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Research Article

The Rough Estimate of the Nuclear Space Occupied by Nucleolar Bodies in Lymphocytes of Patients Suffering from B Chronic Lymphocytic Leukemia and Blood Donors

Smetana K[1*], Mikulenková D[1], Otevřelová P[1], Karban J[2] and Trněný M[2]

[1]*Institute of Hematology and Blood Transfusion, Prague, Czech Republic*
[2]*Department of Hematology and Oncology, 1st Medical Faculty, Charles University, Prague, Czech Republic*

ARTICLE INFO

Article history:
Received: 22 June, 2020
Accepted: 7 July, 2020
Published: 24 July, 2020

Keywords:
Nuclear space occupied by nucleolar bodies
lymphocytes
differentiation
maturation

ABSTRACT

It is generally known that the nucleus : cytoplasmic ratio is a very useful marker for the evaluation of the cell activity and identification. In contrary, the nucleolus : nucleus ratio was less studied. The present study was undertaken to provide more information on that ratio during the differentiation and maturation of human lymphocytes. The ratio of nucleolar bodies to the nuclear body indicated that the size of the nuclear space occupied by nucleolar bodies in B chronic lymphocytic leukemia (CLL) during the cell differentiation and maturation (terminal differentiation) decreased in both untreated and treated patients with the anti-leukemic therapy. However, the nuclear space occupied by nucleolar bodies was apparently characteristic for each differentiation and maturation step. A similar trend was apparent in non-leukemic T lymphocytes of blood donors using *in vitro* de-differentiated lymphocytes as progenitors. During the cell differentiation and maturation, the size reduction of nucleolar bodies of both patients suffering from CLL and blood donors was apparently larger than that of the nucleus. As it was expected, the decreased size of nucleolar bodies was accompanied by the decreasing nucleolar transcription activity expressed by the reduced number of fibrillar centers.

Introduction

It is generally known that the nucleus : cytoplasmic ratio is a very useful marker for the evaluation of the cell activity and identification [1]. However, the nucleolus : nucleus ratio was less studied. On the other hand, in clinical cytology this ratio appeared to be useful for the detection of malignant cells [2-5]. At this occasion it should be mentioned that the nucleolus : nucleus ratio was usually estimated using the "oculometry" and expressed by diameter, volume or area calculations [6, 7]. Recent studies on cultured leukemic granulocytic progenitors indicated that the diameter ratio of nucleolar bodies to nuclear body reflected a remarkable stability of the nuclear space occupied by nucleolar bodies after the treatment with anti-leukemic drugs [8]. The nuclear space occupied by nucleolar bodies also appeared to be characteristic for each differentiation and maturation stage of the leukemic granulocytic lineage [9].

The present study was undertaken to provide more information on the nuclear space occupied by nucleolar bodies in proliferating cells with the differentiation potential, resting cells with the de-differentiation potential and cells representing terminal stages of the cell lineage development. All these cell developmental stages were present in the B lymphocytic lineage of patients suffering from chronic lymphocytic leukemia. The peripheral blood of these patients contained a satisfactory number of cells in all developmental stages for microscopic measurements. A satisfactory number of mature T lymphocytes for microscopic measurements was also present in the peripheral blood of blood donors. Early developmental de-differentiated stages of the non-leukemic lymphocytic T lineage were easily produced by the stimulation of peripheral blood lymphocytes *in vitro* by phytohaemagglutinin [10,

[*]*Correspondence to: Karel Smetana, Professor, M.D., Ph.D., Dr.Sc., Institute of Hematology and Blood Transfusion, U Nemocnice 1, 128 20, Prague, Czech Republic; E-mail: karel.smetana@uhkt.cz; smetana@uhkt.cz*

http://dx.doi.org/10.31487/j.COR.2020.07.10

11]. The simple cytochemical method for the demonstration of RNA in single cells facilitated the simultaneous identification of lymphocytic developmental stages and nucleolar and nuclear body diameter measurements [12, 13].

Moreover, the number of nucleolar fibrillar centers in single cells indicated the nucleolar (ribosomal) RNA transcription activity in studied cells. At this occasion, it should be mentioned that nucleolar fibrillar centers with adjacent regions appearing as silver stained dots seen by light microscopy represented sites of the ribosomal RNA transcription and processing [14-16]. The results of nucleolar and nuclear body measurements indicated that the approximate nuclear space occupied by nucleolar bodies was characteristic and stable for each developmental stage of lymphocytes in B-CLL patients as well as in T lymphocytes of blood donors.

Material and Methods

Lymphocytes were studied in blood smears of 6 patients suffering from B chronic lymphocytic leukemia (state C according to Binet). 3 patients were left untreated and 3 patients were treated with the current anti-leukemic therapy with fludarabine at the time taking samples for the present study. For comparison, lymphocytes were also studied in the peripheral blood smears of 3 blood donors. Since blood smears of these donors did not contain lymphocytic progenitors or a substantial number of immature lymphocytes for measurements, these cells were replaced by stimulated and de-differentiated T lymphocytes with phytohaemagglutinin *in vitro* [10, 11, 17]. To get such cells, isolated lymphocytes of the peripheral blood of donors were cultured for 48 hours in RPMI 1640 medium with inactivated 10% FBS and phytohaemagglutinin (10µg/mL-Sigma) at 37°C in 5% atmosphere with CO_2 [11]. For the microscopy, cytospins were prepared using the cytocentrifuge shandon cytospin 2 (Shandon Southern Products UK).

Nucleolar bodies (NoBs) were visualized in unfixed blood smears and cytospins by a simple cytochemical method for the detection of RNA with methylene blue at pH 5.1 [12, 13]. This procedure also facilitated to see clearly nuclear outlines because of the heavily stained cytoplasm surrounding the nucleus (Figures 1-3). The nucleolar biosynthetic activity was detected by the silver reaction for the detection of nucleolar fibrillar centers**,** which represented morphological markers of the ribosomal RNA transcription in single cells [13-18].

Micrographs captured with a Camedia digital camera C4040 ZOOM (Olympus, Japan) placed on jenalumar microscope (Zeiss, Germany) were magnified and further processed using quick computer photoprogram (Olympus, Japan). Then the mean diameter based on major and minor axis length measurements was calculated for both NoBs and nuclei in single cells of each stage of the lymphocytic development (Figures 1-3) [19]. NoBs : nucleus diameter ratio (NoBs/Nu DmR) was calculated by dividing mean values of the diameter of NoBs by the mean nuclear diameter per cell. Results multiplied by 100 estimated the approximate size of the nuclear region occupied by NoBs. Volumes of nucleolar bodies and nuclei were calculated using volume calculator (Calculator net, Internet 2018). In one and the same cell, mean volume of NoBs was calculated using the formula for the three-dimensional sphere; The mean nuclear volume was calculated according to the formula for the three-dimensional short cylinder with added virtual height 0.2µm [9]. The resulting calculated NoBs : Nu volume ratios (NoBs/Nu VoR)x100 estimated again the approximate size of nuclear space occupied by NoBs in single cells. The results of all measurements and calculations at the single-cell level such as mean and standard deviation were evaluated using Primer of Biostatistics Program, version 1 developed by S.A. Glantz (McGraw-Hill, Canada, 1968).

Results

As it was expected, the measurement results demonstrated that the size of NoBs and nuclei decreased during the cell differentiation and maturation of the lymphocytic lineage in both untreated and treated patients with the anti-leukemic therapy. However, the size reduction of NoBs was larger than that of nuclei. The size of NoBs in mature lymphocytes with ring shaped nucleoli was about 60-70% and in terminal lymphocytes with micronucleoli about 40-45% of nucleolar bodies in less differentiated and immature lymphocytes, i.e. lymphoblasts and prolymphocytes. In contrary, the size reduction of the nucleus in mature lymphocytes with ring shaped nucleoli and terminal lymphocytes with micronucleoli was smaller such as 77-86% of the nuclear size in lymphoblasts and prolymphocytes.

The biosynthetic activity of NoBs of mature and terminal lymphocytes was naturally smaller than in lymphoblasts and prolymphocytes. Lymphoblasts and prolymphocytes possessed multiple fibrillar centers in comparison with mature or terminal lymphocyte mostly containing only one fibrillar center. At this occasion it should be noted again that nucleolar fibrillar centers with adjacent RNA containing components are sites of the ribosomal RNA transcriptions and processing [15, 16, 18]. The diameter of NoBs or nuclei in mature and terminal lymphocytes of blood donors did not substantially differ from that of leukemia patients despite the different phenotypes of these cells. Similarly, the diameter of NoBs and nuclei in lymphoblasts produced by de-differentiation of mature lymphocytes with ring shaped nucleoli of blood donors did not differ from that in leukemic lymphoblasts or prolymphocytes. The number of fibrillar centers in de-differentiated non-leukemic lymphocytes-lymphoblasts was larger than in mature or terminal lymphocytes. Calculated ratios of NoBs to nuclear diameter or volume were smaller in mature and differentiated lymphocytes than in less differentiated and immature lymphoblasts or prolymphocytes of leukemia patients.

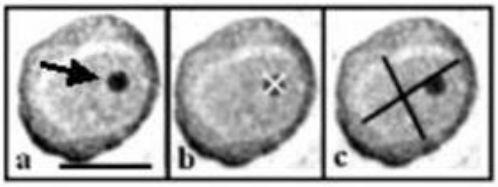

Figure 1: a) Leukemic lymphoblast with one large nucleolus (arrow). **b)** White lines-nucleolar diameter measurement. **c)** Black lines-nuclear diameter measurement. Black bar in the figure a represents 6µm.
NoB: nucleolar body, Nu: nucleus, DmR: diameter ratio.

However, it was characteristic for each of these differentiation or maturation stages. Thus, the estimated size of nuclear space occupied by

NoBs was also characteristic for each differentiation and immature stage. Similarly, calculated NoBs to nuclear diameter or volume ratios were larger in cultured non-leukemic lymphoblasts originating from stimulated peripheral T lymphocytes in comparison with mature and terminal lymphocytes in the peripheral blood of blood donors. For quantitative data (Table 1), illustrating images (Figures 1-3).

Table 1: The diameter of nucleolar and nuclear bodies-the calculated diameter and volume ratios of nucleolar bodies to the nucleus in various stages of the lymphocytic lineage*.

Stage	NoBs M (µm)	Nu M (µm)	NoB/Nu Dm (C)	NoB/Nu Vo(C) ●●	NoFCs >1	Pers	Th
Lybl+Proly	2.1±0.7	9.3±0.4	21.5	35.5	+	CLL	0
	2.0±0.6	9.0±0.6	22.2	25.7	+		+
Lybl stim•	2.2±0.3	8.5±0.6	25.8	48.6	+	BDs	0
Lycyt I	1.4±0.1§ (66.6)	7.6±0.5§ (77.3)	18.4	15.5	0	CLL	0
	1.4±0.1§ (70.0)	7.7±0.3§ (85.5)	18.1	12	0		+
	1.3±0.1§ (59.0)	6.9±0.2§ (81.1)	18.8	14.8	0	BDs	0
Lycyt II	0.9±0.1§ (42.8)	8.0 ±1.2§ (86.0)	11.2	7.1	0	CLL	0
	0.9±1.1§ (45.0)	8.1 ±0.8§ (89.0)	11.3	7.4	0		+
	0.9±0.3§ (40.9)	6.6 ±0.1§ (72.6)	13.6	6.6	0	BDs	0

* - Mean and standard deviation of 500 measurements of the small and large nucleolar and nuclear body diameters in single cells of each stage of the lymphocytic lineage and each group of patients. Mean and standard deviation of >200 measurements in single lymphoblasts of BDs stimulated by phytohaemagglutinin.

• - Measurements in cells of the cytospin periphery. ••- Calculated volumes with the added virtual height (see Material and methods) based on separate diameter measurements. § - Significant difference in comparison with Lybl + Proly and Lybl stim using the t-test (2α=0.05). Lybl + Proly: lymphoblasts and prolymphocytes; Lybl stim: lymphoblasts originating from mature containing "active" nucleoli with multiple fibrillar centers; Lycyt I: "mature" lymphocytes with the ring-shaped nucleolus; Lycyt II: "terminal" lymphocytes with micronucleoli; NoB: nucleolar bodies, Nu-nuclei; M (µm): measured values; C: calculated values; Dm: diameters; Vo: volumes; CLL: patients suffering from chronic lymphocytic lineage; BDs: blood donors; FCs: multiple fibrillar centers; Pers: studied parsons; Th: cytostatic therapy; Numbers in parenthesis-percentage of the nucleolar and nuclear body diameters in lymphoblasts + prolymphocytes.

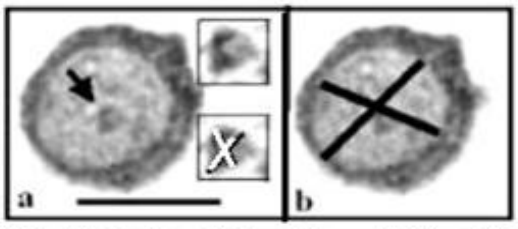

Figure 2: a) Leukemic differentiated and mature lymphocyte with one ring shaped nucleolus (arrow). Differentiated and mature lymphocyte with ring shaped nucleolus (arrow). White lines in the insert-nucleolar diameter measurement. **b)** Black lines-nuclear diameter measurement. Black bar in the figure a represents 5µm.
NoB: nucleolar body, Nu: nucleus, DmR: diameter ratio.

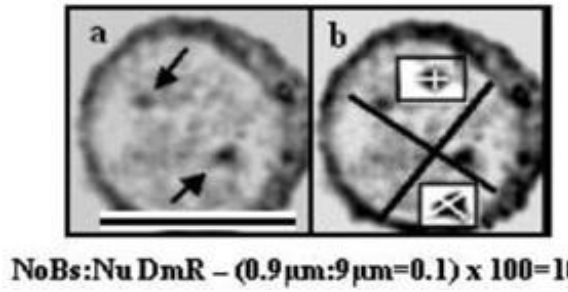

Figure 3: a) Leukemic terminal lymphocyte with two micronucleoli (arrows). **b)** White lines in inserts-nucleolar diameter measurement, black lines-nuclear diameter measurement. Black bar in the Figure a represents 4µm. For other legend (Figure 1).
NoB: nucleolar body, Nu: nucleus, DmR: diameter ratio.

Discussion

The larger reduction of the nuclear space occupied by NoBs during the differentiation and maturation of lymphocytes might accompany the large reduction of the nucleolar biosynthetic activity. Past studies demonstrated that the nucleolar biosynthetic activity during the cell differentiation and maturation decreased earlier than that in extranucleolar nuclear regions [20, 21]. In the present study on leukemic lymphocytes, such decrease of the nucleolar biosynthetic activity during the differentiation and maturation (terminal differentiation) was reflected by the reduced number of nucleolar fibrillar centers and transformation of large nucleoli to ring shaped nucleoli and micronucleoli containing only one fibrillar center [15, 22]. At this occasion it should be mentioned that such differentiation and maturation changes were similar in both untreated and treated patients with the current anti-leukemic therapy at the time of taking samples for the present study. In the present study, large nucleoli in lymphoblasts originating from stimulated non-leukemic lymphocytes of blood donors also possessed multiple nucleolar fibrillar centers. In contrary, mature and terminal lymphocytes of non-leukemic blood donors mostly possessed only one fibrillar center. At this occasion, it should be mentioned again that nucleolar fibrillar centers with surrounding regions are markers of the nucleolar-ribosomal RNA transcription and processing [15, 16, 18].

The above discussed observations based on the estimate of nuclear space containing nucleoli suggested a possibility of the characteristic and stable nucleolus-nucleus morphological and functional equilibrium for each differentiation and maturation step. According to present observations, the nucleolus-nucleus equilibrium for each step of the lymphocytic cell lineage development was similar in leukemic patients

as well as in blood donors. There is also a possibility that such equilibrium just reflects the programmed size of the nucleolar and nuclear bodies in the cell lineage development. From the methodical point of view, it seems to be useful that the calculation of the nucleolus to nucleus ratios might facilitate to estimate the approximate size of the nuclear territory with the NoBs. However, it should be mentioned that the nucleolar or nuclear shape and diameter might be influenced by the specimen preparation for microscopy and measurement technique [23-25]. In addition, the nucleolar and nuclear shape was not always rounded. Such circumstances were partially corrected by the measurement at least two diameters, i.e. long and short axis of each nucleolus and nucleus. For volume calculation, the sphere and short cylinder geometric formula appeared to be most useful.

However, it should be mentioned that the virtual height was added to the short cylinder calculation for studied nuclei measured on smear or cytospin preparations [9]. Since volumetric calculations were also based on diameter measurements, no wonder that the calculated values of NoBs : nucleus volume and diameter ratios exhibited similar trends. The measurements of NoBs were selected instead of whole nucleoli with the perinucleolar chromatin shell. The nucleolar-ribosomal RNA transcription sites were located within NoBs and the increasing width of the perinucleolar chromatin during cell maturation frequently masked small nucleoli in mature or terminal lymphocytes [2, 15, 26, 27].

At the end of the discussion, it should also be mentioned that the presented study would like to indicate that the approximate size estimates of nuclear territory with nucleoli might be a potential further contribution for the evaluation of the cell various states and activities including the differentiation and maturation. In addition, according to the present study, the differentiation and maturation of leukemic B lymphocytes or non-leukemic T lymphocytes follow a remarkable similarity concerning the nucleolar size within the nuclear space and the nucleolar biosynthetic activity. Since similar observations were reported for the leukemic granulocytic lineage it seems to be likely that this phenomenon might be more general for the development of studied cells [9].

Acknowledgement

The study was partially supported by the Research Program of the Institute of Hematology and Blood Transfusion. The authors would like to express their gratitude to physicians and technicians of the Institute of Hematology and 1st Internal Clinic of the General Hospital for the support.

Competing Interests

None.

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