Mean diameter of nucleolar bodies in cultured human leukemic myeloblasts is mainly related to the S and G2 phase of the cell cycle

K. Smetana,[1] K. Kuželová,[1] M. Zápotocký,[2] J. Starková,[2] Z. Hrkal,[1] J. Trka[2]

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

Mean diameter of nucleolar bodies (nucleoli without the perinucleolar chromatin) per cell was studied in human leukemic myeloblasts represented by K 562 and Kasumi 1 cell lines which originated from chronic and acute myeloid leukaemia. The measurement of mean diameter of nucleolar bodies in specimens stained for RNA was very simple. Such approach eliminated the variability of the perinucleolar chromatin discontinuous shell which might influence the measured nucleolar size as suggested by earlier studies. Ageing of K 562 myeloblasts produced a significant decrease of cells in S+G2 phase of the cell cycle accompanied by a significant reduction of mean diameter of nucleolar bodies (MDNoBs) per cell. In contrast, treatment of Kasumi 1 myeloblasts with histone deacetylase inhibitor - Trichostatin A - produced a large incidence of resistant cells in S+G2 phase which were characterised by a large increase of MDNoBs. Thus, MDNoBs in leukemic myeloblasts might be a helpful tool to estimate the incidence of cells in the S+G2 phase at the single cell level in smear preparations when the number of cells is very small.

Key words: leukemic myeloblasts, mean diameter of nucleolar bodies per cell.

Correspondence: Karel Smetana,
Institute of Hematology and Blood Transfusion,
U nemocnice 1, Prague 2, Czech Republic, 128 20
Tel.: +420.2.21977271.
Fax: +420.2.21977249.
E-mail: karel.smetana@uhkt.cz

Classical cytological studies suggested that nucleolar size depends not only on cell maturation and differentiation but also on the cell cycle phase (Gonzalez and Nardone, 1968; Schnedl and Schnedl; 1972; Vendrely and Vendrely, 1959; Wachtler and Stahl, 1993). On the other hand, the information on the nucleolar size in leukemic myeloblasts is very limited and usually not supported by quantitative data. Mean diameter of nucleolar bodies per cell (MDNoBs), i.e. mean diameter of nucleoli without the perinucleolar chromatin in human leukemic myeloblasts, is less known (Smetana *et al.,* 2006a) and the relationship between MDNoBs and the cell cycle of leukemic myeloblasts is unknown. At this occasion it should be also mentioned that the perinucleolar chromatin may influence the nucleolar diameter (Smetana 2006a). The width of the perinucleolar chromatin may vary, forming less or more regular shell around the nucleolus (see Busch and Smetana, 1970, Smetana 2006a).

The present study was undertaken to provide more information on MDNoBs per cell and cell cycle phase of leukemic myeloblasts under two different experimental conditions using a simple ageing (Smetana 2006b) or a cytostatic drug – a histone deacetylase inhibitor, Trichostatin A treatment (Starkova 2004) which both are know to reduce the cell proliferation. The former is known to produce a decrease of mitotic divisions and decreased incidence of cells in the S+G2 phase of the cell cycle. In contrast, a relatively large incidence of cells resistant to the anti-proliferation effect of TSA in the S+G2 phase provides a satisfactory number of nucleoli for MDNoBs measurements. K 562 and Kasumi 1 cell lines were considered to be convenient models for studies of human leukemic myeloblasts *in vitro* (see DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen, 2004; ATCC – American Type Culture Collection, 2006) since the former originated from chronic and the latter from

acute myeloid leukaemia.

According to results of the present study, the decreased MDNoBs was accompanied by a decreased incidence of these cells in S+G2 phase. In contrast, the increased MDNoBs in myeloblasts after TSA treatment was related to the increased incidence of cells in S+G2 phase which were resistant to such treatment. Thus, the MDNoBs in leukemic myeloblasts might be a helpful and complementary tool to estimate the incidence of cells in the S+G2 phase at the single cell level in smear preparations when the small number of cells does not allow other methodical approach.

Materials and Methods

K-562 myeloblasts (European Collection of Animal Cell Cultures (UK) were cultured in RPMI 1640 medium with 10% foetal bovine serum Gibco, USA, supplemented with 100 U/mL penicillin and 50 µg/mL streptomycin in atmosphere containing 5% carbon dioxide at 37°C. Cytospins were prepared using a Shandon II cytocentrifuge (Shandon Southern Products, UK), 6000r/min for 10 min. Control cultures were seeded by dilution in fresh medium to a density 2×10^5/mL three times a week. Ageing cultures were kept for 72 hrs without feeding and were characterised by the cell division arrest and a reduction of translocated AgNORs (see Smetana 2006b).

Kasumi-1 myeloblasts received as a generous gift from Dr. O. Krejčí (Division of Experimental Haematology, Cincinnati Children's Hospital Medical Center, USA) originated from FAB 2 acute myeloid leukaemia (see DSMZ, 2004; ATCC, 2006). These myeloblasts were cultured in RPMI 1640 medium with 20% of foetal bovine serum (Gibco, USA) at 37°C in atmosphere containing 5% carbon dioxide). Aliquots were cultured without or with TSA (Sigma Aldrich, USA) at concentration 120 nM for 24 hrs at 37°C. The used concentration of TSA was determined previously according to its efficiency on cultured leukemic myeloblasts (see Starková 2004). Cytospins of Kasumi-1 myeloblasts were prepared using Universal 16 R centrifuge, rotor 1624 (Hettich, Germany), 800 rpm for 5 min.

The nuclear chromatin structure was visualised in methanol fixed cytospins by acidified methylene blue after hydrolysis with 1N HCl for demonstration of DNA (Busch and Smetana, 1970) to see characteristic apoptotic nuclei (see Biggiogera,

2004; Martelli 2001; Saraste, 1999; Smetana 2000). This procedure was used to evaluate the incidence of apoptotic cells and bodies or mitotic cells. Nucleoli, i.e. RNA containing nucleolar bodies - without the perinucleolar chromatin structure were visualised by acidified methylene blue in unfixed cytospins which were not older than 24 hours (Smetana 1969; Ochs, 1998). Silver stained nucleolar proteins of silver stained nucleolus organiser regions - AgNORs - were visualised by silver reaction in unfixed cytospins older than 1 week to facilitate to see clearly silver particles corresponding to fibrillar centers (Ochs, 1998; Smetana 1999).

Micrographs were taken with a Camedia digital photocamera C-4040 ZOOM (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany). The resulting images were processed using Quick Photoprogram (Olympus, Japan) in combination with L-view and Power Point Microsoft programs (Microsoft, USA). MDNoBs was measured at magnification 4300x on the screen using Quick Photoprogram. The mean diameter for each nucleolus was based on two measurements of the long and minor axis - largest and smallest diameters - which were previously used for the nuclear morphometry as an important parameter (see e.g. Monge 1999, Politi 2003; Setala 1997). Such measurements were simple but were required especially when the nucleolus was not rounded (see Figure 1). Then these both diameters were used for calculation of MODNoBs for each cell and the presented data in Results and Tables represent a summary of at least 200 measurements for each control and experimental group.

The incidence of K-562 myeloblasts in S+G2 phase of the cell cycle. Cells collected by centrifugation were suspended in 70% ethanol, incubated for 30 min at 10°C 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 (10 mM Tris, pH 8, 1mM NaCl, 0.1% Triton X-100, 20 µg/mL propidium iodide and 10 k units of ribonuclease A, see Kuželová 2004). The nuclear fluorescence of propidium iodide was measured using Coulter Epics XL flow cytometer (Beckman, USA).

The incidence of Kasumi-1 myeloblasts in S+G2 phase of the cell cycle. The nuclear DNA in myeloblasts in suspensions was determined using Cycle TEST PLUS DNA Reagent Kit (Becton

Dickinson Immunocytometry Systems, USA) according to manufacturers instructions. Propidium iodide fluorescence bound to nuclei detected by flow cytometer (Becton Dickinson, USA) was analysed with FlowJo (Tree Star, USA) and ModFit (Verity House, Topsham, ME, USA) software applications (see also Hrušák 1998).

The incidence of cells in S+G2 phase was calculated by the deduction of the percentage of mitotic cells determined in cytospins after DNA staining from S+G2/M cells determined by DNA flow cytometry. The presented data in the text and tables represented results of three separate experiments in each studied group.

Results

K 562 myeloblasts

In both control and ageing cultures the number of apoptotic cells and bodies (*not shown*) was limited and did not exceed 5 per cent. The nucleolar coefficient (number of nucleoli per cell, see Gonzalez-Guzman, 1949; Smetana, 2005) in controls was 5.1±0.6 (mean and standard deviation) and did not differ significantly from that in ageing cultures (5.0±0.7). On the other hand, MDNoBs was 3.7 μm in control cells and decreased in ageing ones to 2.6 (see Table 1). In controls the percentage of S+G2 cells was 63.9 and in ageing cultures decreased to 49.5 (Table 1). Thus MDNoBs in ageing cells was 70 per cent of values of the control cultures (Figure 1). Similarly, the percentage of S+G2 cells decreased to 77.4 per cent of values in control cultures (see Figure 1). It should be also noted that the number of AgNORs was small in these cells and mostly translocated to the nucleolar periphery (Figure 2, see also ref. Smetana 2006). At this occasion it should be mentioned that mitotic cells were almost absent, i.e. 0.5±0.3% of cells in ageing cultures in contrast to controls, i.e. 3.0±0.8% (see also Table 1).

Concerning histograms of myeloblasts classified according to MDNoBs, in ageing cultures there was a significantly decreased incidence of cells with MDNoBs larger than 2.5 μm which decreased to 7.5±2.5 from 44.1±4.3 per cent in controls. In contrast, the percentage of cells with MDNoBs smaller than 1.5 μm increased to 17.5±2.5 from 5.8±0.8 per cent in controls. In addition, the percentage of cells with nucleoli with MDNoBs ranging from 1.5 to 2.5 μm in ageing cultures also increased to

Table 1. Control (proliferating) and ageing K 562 myeloblasts. Mean diameter of nucleolar bodies per cell and percentage of S+G2 or M phase cells.

Myeloblasts	Diameter (μm)	S+G2 phase cells (%)	M phase cells (%)
Control	3.7±0.8[a]	63.9±2.9	3.0±0.8
Ageing	2.6±0.9[b]	49.5±2.2[b]	0.5±0.3[b]

[a]Mean and standard deviation; [b]Significant difference in comparison with controls using t-test (*p*<0.001).
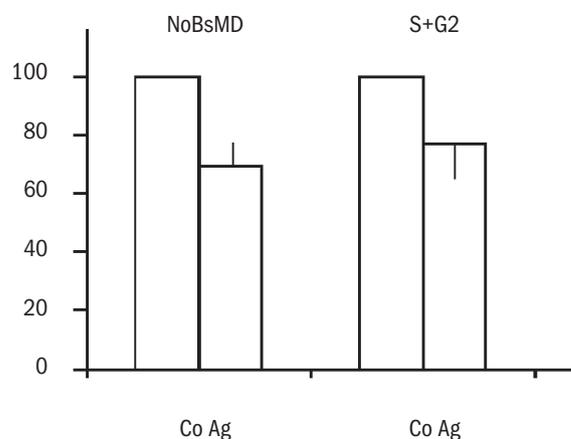
Figure 1. K 562 myeloblasts. The percentage of changes of MDNoBs and S+G2 cells produced by ageing.
MDNoBs: mean diameter of nucleolar bodie per cell, cells in S+G2 phase of the cell cycle determined by flow cytometry; Co: Controls; Ag: ageing cells, Controls values = 100 per cent.
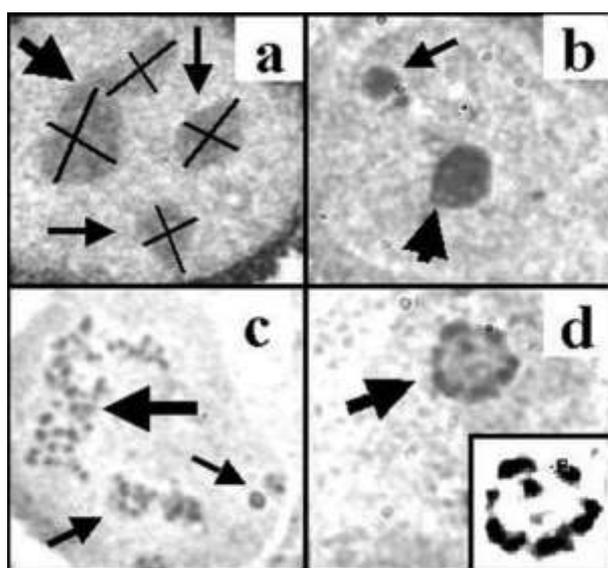
Figure 2. K 562 myeloblasts stained for RNA (a, b) and AgNORs (c,d). Control (a, c) and ageing cells (b, d).Large and small nucleoli - large and small arrows. Black lines in the Figure a indicate how nucleolar diameters were measured. Magnification approx. 3 400x (a-c), 4 400 (d). The black bars in this and following Figure represent 5 μm.

271

75.0±5.0 from 50.0±3.5. Thus, there was a decrease of the incidence of cells with large nucleoli in favour of those which contained the smaller ones. On the other hand, no direct relationship was possible to estimate between MDNoBs and each phase of the cell cycle. Cells with MDNoBs between 1 and 1.5 μm might be in G0+G1 phase, cells with MDNoBs between 1.5 to 2.5 μm possibly were in G1 and S phase and cells with MDNoBs above 2.5 μm might be at the end of the S or in the G2 phase.
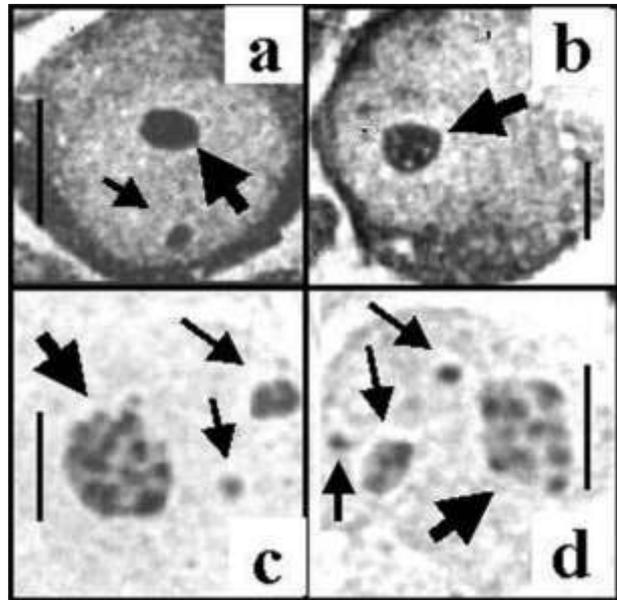
Kasumi 1 myeloblasts

In control cultures, the number of apoptotic cells and bodies (*not shown*) was very small (approximately 2per cent of cells in cultures). The nucleolar coefficient was 2.2±0.2 and MDNoBs was 1.8 μm. The percentage of cells in S+G2 phase was 35.1 and the percentage of mitotic cells in cytospins was 1.5±0.3 (see Table 2). The treatment with TSA produced a marked incidence of apoptotic cells and bodies with characteristic chromatin condensation and fragmentation (about 50 per cent of cells in cultures). In addition, no mitotic cells were observed in cytospins in contrast to controls (see above and Table 2) and thus about 50 per cent of cells remained intact without such chromatin changes. The nucleolar coefficient in TSA treated cells decreased (2.0±0.8) but without a statistical significance. However, in these cells (Figure 3) MDNoBs was apparently enlarged to 2.4 μm (Table 2), i.e. to 133 per cent of values determined for control cells (Figure 4). Similarly, the percentage of unaltered cells in S+G2 phase increased to 51.6±3.0 percent, i.e. to 144 per cent of values found for control cells (see Table 2 and Figure 4). It should be also mentioned that the number of AgNORs in control as well as TSA treated but unaltered and resistant cells was still relatively large and without translocation (Figure 3).

According to histograms of myeloblasts classified according to MDNoBs, in TSA treated cultures there was a significantly increased percentage of cells with MDNoBs larger than 2.5 μm to 24.4±9.1 from 12.5±8.5 in controls. In contrast, the percentage of cells with MDNoBs smaller than 1.5 μm decreased to 14.1±5.8 from 20.5±9.5 per cent in controls. However, the percentage of cells with nucleoli with MDNoBs ranging from 1.5 to 2.5 μm did not significantly change and was similar, i.e. 61.5±9.5 and 67.0±20.5 in controls. Thus, there was a significant increase of the incidence of cells

Table 2. Control (untreated) and TSA treated Kasumi 1 myeloblasts. Mean diameter of nucleolar bodies per cell and percentage of S/G2 or M phase cells.

Cells	Diameter (μm)	S+G2 phase cells (%)	M phase cells (%)
Contro	1.8±0.2[a]	35.1±2.7	1.5±0.3
TSA treated	2.4±0.1[b]	51.6±3.0[b]	0

[a]Mean and standard deviation; [b]Significant difference in comparison with controls using t-test ($p<0.001$).

Figure 3. Kasumi 1 myeloblasts stained for RNA (a, b) and AgNORs (c, d). Control (a, c) and TSA treated but resistant unaltered cells (b, d). Large and small nucleoli – large and small arrows. Magnification approx. 3 000x (a), 2 100x (b), 2 500x (c), 3 000x (d).
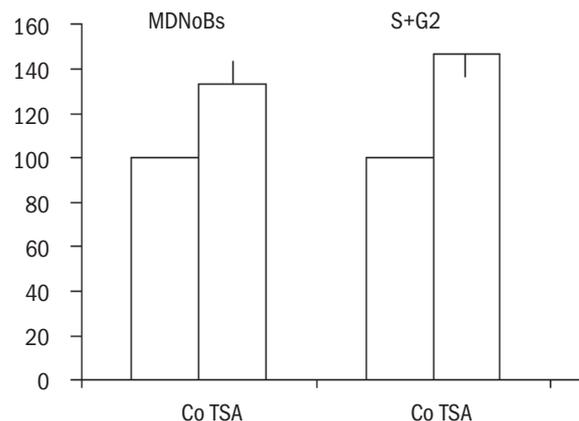
Figure 4. Kasumi 1 myeloblasts. The percentage of changes of MDNoBs and S+G2 cells produced by TSA treatment. TSA: Trichostatin A, for other Legend see Graph 1.

with larger nucleoli and decrease of those which contained the smallest ones. Similarly as in above presented experiments on K 562 cell, it was not possible to establish a direct relationship between MDNoBs and each single phase of the cell cycle. However, it seems to be likely that cells with MDNoBs between 1 and 1.5 µm might be in G0+G1 phase, cells with MDNoBs between 1.5 to 2.5 µm possibly were in G1 and S phase and cells with MDNoBs above 2.5 µm might be at the end of the S or in the G2 phase.

Discussion

The results clearly indicated that MDNoBs in ageing K-562 myeloblasts decreased similarly as their incidence in S+G2 phase of the cell cycle. In contrast, the results also demonstrated that MDNoBs in Kasumi-1 myeloblasts resistant to the TSA increased similarly as the incidence of these cells in that phase. Such observations suggested that MDNoBs exhibited a trend which was similar to the incidence of cells in S+G2 phase of the cell cycle. In addition, under both these experimental conditions no substantial difference was noted in the percentage of changes between directly measured MDNoBs and incidence of S+G2 cells determined by DNA flow cytometry. Thus, it seems to be likely that in the future MDNoBs might be helpful to estimate the incidence of S+G2 cells in smeared peripheral blood and bone marrow biopsies when the number of cells is very limited and unsatisfactory for DNA flow cytometry. Additional studies on clinical material are in progress in this direction at present. On the other hand, in the present study it was not possible to establish a direct relationship between the incidence of myeloblasts classified according to MDNoBs and each single phase of the cell cycle which has been reported in previous studies (Gonzalez and Nardone, 1968; Schnedl and Schnedl, 1972; Wachtler and Stahl, 1993). However, it should be noted that cultures in the present study were intentionally not synchronised to approach to the state *in vivo*.

The measurement of MDNoBs, i.e. mean nucleolar diameter without the perinucleolar chromatin in specimens stained for RNA is very simple. Such approach eliminates the variability of the perinucleolar chromatin discontinuous shell which may influence the measured nucleolar size as suggested by earlier light as well as electron microscopic studies (Busch and Smetana, 1970; Smetana 2006a). The

increased MDNoBs presumably related to the increased incidence of TSA resistant S+G2 cells is in a full harmony with classical cytological studies according to which the nucleolar size increased in S and G2 phase of the cell cycle due to the nucleolar fusion (Gonzalez and Nardone, 1968; Schnedl and Schnedl, 1972; Wachtler 1984). The decreased nucleolar coefficient in TSA resistant cell are also in favour of mentioned supposition. However, in previous studies the mean nucleolar diameter of all nucleoli per cell was not measured and the decreased values of the nucleolar coefficient in the present study were not statistically significant.

The results of the present study also demonstrated that resistant Kasumi 1 myeloblasts to the cytostatic effect of a histone deacetylase inhibitor - TSA - are in the S+G2 phases of the cell cycle. It seems to be possible that these cells might be blocked in these phases of the cell cycle because no mitotic divisions were present in investigated specimens. It should be mentioned that such block in S and especially G2 phase produced by cytostatics is known and a relatively large number of AgNORs supports such speculation (Darzynkiewicz 1976; Gelfant, 1981; Pellicciari 1996; Smetana, 2005). In addition, a possibility exists that such block might represent a transitional state before the cell death as reported previously in the literature (Vávrová 2001).

The reduced MDNoBs and decreased number of K 562 myeloblasts in S+G2 phase of the cell cycle together with the absence of mitoses or decreasing number of translocated AgNORs in ageing cultures are in harmony with the expected and known decrease of proliferation activity of ageing cells (Campisi, 2001; Smetana, 2005; 2006b; Zhao 2000). On the other hand, at the end of the discussion it should be added that changes in MDNoBs accompanying all above mentioned events in both presented experiments might represent an additional and useful morphological marker of the cell activities in blood and bone marrow smears in the future.

References

Biggiogera M, Bottone MG, Scovassi AI, Soldani C, Vecchio L, Pellicciari C. Rearrangment of nuclear ribonucleoprotein (RNP)-containing structures during apoptosis and transcriptional arrest, Biol Cell 2004; 603-15.

Busch H and Smetana K. The nucleolus. Academic Press, New York, 1970.

Campisi J. From cells to organisms: can we learn about aging from cells in culture? Exp Gerontol 2001; 607-18.

Darzynkiewicz Z, Traganos F, Andreeff M, Sharpless, Melamed MR. Different sensitivity of chromatin to acid denaturation in quiescent and cycling cells as revealed by flow cytometry. Histochem Cytochem 1979; 27:475-85.

Gelfant S. Cycling noncycling cell transitions in tissue aging, immunological surveillance, transformation, and tumor growth. Int Rev Cytol 1981; 70:1-25.

Gonzalez P, Nardone RM. Cyclic nucleolar changes during the cell cycle. Exp Cell Res 1968; 50: 599-615.

Kuželová K, Grebeňová D, Pluskalová M, Marinov I., Hrkal Z. Early apoptotic features of K 562 cell death induced by 5-aminolaevulinic acid – based photodynamic therapy. J Photochem Photobiol B:Biology 2004; 73:67-78.

Martelli AM, Zweyer M, Ochs RL, Tazzari PL, Tabellini G, Narducci P, et al. Nuclear apoptotic Changes: An overview. J Cell Biochem 2001; 82: 634-46.

Monge JM, Val-Bernal JF, Buelta L, Garcia-Castrillo L, Asensio L. Selective nuclear morphometry as a prognostic factor of survival of renal cell carcinoma. Histol Histopathol 1999; 14: 119-23.

Ochs RL Methods used to study structure and function of the nucleolus. Methods in Cell Biology 1998; 53:303-21.

Pellicciari C, Bottone MG, Schaack V, Manfredi AA, Barni S. Etoposide at different concentrations may open different apoptotic pathways in thymocytes. Eur J Histochem 1996; 40: 289-98.

Politi EN, Lazaris AC, Kavantzas A, Kountselini H. Comparison between morphometry and immunostaining of malignant cells in non-small cell lung cancer. Annal Quant Cytol Histol 2003; 25: 169-76.

Saraste A. Morphologic criteria and detection of apoptosis. Herz 1999; 24: 189-95.

Schnedel W, Schnedel M. Nukleoluszahl und grőße während des zellzyklus. Z Zellforsch 1972; 126: 374-82.

Setala L, Lipponen P, Kosma VM, Marin S, Eskelinen M, Syrjanen K, et al. Nuclear morphometry as predictor of disease outcome in gastric cancer. J Pathol 1977; 181: 46-50.

Smetana K. Nucleoli in blood cells of hematologic malignancies (Structure, cytochemistry of nucleoli in leukemic, lymphoma and myeloma cells). In: Trends in Leukemia Research (Romero RM ed) Nova Science Publ., Hauppage, New York, 2005, pp. 155-79.

Smetana K, Cajthamlová H, Grebeňová D, Hrkal Z. The 5-aminolaevulinic acid-based photodynamic effects on nuclei and nucleoli of HL-60 leukemic granulocytic precursors. J Photochem Photobiol B: Biology 2000; 59:80-6.

Smetana K, Jirásková I, Perlaky L, Busch H. The silver reaction of nucleolar proteins in the main structural compartments of ring-shaped nucleoli in smear preparations. Acta Histochem 1999;101: 167-83.

Smetana K, Klamová H, Mikulenková D Pluskalová M, Hrkal Z. To the nucleolar size and density in human early granulocytic progenitors – myeloblasts. Eur J Histochem 2006a; 50:119-24.

Smetana K, Klamová H, Pluskalová M, Stőckbauer P, Hrkal Z. To the intranucleolar translocation of AgNORs in leukemic early granulocytic and plasmacytic precursors. Histochem Cell Biol 2006b;125:165-70.

Smetana K, Lejnar J, Potměšil M. A further contribution to the demonstration of RNA and nucleoli of blood cells in smear preparations. Folia Haematol 1969;91: 381-4.

Starková J, Madžo J, Vášková M, Kalina T, Trka J. Histone deacetylase inhibitors are capable to modify leukaemia-specific phenotype of TEL/AML1-positive leukaemic cells. Blood 2004;104-II, 1891, 524a.

Vávrová J, Marekova M, Vokurková D. Radiation-induced apoptosis and cell cycle progression in TP53-defficient human leukemia cell line. Neoplasma 2001; 48:26-33.

Vendrely C, Vendrely R. Localisation de l´acide ribonucleique dans les different tissues et organes de vertèbres. In: Handbuch der Histochemie (Graumann W, Neumann R eds) Vol. 3, part 2, Fischer, Stuttgart, 1959, pp. 84-243.

Wachtler F, Schwarzacher HG, Smetana K. On the fusion of nucleoli in interphase. Europ J. Cell Biol 1984; 34: 190-2.

Wachtler F, Stahl A. The nucleolus: A structural and functional interpretation. Micron 1993;24: 473-505.

Zhao L, Zhang ZY, Tong TJ. Sheng Li Ke Xue Jin Zhan 2000; 31:205-10 – quoted abstract in Medline.