To the Approximate Size of the Nuclear Region Occupied by Nucleolar Bodies During Cell Differentiation and Maturation Using the Human Leukemic Granulocytic Lineage as a Convenient Model

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Summary

The present study was undertaken to estimate the approximate size of nuclear regions occupied by nucleolar bodies during the cell differentiation and maturation. The differentiation and maturation of human leukemic granulocytic cells in patients suffering from the chronic phase of the chronic granulocytic leukemia (CML) represented a convenient model for such study because of the large number of cells for the diameter measurements at the single cell level. Early and advanced differentiation or maturation stages of these cells are well defined and nucleolar bodies and nuclear outlines are easily seen by simple cytochemical methods for the visualization of RNA and silver stained proteins in smear preparations. During the cell differentiation and maturation, the estimated size of the nuclear region occupied by nucleolar bodies decreased in both untreated and treated patients with the anti-leukemic therapy. However, the size reduction of nucleolar bodies in differentiated and mature cells was larger than that of the nucleus. In addition, the results also indicated that the nuclear region occupied by nucleolar bodies was characteristic for each differentiation and maturation stage of the granulocytic cell lineage and was not substantially influenced by the anti-leukemic therapy of CML patients.

Key words

Nuclear region • Nucleolar bodies

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Introduction

It is generally known that the nuclear–cytoplasmic ratio is a very important tool for the cell identification including differentiation or proliferation state. On the other hand, the relationship between the nucleolar and nuclear size was less studied although some previous studies provided useful information on the knowledge of malignant cells already in the last and even in the present century (Foot 1937, McGrew 1965, Busch and Smetana 1970, Tone *et al*. 2016).

The present short study was undertaken to provide information on the nucleolar and nuclear mean diameter to estimate the approximate size of nuclear regions occupied by nucleolar bodies (NoBs) during the cell differentiation and maturation. The simple cytochemical procedures for the visualization of RNA and silver stained proteins facilitated to see clearly the nuclear outline and large as well as very small NoBs in all developmental stages of the granulocytic cell lineage (Smetana *et al*. 1969, 1999, Ochs 1998). Early and late differentiation as well as maturation stages of neutrophils in patients suffering from chronic myelocytic leukemia (CML) represented a convenient model for such study because of the satisfactory number of cells for the nucleolar and nuclear diameter measurements at the single cell level. In addition, there is not a general morphological difference of differentiation and maturation stages between CML and non-leukemic granulocytic lineage (Rundles 1983)

Definitions of cell differentiation and maturation in the earlier and recent hematological literature were not

rigorously respected (Astaldi and Lisiewicz 1971). Therefore, in the present study, the term "differentiation" reflected developing and proliferating stages of the studied cell lineage, i.e. "the mitotic compartment" (Cline 1975). Thus myeloblasts are early differentiation progenitors and myelocytes last differentiation stages of the granulocytic development. The term "maturation" was used for the development of fully differentiated and terminal stages of the granulocytic lineage without the proliferation potential such as metamyelocytes, bands and segmented granulocytes, i.e. for the "maturation compartment" (Cline 1975). Granulocytes with segmented nuclei were selected as terminal. steps of the granulocytic development.

In the present study it was apparent that using the computer image processing and diameter measurements, the size of nuclear regions occupied by nucleolar bodies (NoBs) decreased during the differentiation and maturation. However, the nuclear region occupied by NoBs was constant and characteristic for each developmental stage and was not substantially influenced by the anti-leukemic therapy of CML patients.

Table 1. Mean diameter of nucleolar bodies and nucleus, NoBs: nucleus diameter and volume ratios in differentiating and maturing cells of the granulocytic lineage in CML patients[§]

Stage	NoBs Dm [µm](M)	Nu	NoBs/Nu DmR(C)	NoBs/Nu VoR (C)	Th
Myeloblast	1.7±0.2	13.0.±1.7	14.0	18.5	0
	1.5±0.2	11.0±0.9	13.6	17.6	+
Myelocyte	1.0±0.1*(58.8)	9.6±0.7*(79.3)	10.4	7.2	0
	1.0±0.1*(66.6)	9.1±1.6*(82.7)	10.9	8.2	+
Segment[+•]	0.8±0.1*(47.0)	9.7±1.1*(80.1)	8.2	3.5	0
	0.8±0.1*(53.3)	10.0±1.5*(90.9)	8.0	3.3	+

[§] Mean and standard deviation based on 180 measurements of long and short axis in 50-100 single cells of each patient in the group of 4 untreated and 4 treated patients with the anti-leukemic therapy, * Significantly different from early granulocytic progenitors – myeloblasts using t-test (2α=0.05), • Nucleoli and nuclei stained for better selective visualization with silver reaction, [+] Mean diameter of a single nuclear segment calculated from the diameter of all segments in one cell. Segment – mature granulocyte with segmented nucleus, Myelocyte – last differentiation stage of granulocyte precursors, Myeloblast – early differentiation stage of granulocyte progenitor, R – ratio, Dm – diameter, Vo – volume, (M) – measured values, C – calculated values, Th – therapy with the anti-leukemic drug (imatinib), numbers in brackets – diameter percentage of nucleolar bodies or nuclei in comparison with myeloblasts

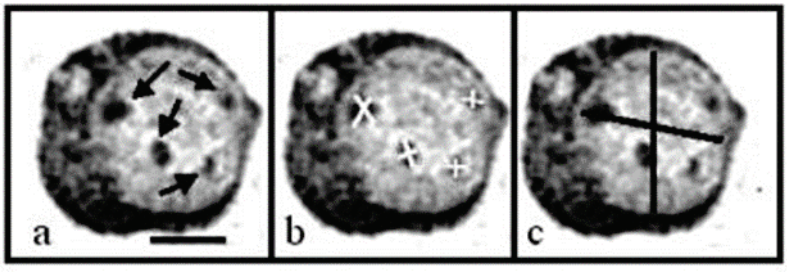

Fig. 1. Granulocytic early progenitor – myeloblast. (**a**) Nucleoli – black arrow. (**b**) White lines indicate the diameter measurement of NoBs. (**c**) Black lines indicate the nuclear diameter measurements. The bold black line in (a) indicates 10 µm. The illustrating calculation of the NoB/Nu DmR is (2µm:14µm=0.14) x 100=14.0

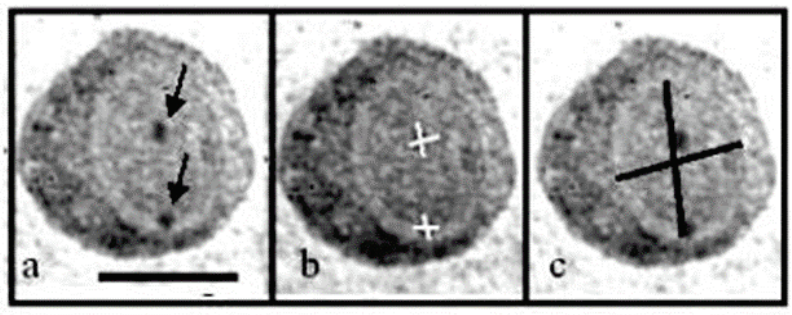

Fig. 2. Differentiated granulocytic precur-sor – myelocyte. (**a**) Small nucleoli (micro-nucleoli) – black arrows. (**b**) The diameter measurement of NoBs – white lines. (**c**) The nuclear diameter measurement – black lines. The bold black line in (a) indicates 8 µm. The illustrating calculation of the NoBs/Nu DmR – (1.1µm:9.2=0.11) x 100=11.0.

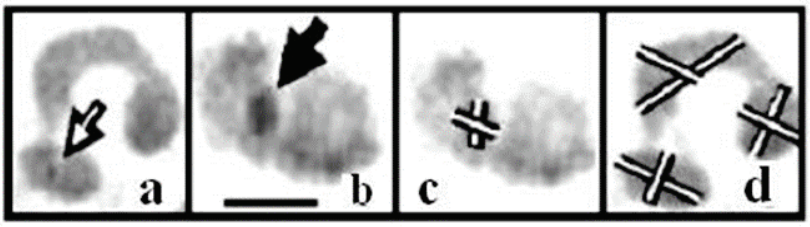

Fig. 3. (**a**) Differentiated and fully mature granulocyte with segmented nucleus and one small NoB (arrow). (**b**) Magnified nuclear segment with the small NoB (arrow). That NoB with lines of the measurement (**c**). (**d**) Nuclear segments with lines of the diameter measurements. The silver reaction facilitates the visualization of nuclear segments and especially NoB. Figs b,c,d were processed and bleached (c,d) using computer program for more distinct visualization of measured lines. The bold black line in (b) indicates 3 μm. The illustrating calculation of the NoBs/Nu DmR – (0.9μm:9.3μm=0.09) x 100=9.0.

Methods

The nucleolar and nuclear diameters were measured in single cells of the granulocytic lineage. These cells were studied in bone marrow smears of 8 patients with the chronic phase of Ph+ chronic myelocytic leukemia (CML). It must be added that studied bone marrow smears of CML patients were originally taken for diagnostic purposes approved by the leading authorities of the Institute.

All studied patients exhibited common characterristics of the chronic phase of CML such as laboratory markers including the cytology, genetics and FACS phenotyping. At the time of taking samples for the present study 4 patients were without the anti-leukemic therapy. 4 patients received the current "specific" anti-leukemic therapy with imatinib mesylate for 3 months before and at the time of taking samples for the present study. In patients treated with this therapy the cytological examination of bone marrow smears indicated a markedly reduced granulocyte to erythroid ratio (see Results).

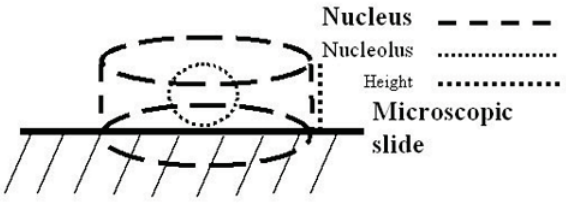

Fig. 4. Scheme of the smeared nucleus and nucleolus on the microscopic slide represented by a sphere (Nucleolus) and a short cylinder (Nucleus) with added virtual height (Height) for volume calculation.

NoBs and nuclear outlines were visualized in unfixed bone marrow smears by a simple but sensitive method for the demonstration of RNA using methylene blue buffered with McIlvain´s buffer to pH 5.3 (Smetana *et al.* 1969, Ochs. 1998). NoBs and nuclear outlines of segmented mature granulocytes were also visualized by the silver reaction for the demonstration of main nucleolar proteins (Ochs 1998, Smetana *et al.* 1999) to facilitate more precise measurement.

Micrographs were captured with a Camedia digital camera C4040 ZOOM (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany). The double adapter on the microscope increased the magnification of captured images transferred to the computer screen. The increased magnification and contrast by image processing (Quick Computer Photoprogram, Olympus, Japan) facilitated easy measurements of the major and minor axis length of both NoBs and nuclei in single cells (Politi *et al.* 2003, Fig. 1-3). NoBs: nucleus diameter ratio (NoBs/Nu DmR) was calculated by dividing mean values of the diameter of NoBs by mean nuclear diameter per cell and multiplied by 100. Then the results estimated the approximate size – percentage – 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 three-dimensional sphere; mean nuclear volume was calculated according to the formula for the three-dimensional short cylinder with added virtual height of 0.1μm (Fig. 4). The resulting calculated NoBs : Nu volume ratios (NoBs/Nu VoR) estimated again the approximate size of nuclear regions 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 Biostatistic Program, version 1 developed by S.A. Glantz (McGraw-Hill, Canada, 1968).

Results

Quantitative data and illustrating figures are in the Table 1 and Figs 1-4.

Myeloid : erythroid ratio.

According to the cytological examination of bone marrow smears the leukocyte to erythroid ratio in

patients untreated with the anti-leukemic therapy was very high and variable, i.e.20.8±11.9 : 1, variation coefficient 57.2 %. In patients treated with the anti-leukemic therapy the leukocyte to erythroid ratio was apparently smaller and less variable, i.e. 4.8±1.8 : 1, variation coefficient 37.5 %. In these patients this ratio was very similar to non-leukemic persons (Rundles 1983).

NoBs : Nu diameter ratio in the granulocytic lineage of patients without the anti-leukemic therapy.

As it was known and expected, the size of NoBs and nuclei was largest in early granulocytic progenitors – myeloblasts (Fig. 1). In differentiated and fully mature cells such as myelocytes and segmented granulocytes the size of both NoBs and nuclei including nuclear segments was significantly smaller (Figs 2, 3). However, the size reduction of NoBs in differentiated and mature cells appeared to be larger (~ 40-50 % in comparison with that of nuclei (~ 10-20 %, see also Table 1). Thus the resulting NoBs/Nu diameter ratios were larger in less differentiated granulocytic progenitors than in differentiated or mature cells because NoBs occupied larger nuclear regions.

NoBs : Nu diameter ratio in the granulocytic lineage of patients treated with the anti-leukemic therapy.

Similarly as in patients untreated with the anti-leukemic therapy, in patients receiving that therapy, nuclear regions occupied by NoBs in differentiated and mature cells also diminished and reached the smallest size in fully mature "terminal segmented granulocytes". On the other hand, the NoBs/Nu diameter ratios reflecting the approximate size of nuclear regions occupied by NoBs were constant for each developmental stage regardless the anti-leukemic therapy. At this occasion it should be mentioned that studied cells might just "survive" the anti-leukemic treatment of CML patients and the effect of this therapy was reflected by the reduction of the bone marrow myeloid: erythroid ratio (see above). The approximate size of nuclear regions occupied by NoBs after the anti-leukemic therapy and in controls was also noted in cultured myeloblasts of established leukemic cell lines (Smetana et al. 2018).

NoBs : Nu volume ratio

The approximate size of nuclear regions occupied by NoBs expressed by NoBs/Nu VoRs generally followed similar trends as the above reported results of NoBs/Nu DmR calculations. The small differences were due the used different calculation formulas for three-dimensional geometrical shapes (Fig. 4). At this occasion it should be mentioned that a virtual height of measured nuclei was added to the measured diameters in bone marrow smears (Fig. 4).

Discussion

The present study provided missing information on NoBs/Nu ratios in less differentiated progenitors (myeloblasts) as well as in fully differentiated precursors (myelocytes) and mature cells (granulocytes with segmented nuclei). These ratios in various differentiation and maturation stages of the studied leukemic lineage indicated that the size of nuclear regions occupied by NoBs diminished during the cell differentiation and maturation. The decreased NoBs/Nu ratios in differentiating and maturing cells also reflected a larger size reduction of NoBs than that of the whole nucleus. In differentiating and maturing cells such large reduction of the nucleolar size is accompanying the known larger decreasing reduction of the nucleolar biosynthetic activity in comparison with nuclear extranucleolar regions (Grasso *et al.* 1963, Busch and Smetana, 1970, Smetana and Likovský 1984, Homáček *et al.* 2017, Stępiński 2018). On the other hand, it should be mentioned that the size of nuclear regions occupied by NoBs was characteristic and stable for each differentiation and maturation stage of the studied leukemic cell lineage because of the apparently changed the nucleolar and nuclear biosynthetic equilibrium. A similar development was also noted in the lymphocytic lineage (Smetana *et al.* 2017).

From the methodical point of view, the computer assisted diameter measurements of NoBs and nuclear outlines were simple procedures for the calculation of the NoBs/Nu ratios. Then the calculated NoB/Nu ratios facilitated the approximate estimation of the size – percentage – of the nuclear region occupied by NoBs. However, it should be considered that nucleolus : nucleus ratio might be also influenced by the cell preparation and "spreading". Moreover, the nucleolar and nuclear shape might be variable and not exactly rounded (Tocco *et al.* 2018).

The calculation of the NoBs/Nu VoRs seems to be less precise than the NoBs/Nu DmR. In cell smears used in the present study the smeared NoBs and nuclei to

some extent resembled geometrical three-dimensional shape of spheres (NoBs) and short cylinders (nuclei, Fig. 4). However, the addition of a virtual height was necessary for the short cylinder volume calculation. Nevertheless, the calculations of NoBs/Nu VoRs naturally exhibited similar trends during the differentiation and maturation to the NoBs/Nu DmR since they were based on the diameter measurements. On the other hand, both NoBs/Nu DmR and VoRs facilitated the approximate estimate of the nuclear region size occupied by NoBs in all differentiation and maturation stages of the studied granulocytic lineage. However, the calculation of the NoBs/Nu DmR was faster, more simple and easy.

In addition, the measurement of whole nucleoli instead of NoBs would be also influenced by the increasing width of the perinucleolar heterochromatin during the differentiation and maturation of studied cells (Smetana *et al.* 2013). The results of such measurements would mask the real size of the NoBs and especially of those, which are smaller than 1μm in differentiated or mature cells.

At the end of the discussion it has to be concluded that the approximate size of the nuclear region occupied by nucleolar bodies is decreasing in the course of differentiation and maturation of leukemic granulocytes but is characteristic and stable for each differentiation and maturation stage regardless of the anti-leukemic therapy. Such stability is also supported by the relatively small variation coefficient of measured diameters of NoBs and nuclei. The variation coefficient NoBs did not exceed 14 % and that of nuclei 18 % of the measured mean values in all developmental stages of the granulocytic lineage in both untreated and treated patients with the anti-leukemic therapy.

Conflict of Interest

There is no conflict of interest.

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