A Karyometric Note on Nucleoli in Human Early Granulocytic Precursors

(nucleolar size / human early granulocytic precursors)

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Abstract. The diameter of nucleoli was measured in human bone marrow early granulocytic precursors after visualization by a simple cytochemical method for demonstration of RNA. Such method facilitated to clearly see nucleolar bodies without perinucleolar chromatin, including those of micronucleoli. The bone marrow of patients suffering from chronic myeloid leukaemia (untreated with cytostatics) provided a satisfactory number of both myeloblasts and promyelocytes for nucleolar measurements because of prevailing granulopoiesis. The direct nucleolar measurement was carried out on digitized and processed images on the screen at magnification 4,300x. It seems to be likely that the nucleolar size is directly related to the number of nucleoli per cell. The largest nucleoli were present in both myeloblasts and promyelocytes that possessed a single nucleolus. In contrast, the nucleolar diameter was significantly smaller in cells with multiple nucleoli. However, in cells with small multiple nucleoli, one of them was always larger and dominant with a large number of AgNORs. Such large nucleoli are possibly visible in specimens stained with panoptic procedures or methods staining nuclear chromatin or DNA. It should also be mentioned that both myeloblasts and promyelocytes mostly possessed two nucleoli with the mean diameter close to 1.5 µm. The incidence of early granulocytic precursors classified according to the nucleolar number and size strongly suggested that the various nucleolar number and nucleolar size in these cells might be related to the different stage of the cell cycle and might also explain their heterogeneity.

Introduction

It is generally accepted that nucleoli are multifunctional cell organelles involved directly or indirectly in cell proliferation, differentiation, maturation, ageing, programmed death and cell cycle (see Pederson, 1998; Wachtler and Stahl, 1993; Olson et al, 2000; Martelli et al., 2001; Biggiogera et al., 2004; Hernandez-Verdun and Louvet, 2004; Smetana, 2005). In addition, it has also been shown that nucleoli react to various factors influencing the cell behaviour including cytostatics (Busch and Smetana, 1970; Daskal, 1979; Busch, 1997). On this occasion it should be mentioned that various nucleolar activities are expressed by the nucleolar size and structure (see Smetana, 2002). Previous studies have demonstrated that the nucleolar size decreases during granulocytic differentiation and maturation (see Vendrely and Vendrely, 1959; Busch and Smetana, 1970). However, the information on the nucleolar size within early stages of the granulocytic development such as myeloblasts and particularly promyelocytes is still very limited. In addition, in various publications there is a broad variation of the nucleolar size and number. Such differences are possibly due to different approaches to the nucleolar visualization and the small number of bone marrow myeloblasts and promyelocytes for nucleolar measurements.

The present study was undertaken to provide more extensive and complementary information on the nucleolar diameter in myeloblasts and promyelocytes using a selective method for the nucleolar visualization. Such method based on RNA staining clearly demonstrates only nucleolar bodies without the perinucleolar chromatin that frequently masks small nucleoli (see Smetana 2002). The chronic phase of myeloid leukaemia is very convenient because the increased granulopoiesis provides a satisfactory number of myeloblasts and promyelocytes in the bone marrow for nucleolar measurement. In addition, there is a remarkable similarity of nucleoli in non-leukaemic and leukaemic granulocytic precursors (Vendrely and Vendrely, 1959; Smetana et al., 1998) especially in patients who were not treated with the cytostatic therapy. It should also be mentioned that electron microscopy is less convenient for measurements of the nucleolar size because only a limited number of studied cells and cell components is present in ultrathin sections (Low and Freeman, 1958).

Material and Methods

Nucleoli were visualized by acidified methylene blue for demonstration of RNA and silver reaction for demonstration of silver-stained nucleolus organizer regions

Received April 12, 2006. Accepted April 27, 2006

This study was supported in part by the Ministry of Health of the Czech Republic – Research Project VZ 0002373601.

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Abbreviation: AgNORs – silver-stained nucleolus organizer regions.

(AgNORs) in unfixed bone marrow smears (Smetana et al., 1969; Ochs, 1998; Smetana et al., 1999a). Nuclear chromatin structures were visualized by acidified methylene blue after HCl hydrolysis in methanol-fixed smears for demonstration of DNA (Busch and Smetana, 1970). Bone marrow smears were taken from diagnostic biopsies of four patients suffering from chronic phase of myeloid leukaemia with the approval of the ethic committee of the Institute. Investigated patients at the admission to the clinical department of the Institute were untreated with any cytostatic therapy. It should be mentioned that granulopoiesis in the bone marrow specimens was dominant and prevailed in comparison with the erythroid lineage. The ratio of granulocytic to erythroid lineage in these patients was always higher than 5 : 1 (see Rundles, 1983, and Results).

Micrographs of studied cells in cell monolayer portions of bone marrow smears were taken with a Camedia digital photocamera C-4040 ZOOM (Olympus, Tokyo, Japan) placed on a Jenalumar microscope (Zeiss, Jena, Germany) with special mechanical adapters. The resulting images were processed (see Skinner et al., 1993) with Quick Photoprogram (Olympus) in combination with L-view and Power Point Microsoft programs (Microsoft, Redmond, WA, USA). Nucleolar diameters were measured directly on the screen at magnification 4,300x using Quick Photoprogram. The diameter of elongated nucleoli was calculated from measured largest and smallest diameter divided by 2. The mean nucleolar diameter based on measurements of all distinct nucleoli per cell and large nucleoli in multinucleolated cells was calculated for each myeloblast and promyelocyte. All reported measurements were expressed by mean values and standard deviation (SD).

Results

The incidence of bone marrow early granulocytic precursors and methodological notes to the nuclear and nucleolar visualization

As was expected, bone marrow of investigated patients contained only a small percentage of early granulocytic precursors, i.e. 2.30 % (SD 1.08) of myeloblasts and 6.17 % (SD 4.10) of promyelocytes. However, in spite of the small percentage of these cells, the markedly increased granulopoiesis provided the possibility to measure their satisfactory number. Such increased granulopoiesis was expressed by its increased ratio to erythropoiesis. The median of the ratio of granulo- to erythropoiesis in investigated bone marrow specimens was 10.96 : 1 with mean values 15.07 (SD 11.37) : 1.

The light microscopic selective visualization of nucleoli was achieved using a simple cytochemical method for demonstration of RNA-containing structures, which made it possible to clearly see nucleolar bodies regardless of their size without the perinucleolar chromatin (Fig. 1). As was published previously, after visualization of the nuclear chromatin with both panoptic staining procedures or cytochemical methods for demonstration of DNA, it was frequently impossible to distinguish small nucleoli from nuclear interchromatin regions or small "postnucleolar chromatin clumps" (see Undritz, 1972; Smetana, 2002, Fig 2). The distinct perinucleolar chromatin, however, facilitated clear visualization of larger nucleoli (Fig. 2, see also Undritz).

The nucleolar coefficient and diameter in myeloblasts and promyelocytes

The mean values of the nucleolar coefficient in myeloblasts was 2.25 (SD 0.17). According to the histogram, myeloblasts mostly possessed two nucleoli (Table 1). The mean values of the nucleolar coefficient determined for promyelocytes were very similar, i.e. 2.27 (SD 0.07), and most of these cells also possessed two nucleoli (Table 1). The mean diameter of nucleoli in myeloblasts was 1.40, SD 0.01 μm. As was expected, the mean nucleolar diameter in promyelocytes was slightly but significantly smaller, i.e. 1.26, SD 0.10 μm ($P < 0.04$ using t-test). The largest nucleoli with mean values of the diameter 2.22 and 2.19 μm were present in both myeloblasts and promyelocytes that were characterized by the presence of a single nucleolus (see Table 2). It seemed to be interesting that the mean diameter of nucleoli in both myeloblasts and promyelocytes significantly decreased with the increasing number of nucleoli per cell (Table 2).

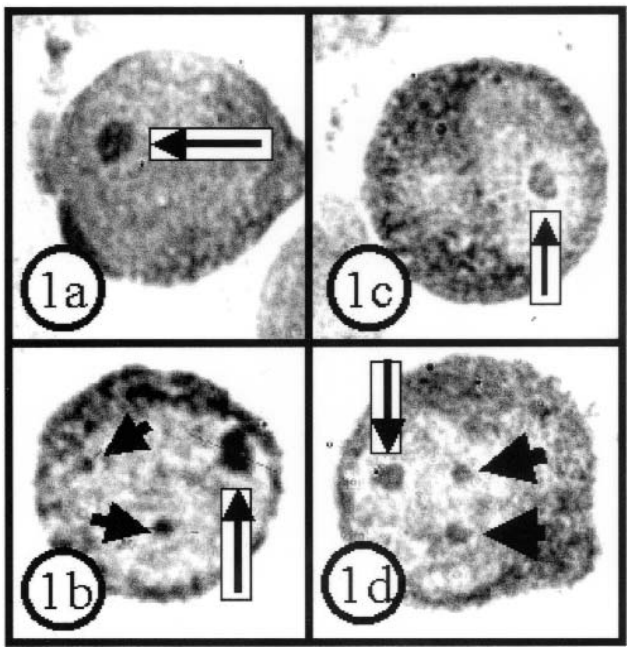

Fig. 1. Myeloblasts (1a, 1b) and promyelocytes (1c, 1d) in specimens stained for RNA. Note the presence of a large single nucleolus (large arrows) in mononuclear cells (1a, 1c). In multinucleolar cells (1b, 1d) one nucleolus is always larger (large arrows) than other nucleoli (short arrows). Magnification approximately 2,100x (1a, 1b), 1,500x (1c), 1,900x (1c)

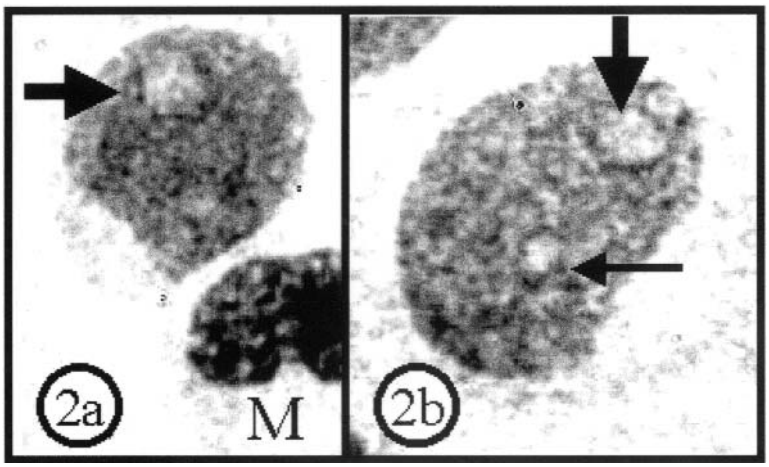

Fig. 2. A myeloblast (2a) and a promyelocyte (2b) with fine chromatin structure in specimens stained for DNA. Distinct but unstained large nucleoli (large arrows) and a smaller nucleolus (thin arrow) are surrounded by a stained and distinct layer of the perinucleolar chromatin. Some of small light regions in the chromatin structure might represent both small unstained nucleoli and interchromatin regions. Note a metamyelocyte (M, 2a) with highly condensed chromatin structure. Magnification approximately 2,600x (2a), 3,125x (2b)

Table 1. The incidence (percentage) of cells classified according to the number of nucleoli (based on 250 measurements)

Cells	Number of nucleoli per cell			
	1	2	3	4 and more
Myeloblasts	**27.37** (8.57)[a]	**43.77** (9.28)[*°]	**17.76** (9.96)	**10.92** (6.40)
Promyelocytes	**15. 97** (8.21)	**41.18** (3.99)[*•]	**31.30** (7.41)	**11.61** (2.55)

[a] Mean and standard deviation
[*] Significant difference in comparison with cells containing a single nucleolus using t-test ($P < 0.04$)
[°] Significant difference in comparison with cells containing more than two nucleoli using t-test ($P < 0.01$)
[•] Marginally significant difference in comparison with cells containing three nucleoli ($P = 0.057$) and significant difference in comparison with cells containing four and more nucleoli ($P < 0.001$) using t-test

Table 2. The nucleolar mean diameter (in μm) in myeloblasts and promyelocytes classified according to the number of nucleoli (based at least on 250 measurements)

Cells	Number of nucleoli per cell			
	1	2	3	4 and more
Myeloblasts	**2.22** (0,36)[a]	**1.58** (0.13)[*]	**1.40** (0.05)[**]	**1.04** (0.09)[***]
Promyelocytes	**2.19** (0.26)	**1.60** (0.12)[*]	**1.19** (0.12)[**]	**1.01** (0,05)[***]

* significant difference in comparison with cells containing a single nucleolus using t-test ($P < 0.01$)
** significant difference in comparison with cells containing a single nucleolus or two nucleoli using t-test ($P < 0.04$)
*** significant difference in comparison with cells containing a single nucleolus or two or three nucleoli using t-test ($P < 0.02$)
For other legend see Table 1

It must also be mentioned that in myeloblasts and promyelocytes containing two or more nucleoli, one nucleolus was always larger (Fig. 1b, c). Such nucleoli possibly correspond to dominant nucleoli with a larger number of AgNORs. In the present study large nucleoli in myeloblasts with multiple nucleoli contained in average 7.71 (SD 2.43) and small nucleoli 2.92 (SD 1.56) AgNORs. In comparison with these myeloblasts, large nucleoli in multinucleolated promyelocytes possessed a decreased number of AgNORs (5.40, SD 1.44) which, however, was still larger than that in remaining smaller ones (1.95, SD 0.82). It should also be noted that the diameter of larger nucleoli decreased with the increasing number of nucleoli per cell and was significantly smaller in cells with three and more nucleoli per nucleus (Table 3). In addition, the possibility exists that such larger nucleoli are visible in specimens with stained chromatin by panoptic staining procedures or cytochemical methods for demonstration of DNA allowing clear distinction of small nucleoli (Fig. 2).

Discussion

The present study provided complementary information on human early granulocytic precursors and contributed to the present knowledge on the nucleolar size and number in less differentiated and immature cells. As was expected (see Vendrely and Vendrely, 1959), the mean nucleolar diameter was larger in myeloblasts than in promyelocytes. On the other hand, in both myeloblasts and promyelocytes, single nucleoli were always larger than the multiple ones. Such observation suggests that the large size of single nucleoli and small size of multiple nucleoli within both these early stages of the nucleolar development is not related to their further differentiation but might be related to the cell cycle. It has been shown that small multiple nucleoli are characteristic for the G1 and early S phase of the cell cycle. Then, when the cell cycle progresses, small multiple nucleoli fuse and form large single nucleoli characteristic for the late S and G2 phases (Schnedel and Schnedel, 1972; Gonzalez and Nardone, 1968; Wachtler et al. 1984; Wachtler and Stahl, 1993). Therefore, it is possible that myeloblasts and promyelocytes with multiple small nucleoli might be

Table 3. The mean nucleolar diameter (in μm) of largest nucleoli in myeloblasts and promyelocytes with two or more nucleoli (based on 250 measurements)

Cells	Number of nucleoli per cell		
	2	3	4 and more
Myeloblasts	**1.84** (0.10)[a]*	**1.85** (0.11)*	**1.37** (0.38)*
Promyelocytes	**1.93** (0.36)	**1.43** (0.17)*	**1.29** (0,17)*

* significant difference in comparison with cells containing a single nucleolus using t-test ($P < 0.03$)

For other legend see Table 1

in the postmitotic stages of the cell cycle. In contrast, myeloblasts and promyelocytes with single large nucleoli might represent cells in premitotic stages. On this occasion it should be mentioned that under experimental conditions, the decreased nucleolar size in myeloblasts was accompanied by a significant increase of cells in G0/1 phase of the cell cycle (see Smetana et al., 2005; 2006). Thus, heterogeneity of bone marrow early granulocytic progenitors might be due, at least in part, to different states of these cells in the cell cycle. Moreover, the marked similarity of the nucleolar size distribution between both early stages of the granulocytic development supports such speculations. It should also be added that nucleoli of these cells in the present study were not influenced by the cytostatic therapy, which may substantially modify nucleolar biosynthetic activities reflected by marked changes of the nucleolar morphology and components (Busch and Smetana, 1970; Daskal, 1979; Busch, 1997).

The present study also demonstrated that in both myeloblasts and promyelocytes with small multiple nucleoli one of the nucleoli was always larger than the rest. Such larger nucleoli are possibly dominant similarly as in proerythroblasts (Smetana et al., 1999b), and possess a larger number of AgNORs than small nucleoli. It should be mentioned that AgNORs are considered to be markers of the nucleolar rRNA transcription (see Smetana, 2005). On the other hand, the function of the dominant nucleolus in the cell has not been satisfactorily clarified yet. From the methodological point of view it seems to be likely that these large nucleoli in multinucleolated myeloblasts and promyelocytes are clearly visible regardless of the procedure used for staining of blood cells including the panoptic or chromatin stains (Undritz, 1972). On the other hand, small nucleoli may not be visible and are masked by chromatin or may not be distinguished from other nuclear components after such procedures (Undritz, 1972; Smetana, 2002). Thus, the use of such procedures may explain differences in the literature in the number and size of nucleoli of granulocytic early progenitors.

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