. Statistical distribution of different pH3S10 immunolabeling patterns in cultivated Raji and K562 cells

	IHC pattern, %		
	negative	dot-type	mitotic
<i>Raji</i>			
Total	87.5	4.3	8.2
G ₀ /G ₁	96.4	2.7	0.9
S	92.6	3.9	3.4
G ₂ /M	75.6	5.6	18.8
<i>K562</i>			
Total	82.8	9.7	7.5
G ₀ /G ₁	97.4	2.0	0.6
S	92.1	5.2	2.7
G ₂ /M	67.4	20.9	11.7

Logarithmically growing cell cultures and sorted cell cycle fractions following Hoechst 33342-based DNA flow cytometry were evaluated in the microscope. The percentage of the pH3S10 pattern was determined in digital images ($\times 40$ magnification) following immunocytochemistry. The sum of 3 parallel experiments is presented.

interphase cell nuclei to the fully labeled packed mitotic figures (Fig. 1). The majority of the cells remained completely negative in the total unsorted populations grown in logarithmic phase (87.5 $\pm$ 3.6 and 82.8 $\pm$ 5.4 in Raji and K562 cells, respectively), while cells with variable amounts of pH3-related dots were easy to recognize (Table 1).

H33342-based DNA histograms obtained by flow cytometry enabled to identify and to set a gate on the different phases of the cell cycle in cultivated Raji and K562 cell lines. Three different fractions could be obtained by sorting representing cell populations enriched in G₀/G₁, S, and G₂/M phases. Immunolabeling presented a differential positivity for both of the dot-type and the mitotic pH3S10 pattern in the different cell fractions (Table 1). While the G₂/M fraction clearly presented the highest rates for pH3S10 positivity referring to the mitotic pattern (18.8 $\pm$ 6.6 and 11.7 $\pm$ 5.1 for Raji and K562, respectively), an accumulation of dot-type (premitotic) labeling was also observed. Interestingly, the dot-type labeling was the dominant pattern in the G₂/M fraction in K562 cultures (20.9 $\pm$ 8.7), which reached much higher values than in Raji cells (5.6 $\pm$ 1.4). The relative difference was also addressed by the calculation of the premitotic/mitotic ratio using the 2 labeling patterns. This presented a major difference between the evaluated cell types in the same sense (1.78 vs. 0.29, relative values) (Table 1).

Fig. 1. Gradual increase in pH3S10-related labeling in K562 cells in line with cell cycle progression. **a** Dominant negative fraction typical for the G_0/G_1 and the S phase lacking immunolabeling. **b–g** G_2 phase fraction presenting with characteristic dot-type nuclear immunopositivity. **h–j** Cells immediately before mitotic entry show diffuse chromatin labeling, while the nuclear membrane

is still intact. **k–n** M phase (prophase and metaphase) cells featured by condensed robust chromatin mass intensely labeled by H3S10. Immunocytochemistry was performed by the use of the anti-pH3 (S10) antibody on cytopsin preparations. Hematoxylin counterstaining. Magnification $\times 40$.

pH3S10 Immunostaining in Reactive and Neoplastic B-Cell Lymphoproliferations

pH3S10 IHC was performed to compare cell kinetics and mitotic transition in reactive and neoplastic lymphoproliferations in archived lymph node tissue samples. The use of the histone H3 phosphorylation-specific antibody clearly enabled the differentiation of the highly intense, solid labeling of the mitotic figures from the isolated dot-type reaction of the premitotic/ G_2 phase cells featured with intact nuclear membrane (Fig. 2). Cell proliferation activity expressed by the Ki-67 index, the mitotic index and the mitotic count determined by pH3 IHC were highly different and characteristic in reactive and neoplastic B-cell proliferations (Table 2). As expected, the highest cell proliferation and mitotic rates were detected in Burkitt's lymphoma, followed by reactive germinal centers and the other forms of aggressive lymphoma (DLBCL and lymphoblastic lymphoma). Small lympho-

cytic lymphomas representing indolent lymphomas with most benign behavior appeared with generally low proliferation/mitotic values. Interestingly, follicular lymphoma parameters showed a gradual increase with the histological grade. Importantly, the number of mitotic figures selectively demonstrated by pH3S10 IHC proved to be 1.5–4.3 times higher than the conventional mitotic index ($p < 0.005$ in all sample groups) due to the specific display independent of the variations in the morphological appearance. The distribution of the premitotic and mitotic pH3S10 labeling and its relation to the conventional cell proliferation values is indicated in Table 2.

In the present evaluation we paid special attention to the cell fractions presenting with a nuclear dot-type pH3S10 labeling, reflecting the ongoing histone H3 phosphorylation within the limited time frame of late G_2 /M phase transition. We observed a surprisingly high fraction of dot-type premitotic pH3S10 positivity in any of

Fig. 2. pH3S10 immunohistochemistry in the reactive germinal center (a) and in diffuse large B-cell lymphoma (DLBCL) (b). High-frequency dot-type premitotic staining pattern and mitotic figures (dark dot in b) were observed in reactive centroblasts. Similar mitotic frequency but almost complete lack of dot-type premitotic labeling pattern typical of DLBCL indicates the differences in the mitotic transition kinetics. Magnification $\times 40$.

the nonneoplastic germinal centers counted (mean $1,321.7 \pm 356.5/10$ HPF). In contrast, this pattern was strongly suppressed, frequently nearly missing in all neoplastic lymphoproliferations, independent of the mitotic counts (mean $140.0 \pm 188.7/10$ HPF) ($p < 0.001$). Accordingly, the relative proportion of the dot-type labeling pattern compared to the mitotic pattern (premitotic/mitotic ratio) dramatically decreased in lymphoma (mean 4.6 ± 0.6 vs. 1.2 ± 0.6) ($p < 0.005$), irrespective from the lymphoma type, cell of origin or dignity. The rate of premitotic/ G_2 pattern in lymphomas was basically dependent

on the cell proliferation activity measured by the mitotic index or the Ki-67 index (Table 2), but the correlation was generally weak (CI = 0.55). Interestingly, the lowest premitotic/mitotic rate (0.7 ± 0.2) was measured in Burkitt lymphoma, a neoplasia featured with the highest proliferative potential (mean Ki-67 index 94.3 and mitotic index 236.8/10 HPF). Increase in the grade of follicular lymphoma resulted in rising mitotic activity and premitotic rates but the premitotic/mitotic ratio remained suppressed with a range (between 0.9 ± 0.4 and 2.3 ± 1.4) far below the values calculated for reactive lymphadenopathies ($p < 0.005$).

Discussion

Phospho-histone H3 IHC was reported as an accurate tool to determine mitotic activity in histological conditions [12, 26]. Phosphorylation dependent conformational change at the N-terminal serine 10 of histone H3 enabled the development of specific antibodies with high affinity to the modified pH3S10 nucleosome protein [27, 28]. The intense staining was reported to be beneficial to demonstrate and count mitotic figures in histological sections in earlier studies. The specific presentation enabled the precise grading in different cancer types, such as breast carcinoma, astrocytoma, meningioma [19, 29–31] and follicular lymphoma by our group [20]. Beside intense labeling of the condensed chromatin during mitosis, premitotic cells can be frequently identified in histological sections with increasing amounts of intranuclear pH3S10 labeling. However, premitotic cells representing the phase of progressive chromatin condensation just prior to mitotic entry have not been considered in histological studies so far. In the present approach we analyzed the full morphological spectrum of histone H3 phosphorylation beginning in the G_2 phase with the earliest signs of chromatin modification occurring as single IHC-positive dots. Simple cell cycle measurements using cultivated K-562 (myeloid leukemia) and Raji (Burkitt lymphoma) cells clearly showed that the dot-type pattern of the pH3S10 labeling is enriched during progression through the late G_2 phase, although the proportion and dynamics can be highly variable according to the biological setup of the cell. Together with earlier results [22, 32, 33], this observation strongly supports that cells with “dot-type” pH3S10 morphology can be considered as mitotic precursors approaching the G_2/M phase transition. The increase in the number of intranuclear dots and later the occurrence of large immunopositive clumps indicates the

Table 2. Cell proliferation (Ki-67%), mitotic index (MI), and pH3S10 IHC staining characteristics in reactive and neoplastic B-cell lymphoproliferations

	<i>n</i>	Ki-67-index, %	MI/10 HPF	pH3S10 MI/10 HPF	pH3S10 premitotic	pH3S10 G ₂ /M
Reactive GC	18	77.8±5.3	134.1±25.0	286.4±89.9	1,321.7±356.5	4.6±0.6
DLBCL						
Total	44	63.6±16.1	50.0±27.8	108.8±68.9	140.4±88.7	1.3±1.1
Non-GC	31	63.8±14.8	51.3±22.1	114.9±66.5	156.2±79.1	1.4±1.0
GC	13	67.2±15.3	55.8±23.7	119.5±65.7	115.8±57.1	1.0±1.0
Burkitt lymphoma	6	94.3±2.4	236.8±67.2	306.0±84.3	213.4±39.4	0.7±0.2
ALL/LBL	5	56.6±10.3	143.3±27.5	279.6±42.4	405.3±178.2	1.5±0.6
Follicular lymphoma						
Grade 1	5	4.3±1.1	3.6±1.8	15.6±8.6	23.6±8.3	1.5±0.5
Grade 2	5	15.8±6.9	17.6±9.4	35.2±17.2	82.0±50.4	2.3±1.4
Grade 3a	6	23.8±18.1	56.0±32.6	84.8±30.1	74.8±47.4	0.9±0.6
Grade 3b	8	37.0±10.4	93.2±32.6	154.4±73.6	137.6±68.2	0.9±0.4
SLL	10	4.6±2.5	15.9±9.8	34.7±13.5	31.7±13.9	0.9±0.2

The premitotic/mitotic ratio determined by the differential pH3S10 staining pattern proved to be significantly higher in reactive germinal centers (GC) suggesting a G₂ blockade prior to entering the mitotic process. HPF, high-power field; ALL, acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; SLL, small lymphocytic lymphoma.

ongoing reorganization of the chromatin and the formation of intact chromosomes which is followed by the dissolution of the nuclear membrane. Earlier data suggest that histone H3 phosphorylation begins in the centromeric heterochromatin of individual chromosomes [22] which explains the gradual occurrence of multiple pH3-positive intranuclear dots in cell preparations and histological sections.

Reorganization of chromatin seems to be highly regulated and includes a series of indispensable steps. However, little is known about the temporal kinetics of the cyclic processes and their relation to parallel biological mechanisms. The length of the G₂ phase and the kinetics of chromatin reorganization highlighted by pH3S10 are obviously influenced by variables, such as the type and actual functionality of the cell. The careful analysis of premitotic pH3S10 labeling by IHC uncovered a significant difference in the S10 phosphorylation kinetics between reactive and neoplastic B-lymphoid cells (4.6 ± 0.6 vs. 1.2 ± 0.6). This feature was directly evident within histological slides where partial lymphomatous infiltration was observed in the presence of follicular hyperplasia of the residual lymph node parenchyma. Based on our observation it is of particular interest that antigen-driven immunoactivation of B-cell proliferation, as present in the normal germinal center reaction, is associated with an unexpectedly high proportion of G₂ phase cells. The biological function of this relative enrichment calls for explanation.

The massive B-cell growth due to antigen stimulus in the germinal center is physiologically followed by a simultaneous clonal selection process [1]. We speculate that large masses of activated, low-affinity immunoglobulin rearranged B-cell clones are susceptible to exit the cell cycle and undergo apoptosis following a period of passive queuing in the G₂ phase. In contrast to the physiological process, the proportion of the G₂ cells with a premitotic pH3S10 staining pattern proved to be significantly reduced in all forms of lymphomas studied, and this was irrespective of the growth rate (G₂/M between 0.7 ± 0.2 and 2.3 ± 1.4). This suggests an enhanced G₂ phase transition due to the abrupt chromatin phosphorylation in neoplastic B cells induced in an antigen-independent fashion. The acceleration of mitotic transition seems to be complex and can be affected by highly diverse pathways including p53 deficiency, unscheduled G₂/M checkpoint regulator or mitotic kinase activity [10, 34, 35]. Defect TP53 is frequently contributing to the dysregulation of G₂/M checkpoint function reported in aggressive lymphomas [36]. Histone H3 phosphorylation timing on the other hand is dependent on Aurora kinase expression that is overrepresented in a significant portion of DLBCL, according to our earlier observations [37]. Moreover, phosphorylation efficacy is determined on protein phosphatase 1 activity, a major antagonist of Aurora B kinase, which may both interfere with histone H3 modification or induce early termination of the cell division process

[38, 39]. Altogether, simultaneous synergic and antagonistic effects are responsible for the actual timing and quantitative distribution of intranuclear pH3 levels in a given cancer cell type. An interesting point for future studies would be to establish the link between G₂/M transition dynamics and TP53, Aurora B and protein phosphatase 1 functionality in lymphoid neoplasias.

Although the exact mechanisms and the significance of histone H3 phosphorylation status in lymphatic cells

requires further clarification, our observations indicate significant changes in selected pathological conditions. IHC using phosphorylation-specific antibodies is well applicable to present dynamic chromatin modifications and highlights measurable differences regarding chromatin activity, reorganization and G₂ phase progression in proliferating cells.

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