

## On the nucleolar size and density in human early granulocytic progenitors, myeloblasts

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Human myeloblasts were studied in bone marrow of patients suffering from chronic phase of chronic myeloid leukaemia to provide more information on the nucleolar diameter in these early granulocytic progenitors. These cells are a convenient model for such study since the number of myeloblasts in diagnostic bone marrow smears of investigated patients is larger than in not-leukemic persons because of the increased granulopoiesis. The nucleolar diameter was measured in myeloblasts after various cytochemical procedures such as methods for visualisation of RNA, DNA and proteins of AgNORs using digitized images and image processing. The results clearly demonstrated that values of the nucleolar diameter depended on the procedures used for visualising nucleoli. It seems to be also clear that a close relationship exists between the diameter of nucleoli and their number since the larger the number of nucleoli per cell the smaller their mean size. However, one of multiple nucleoli present in the nucleus is usually significantly larger. Moreover, the possibility exists that the variability of nucleolar diameter of leukemic myeloblasts and thus the heterogeneity of these cells might depend on various stages of the cell cycle as supported by nucleolar measurements on aging leukemic myeloblasts (K 562 cells) *in vitro*. Since the staining density of small and large nucleoli did not differ substantially after staining for RNA, it seems to be likely that the nucleolar size is directly related to the total RNA content in myeloblasts. In addition, karyometry combined with RNA cytochemistry still appears to be an useful tool to study nucleoli at the single cell level.

Key words: nucleolar diameter, human myeloblasts, nucleolar visualisation.

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Recent studies demonstrated that nucleoli are multifunctional cell components involved in the regulation of the cell resting state, proliferation, length of the cell cycle and aging (see Olson *et al.*, 2000; Pederson, 1992; Smetana, 2005). It is also known that the nucleolar size may reflect the nucleolar biosynthetic and functional activities and decreases during differentiation and maturation of granulocytic progenitors (see Smetana, 2005; Vendrely and Vendrely, 1959). However, previous studies also demonstrated that the size of nucleoli increases during the cell cycle in parallel with the increase in nucleolar biosynthetic activities reaching maximal values at the S and G<sub>2</sub> phase because of the fusion of small nucleoli (see Gonzalez and Nardone, 1968; Schnedel and Schnedel, 1972; Wachtler *et al.*, 1984; Wachtler and Stahl, 1993). On the other hand, the knowledge on the nucleolar diameter in the stage of early granulocytic progenitors – myeloblasts – is very limited or unknown including its variability. In addition, the nucleolar size of nucleoli in one and the same nucleus may be apparently different but mean values of the nucleolar diameter per nucleus of myeloblasts were not reported (see Smetana 2005). Such a lack of reliable data may be due to the fact that some of the methods used for the visualisation of nucleoli do not demonstrate clearly the nucleolar body which frequently is masked by the surrounding chromatin structures (see Smetana, 2002).

The present study was undertaken to provide more information on mean values of the nucleolar diameter in myeloblasts using three different methods for nucleolar visualisation at light microscopy. It is worth mentioning that electron microscopic measurements are less suitable because only a relatively small portion of the measured cell components – i.e. nucleoli – may be present in ultrathin sections depending on the cell position (see Low and Freeman, 1958; Smetana, 2002). The stan-

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standardised cytochemical method visualising clearly the nucleolar body based on its RNA content and the improved approach using image processing facilitated a more precise measurement of the nucleolar diameter at very high light microscopy magnifications. It should be added that myeloblasts of patients suffering from the chronic phase of the chronic myeloid leukaemia represented a convenient model for nucleolar diameter measurements since they were present in diagnostic bone marrow smears in a larger number than in not-leukemic persons because of the increased granulopoiesis.

## Materials and Methods

Nucleoli were visualised by acidified methylene blue for demonstration of RNA and by the silver reaction for AgNORs on unfixed smears (Ochs, 1998; Smetana *et al.*, 1969; Smetana *et al.*, 1999) of patients suffering from chronic phase of myeloid leukaemia or cytopins of cultured K 562 cells which originated from a patient suffering from the same disease (European Collection of Animal Cell Cultures, UK). DNA (corresponding to the nuclear and perinucleolar chromatin structures) was visualised in methanol fixed bone marrow smears of the same patients and on cytopins of K 562 cells by acidified methylene blue after hydrolysis with HCl (see Busch and Smetana, 1970). Bone marrow smears were taken from diagnostic biopsies with the approval of the ethic committee of the Institute. It should be mentioned that granulopoiesis in the bone marrow specimens was dominant and prevailed in comparison with the erythroid lineage, the ratio of the granulocytic to erythroid lineage being higher than 3:1 in these patients. K 562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 50 µg streptomycin in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Control cultures were seeded by dilution in fresh medium to a density 2×10<sup>5</sup>/mL three times a week. The aging cultures were kept for 72 hours without feeding and were characterised by the cell division arrest. Cytopins were prepared using a Shannon II cytocentrifuge (Shandon Southern Products, UK) – 6000 RPM for 10 min.

Micrographs were taken with a Camedia digital photcamera C-4040 ZOOM (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany). The resulting images were processed with Quick Photoprogram (Olympus, Japan) in combination

with L-view and Power Point Microsoft programs (Microsoft, USA). Nucleolar diameters were measured at 1500x magnification using Quick Photoprogram. The mean nucleolar diameter based on measurements of all distinct nucleoli was calculated for each myeloblast. The nucleolar density in myeloblasts stained for RNA in the cell monolayer portions of thin bone marrow smears was measured using the NHI Image Program (National Institutes of Health, USA) and expressed in arbitrary standardized units. Captured colour images were transformed to the corresponding degrees of grey scale copies. The nuclear background in arbitrary units was subtracted in each measurement for the possibility to compare measurements in different bone marrow smears and different portions of the same smear. All the data of size and density measurements were expressed as the mean values and standard deviation (SD); at least 100 measurements were considered for each investigated group

Cell cycle analysis: K 562 cells collected by centrifugation were suspended in 70% ethanol at 10°C for 30 min and then kept at -20°C for 5-7 days. Then, samples washed in PBS at room temperature were incubated in modified Vindelov's propidium iodide solution (10mM Tris, pH 8, 1mM NaCl, 0.1% Triton X-100, 20 µg/mL propidium iodide and 10 K units of ribonuclease A (Kuzelová *et al.*, 2004). The red fluorescence was measured using Coulter Epics XL flow cytometer (Beckman, USA).

## Results

### ***Diameter of nucleoli in myeloblasts measured after various visualisation***

Nucleoli visualised by a simple cytochemical procedure for the demonstration of RNA appeared as intensely stained bodies with the exclusion of perinucleolar chromatin (Figure 1). Since the nuclear chromatin was unstained, nucleolar bodies appeared very distinct including micronucleoli whose size was even smaller than 1 µm. The mean values of the nucleolar diameter (see Table 1) were significantly smaller (2.2 µm) in comparison with those recorded in specimens stained for AgNOR proteins (2.7 µm) or for DNA (2.9 µm). In fact, the larger nucleolar diameter measured in specimens stained for AgNOR proteins combined with phase contrast microscopy (Figure 2) and especially for DNA (Figure 3) included both the nucleolar body and the perinucleolar chromatin. In addition, in the

**Table 1. Nucleolar mean diameters  $\pm$  SD in myeloblasts measured in specimens stained with the various visualisation procedures used.<sup>a</sup> The values are expressed in  $\mu$ m.**

Staining for	RNA	AgNOR proteins	DNA
Mean diameter	2.2 $\pm$ 0.4	2.7 $\pm$ 0.6*	2.9 $\pm$ 0.7*

<sup>a</sup>Based on 150 measurements; \*significantly different in comparison with values found in specimens stained for RNA using t-test ( $p < 0.005$ ).

specimens stained for DNA, nucleoli were identified mainly based on to the perinucleolar chromatin and, frequently, small nucleoli could not be distinguished from the interchromatin regions (Figure 3). It is therefore likely that some of the small nucleoli in those specimens were not included in the measurements.

### Number and diameter of nucleoli in myeloblasts in specimens stained for RNA

The number of nucleoli in bone marrow myeloblasts usually ranged from 1 to 4 and the values of the nucleolar coefficient (number of nucleoli per cell) between 1.5 and 3.4 (mean 2.37, SD 0.50). The nucleolar diameter in myeloblasts ranged between 1 and 3  $\mu$ m (mean value 2.2, SD 0.5  $\mu$ m). According to the histograms of the nucleolar number and diameter, the mean diameter of nucleoli was decreasing with the increasing number of nucleoli per cell (Table 2). The largest mean values of the nucleolar diameter per cell were present in myeloblasts with one (2.6  $\mu$ m) or two nucleoli

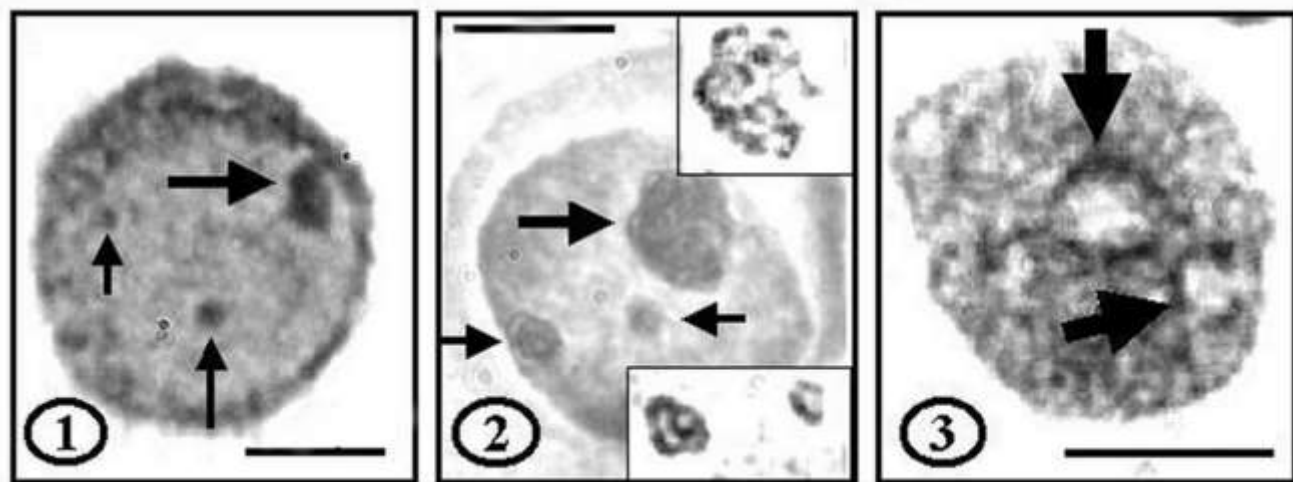
**Table 2. Nucleolar mean diameter and mean density  $\pm$  SD of nucleolar bodies per cell in myeloblasts classified according to the number of nucleoli.**

Myeloblasts with	Diameter in $\mu$ m <sup>a</sup>	Density <sup>b</sup>
1	2.65 $\pm$ 0.40	81.5 $\pm$ 23.0
2	1.60 $\pm$ 0.16*	89.5 $\pm$ 16.0
3	1.38 $\pm$ 0.23**	85.6 $\pm$ 16.6
4 and more nucleoli	1.15 $\pm$ 0.08***	76.3 $\pm$ 20.0

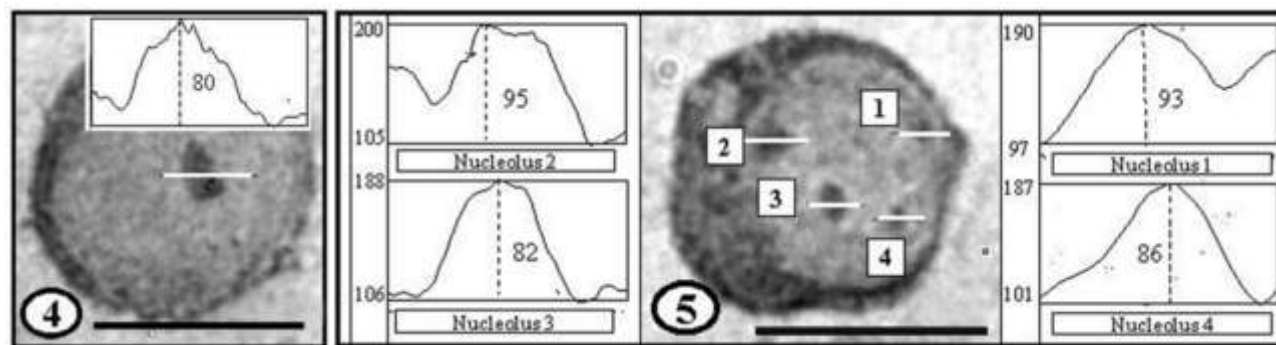
<sup>a</sup>Based on at least 120 measurements; <sup>b</sup>in arbitrary units calculated with subtraction of nuclear background density; based on 100 measurements; \*significantly different ( $p < 0.004$ ) from myeloblasts containing 1, \*\*significantly different from myeloblasts containing 1 or 2 nucleoli; \*\*\*significantly different from myeloblasts containing 1, 2 and 3 nucleoli using t-test.

(1.6  $\mu$ m). In contrast, smaller mean values were found in multinucleolar cells, i.e. 1.3  $\mu$ m in myeloblasts with three and 1.1  $\mu$ m in myeloblasts with four or more nucleoli. It should be mentioned that in multinucleolar cells one of the nucleoli was usually dominant in size, i.e. larger than 1.5  $\mu$ m (Figures 1-3). Such phenomenon was also observed in proerythroblasts in which such large dominant nucleolus possessed a larger number of AgNORs than smaller nucleoli present in the same nucleus (Smetana *et al.*, 1999)

It was also interesting that the nucleolar diameter of nucleoli in control and proliferating cultured K 562 myeloblasts was significantly larger (2.3, SD 0.1  $\mu$ m) than in aging cells (1.3, SD 0.2  $\mu$ m). Aging cultures of these cells also possessed a larger incidence of G0/G1 cells (51.7%, SD 11.1) in comparison with controls (43.1%, SD 8.4; see also Smetana *et al.*, 2006).



**Figure 1.** One large nucleolus (large arrow) and two small nucleoli (small arrows) stained for RNA. The bar is for 5  $\mu$ m. **Figure 2.** One large nucleolus (large arrow) and two small nucleoli (small arrows) stained for AgNORs observed by phase contrast microscopy to better visualize the nucleolar size. Insets: nucleoli as seen by transmission microscopy without phase contrast to see more clearly silver stained particles and trabecular structures. The bar is for 5  $\mu$ m. **Figure 3.** Nuclear chromatin structure after staining DNA. Nucleoli (arrows) are visible because of the distinct perinucleolar chromatin. The bar is for 5  $\mu$ m.



**Figure 4.** The density of one single nucleolus stained for RNA. The large bar is for 10 µm. The small bar indicates the measured nucleolar region. **Figure 5.** The density of four nucleoli with different diameter which are stained for RNA. The large bar is for 10 µm. The small bar indicates the measured nucleolar region.

### ***Nucleolar density in myeloblasts stained for RNA***

The density of nucleolar bodies was slightly variable (see Table 2 and Figures 4,5). However, the mean values determined for myeloblasts - classified according to the number of nucleoli per cell - were similar and did not show significant differences (Table 2). Observed variations might be due to the small differences in the positivity of cells and the nuclear background in various bone marrow smears. Such differences were not apparent by a subjective microscopic evaluation but were detected using the computerised densitometric program when the nuclear density background was subtracted from the density of the nucleolus (see Figure 5). On the other hand, the similar density reflecting the RNA content and diameter diversity suggests that the total nucleolar RNA content in myeloblasts is directly related to the nucleolar size.

### **Discussion**

From the methodical point of view, the present study demonstrates that the nucleolar diameter should be measured in specimens stained with methylene blue under conditions for the selective visualisation of RNA and nucleolar bodies with the exclusion of the perinucleolar chromatin. The other procedures used to visualize either DNA or the silver staining proteins combined with phase contrast microscopy did not show clearly nucleolar bodies. In particular, it was frequently impossible to distinguish small nucleoli from interchromatin regions or other nuclear components, so that only large and distinct nucleoli were actually measured in specimens processed for DNA or silver staining proteins detection.

The results of the present study also indicated

that the average density of nucleoli in mononucleolar myeloblasts (with larger nucleoli) and multinucleolar myeloblasts (with smaller nucleoli) was not significantly different in specimens stained for RNA. Such observation may suggest that the decreasing content of nucleolar RNA in myeloblasts seems to be rather related to the decreased nucleolar size (see Vendrely and Vendrely, 1959). At this occasion it should be mentioned that the evaluation of the nucleolar density at the single cell level was carried out by computerised densitometry with the subtraction of the nuclear density background. Without this approach the comparison of specimens stained at different time would be impossible and the results not comparable despite the standardisation of the cytochemical procedure used.

The results also demonstrated that in myeloblasts the increased number of nucleoli was accompanied by their decreased size. The decreasing nucleolar size is a generally known phenomenon which occurs during the differentiation and maturation of nucleolated blood cells (Smetana, 2002; 2005; Vendrely and Vendrely, 1959). However, such phenomenon was noted in further differentiating and maturing stages of the granulocytic development and not in granulocytic early progenitors - myeloblasts (Busch and Smetana, 1970; Vendrely and Vendrely, 1959). On the other hand, a possibility exists that in myeloblasts the small number of nucleoli per cell characterised by their large size might be also related to the progress of the cell cycle. It is known that during the cell cycle the nucleolar number decreases in parallel with the increasing nucleolar size due to nucleolar fusion (Gonzalez and Nardone, 1968; Schnedel and Schnedel, 1972; Wachtler *et al.*, 1984; Wachtler and Stahl, 1993).

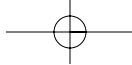
In the present study, the nucleolar diameter of nucleoli in control and proliferating cultured myeloblasts was significantly larger than in aging cultures with the increased incidence of G0/G1 cells. Thus the variability of the nucleolar number and size of bone marrow myeloblasts might reflect not only their progressing maturation but also various stages of their cell cycle at the single cell level. This conclusion might contribute to the elucidation of the various size and number of nucleoli in myeloblasts reported in various monographs and seems to be also consistent with the generally known nucleolar heterogeneity of malignant and leukemic blastic cells (see Bessis, 1973, Busch and Smetana, 1970; Undritz, 1972). However, more clinically oriented studies are required in this direction.

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