Original Article

To the Density and Distribution of Heterochromatin in Differentiating, Maturing and Apoptotic Cells Represented by Granulocytic, Lymphocytic and Erythrocytic Precursors

(heterochromatin density / differentiating and maturing cells)

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Abstract. The present study was undertaken to provide more information on the density and distribution of heterochromatin in early and advanced stages of the granulocytic, lymphocytic and erythroid development. Heterochromatin was visualized using a simple cytochemical method for the demonstration of DNA followed by computer-assisted densitometry of the digitized images. The largest heterochromatin density in early proliferating stages of all studied blood cell lineages was noted in the perinucleolar region and centrally located chromocentres. In contrast, the heterochromatin density at the nuclear membrane was significantly lower. In advanced non-proliferating stages or apoptotic cells the heterochromatin density increased and was similar in all nuclear regions, i.e. in the perinucleolar regions, chromocentres, and at the nuclear membrane. Thus, such observations indicated that the heterochromatin condensation in the perinucleolar region and chromocentres, i.e. in "gene-rich nuclear regions", of differentiating and maturing progenitors of blood cells preceded that at the nuclear periphery.

Introduction

The subnuclear structure apparently reflects the spatial organization of the machinery for gene expression or silencing (Zajdi et al., 2007). It is generally known that cell differentiation and maturation is accompanied by chromatin condensation related to the decline of nuclear biosynthetic activities in the DNA and RNA synthesis (Frenster, 1974). Granulocytic, lymphocytic and nucleated erythrocytic precursors represent very convenient models for such studies. The increasing chromatin condensation reflected by the increasing incidence of heterochromatin in the interphase nucleus is considered to be ontogenetically determined. It is characteristic for advanced or terminal stages of nucleated blood cells and is in harmony with the decrease or loss of the proliferation activity (Bessis, 1972; Alcobia et al., 2000). On the other hand, no information on quantitative data on the density of heterochromatin in various nuclear regions has been reported in the literature.

The present study was undertaken to provide more information on the density and distribution of the condensed chromatin – heterochromatin – in granulocytic, lymphocytic and erythroblastic early and advanced developmental stages using a simple cytochemical method for the demonstration of DNA followed by computer-assisted densitometry. For comparison, the density of heterochromatin was also studied in cultured HL-60 myeloblasts after induction of the apoptotic process, which is known to be accompanied by the characteristic chromatin condensation and fragmentation (Smetana et al., 2000).

Material and Methods

The nuclear chromatin structures of lymphocytic, granulocytic and nucleated erythrocytic precursors in methanol-fixed bone marrow smears were visualized by DNA staining with acidified methylene blue after 1 N HCl hydrolysis at 60 ºC under conditions extracting histones and RNA (see Smetana et al., 1967; Busch and Smetana, 1970). Lymphocytic early precursors were represented by lymphoblasts. Late developmental stages of lymphocytes were represented by "sleeping cells", i.e. by small mature lymphocytes. Granulocytic early precursors were represented by proliferating precursor stem cells – myeloblasts. The late precursors without the ability to proliferate and divide were represented by metamyelocytes. Early precursors of the erythrocytic lineage were represented by proliferating precursor stem cells – proerythroblasts. Late developmental stages of erythroblasts without capability to proliferate and divide were represented by late erythroblasts. Granulocytic and erythrocytic precursors were studied in patients suffering from the chronic phase of chronic myeloid leukaemia. Cells of the lymphocytic lineage were studied in

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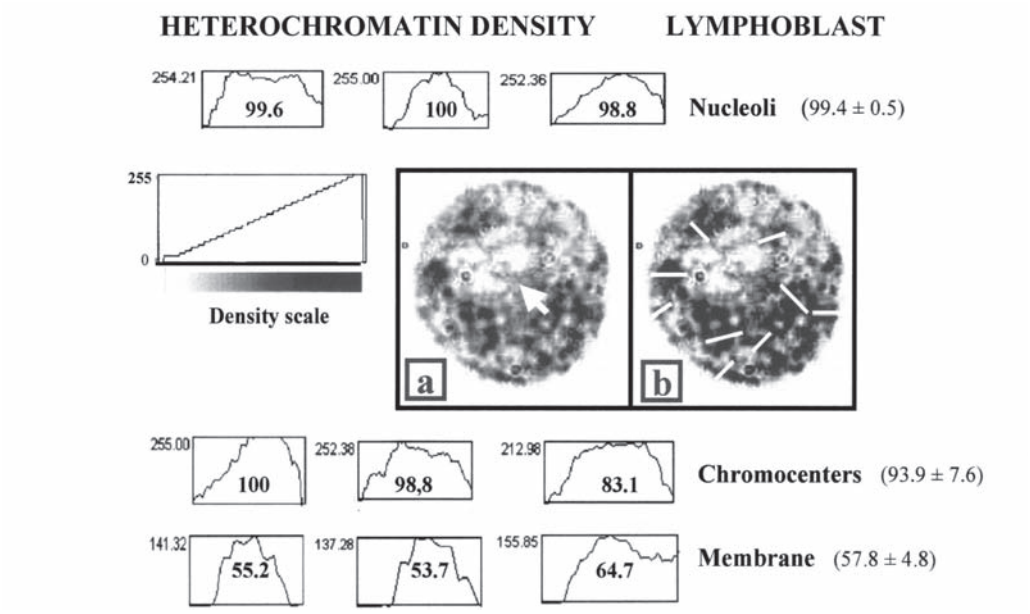

Fig. 1. Heterochromatin density values in a bone marrow lymphoblast (a, b). Thick bars (see b) indicate measured lines. Mean heterochromatin density values for three nuclear regions in the measured cell are in brackets. Nucleoli – perinucleolar regions. Membrane – nuclear regions at the inner surface of the nuclear membrane. Nucleolus – arrow. Magnification approximately 4300×.

patients with B chronic lymphocytic leukaemia. In both these haematological malignancies, the morphology of early and late differentiation and maturation stages of studied lineages is similar to that in normal bone marrow, but their increased number in bone marrow specimens is satisfactory for the present measurements (Bessis, 1972; Cline, 1975; Smetana et al., 1998). Bone marrow samples were prepared by simple smearing of small tissue fragments from thin needle biopsies (Undritz, 1972) and originated from diagnostic biopsies of patients untreated with cytostatics with the approval of the ethics committee of the Institute.

The apoptotic process in HL-60 myeloblasts originating from acute myeloid leukaemia (European Collection of Animal Cell Cultures, Salisbury, UK) was induced by the 5-aminolaevulinic acid-based photodynamic effect. Such effect produced characteristic apoptotic changes of nuclear structures, which were morphologically reflected by chromatin condensation and fragmentation (Smetana et al., 2000). In that procedure, cells sensitized by 5-aminolaevulinic acid for 4 h at 37°C were exposed to broad-spectrum blue light for 1 h and subsequently incubated in the dark at room temperature for 1 h. HL-60 myeloblasts representing proliferating cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum without the sensitizer and exposure to blue light. Harvested cells were prepared for the light microscopic observations using a Shandon II cytocentrifuge (Shandon Southern Products, Runcorn, Cheshire, UK) – 6.000 RPM for 10 min.

Micrographs were taken with a Camedia digital photocamera C.4040 ZOOM (Olympus, Tokyo, Japan) placed on a Jenalumar microscope (Zeiss, Jena, Germany) equipped with a double adapter to provide larger magnification of the resulting images. The images were then processed with Quick Photoprogram (Olympus) in combination with Power Point Microsoft programs (Microsoft, Farmington, CT). The heterochromatin density in perinucleolar regions, chromocentres and at the nuclear membrane was measured using the NIH Image Program – Scion for Windows (Scion Corp., Frederick, MD) after image conversion to the grey scale and expressed in arbitrary units, which were standardized and calculated according to the following formula: measured heterochromatin density in the investigated nuclear region was divided by the largest – maximal – density of the perinucleolar chromatin. At least three density measurements were carried out for three generally recognized nuclear heterochromatin regions, i.e. perinucleolar region, chromocentres, and at the nuclear membrane in each measured cell (see Comings, 1980, Fig. 1). It should also be mentioned that the perinucleolar chromatin region was always very prominent (Busch and Smetana, 1970). However, when in advanced stages of studied cell lineages (metamyelocytes and late erythroblasts) perinucleolar regions were not distinct, the maximal perinucleolar heterochromatin density value of the perinucleolar region in these cells was replaced by that in the early stage that was present in the same portion of investigated specimens. Such calculation and standardization of arbitrary density units facilitated the comparison of results in various portions of bone marrow smears or cytospins that occasionally exhibited various artificial densities due to preparation techniques. This approach decreased artificial measurements and thus pro-

vided better results than the background adjusted to zero, which depended on the investigator.

Results

Early differentiation stages of the bone marrow lymphocytic, granulocytic and erythroblastic lineages

Lymphoblasts, myeloblasts and proerythroblasts with large nucleoli representing proliferating pools of lymphocytic, granulocytic and erythroblastic lineages were characterized by the presence of a very condensed and distinct heterochromatin in perinucleolar regions and chromocentres (Table 1). On the other hand, the heterochromatin density at the nuclear membrane in all of these cells was significantly lower (Table 1).

Mature or late differentiation and non-proliferating stages of the bone marrow lymphocytic, granulocytic and erythroblastic development

The heterochromatin density in the perinucleolar region was measured only in small mature lymphocytes that still possessed small but distinct nucleoli (see Busch and Smetana, 1970). In these cells the heterochromatin density in all measured chromatin regions such as in the perinucleolar region, chromocentres, and at the nuclear membrane was very similar (Table 1). The measurement of heterochromatin density in the perinucleolar region in metamyelocytes or late erythroblasts was not possible. Nucleoli in these cells are small and reflect the cessation of the nucleolar biosynthetic activities. The identification of such nucleoli ("micronucleoli") is possible only in specimens stained for RNA or AgNORs (Busch and Smetana, 1970; Smetana, 2002). On the other hand, the heterochromatin density in chromocentres and at the nuclear membrane was not substantially different (Table 1).

Cultured proliferating and apoptotic HL-60 myeloblasts

HL-60 cells representing cultured myeloblasts also exhibited large heterochromatin density in perinucleolar regions and chromocentres, similarly as myeloblasts in the bone marrow (Table 1). On the contrary, when cultured myeloblasts entered the apoptotic process, the heterochromatin density at the nuclear membrane did not differ from that in the perinucleolar region or chromocentres (Table 1). The latter, however, were not frequently seen because of the fusion and/or fragmentation of altered chromatin structures (see Smetana et al., 2000).

Discussion

The presented results clearly indicated that in early differentiation and maturation stages, i.e. in morphologically defined precursor stem cells of granulocytic, lymphocytic and erythrocytic lineages (Astaldi and Lisiewicz, 1971; Bessis, 1972; Cline, 1975), the heterochromatin density in perinucleolar regions and centrally located chromocentres was larger than that at the nuclear membrane. Such difference decreased and disappeared with further differentiation and maturation progress. In advanced differentiation and maturation stages the density of heterochromatin at the nuclear membrane increased and was similar to that in other intranuclear regions. On this occasion it should be noted that these cells are known to lose the ability to proliferate (Astaldi and Lisiewicz, 1971; Bessis, 1972; Cline, 1975). Thus, the chromatin condensation in the heterochromatin around nucleoli and in centrally located chromocentres preceded that at the nuclear membrane. As was expected, the density of heterochromatin at the nuclear membrane also increased in cultured apoptotic HL-60 myeloblasts. Maximal values of the heterochromatin density at the nuclear membrane were noted in

Table 1. Heterochromatin density in the perinucleolar region, chromocentres and at the nuclear membrane of proliferating early and non-proliferating late precursors of lymphocytes, granulocytes and erythroblasts[+]

Perinucleolar region	Chromocentres	Nuclear membrane	Cells	Proliferation
90.3 ± 6.4[a]	**91.1** ± 8.0	**81.8** ± 8.9*	lymphoblasts	+
89.9 ± 3.5	**92.6** ± 2.7	**70.3** ± 5.7*	myeloblasts	+
88.2 ± 9.4	**91.8** ± 7.5	**77.6** ± 11.6*	HL-60 myeloblasts	+
90.6 ± 3,0	**87.0** ± 2.8	**83.5** ± 2.0*	proerythroblasts	+
93.8 ± 2.4	**95.6** ± 4.0	**90.5** ± 6.9	lymphocytes	0
	98.5 ± 3.9	**95.4** ± 3.4	metamyelocytes	0
90.2 ± 3.0		**94.1** ± 11.4	HL-60 myeloblasts-apoptotic	0
	110.6 ± 8.1	**112.5** ± 5.0	late erythroblasts	0

Legend

[+] – based on more than 90 measurements for each group of cells

[a] – **Mean** and standard deviation

***** – Significant difference from perinucleolar region and chromocentres using *t*-test ($P < 0.006$)

terminal maturation stages of the erythroblastic development representing non-proliferating, terminal pre-apoptotic cells just before the loss of the nucleus (Bessis, 1972, Biggiogera et al. 1999; 2004).

The interpretation of the above-described observation is difficult since no similar observations on the heterochromatin density were reported previously. The larger density of the heterochromatin around nucleoli and within the cell nucleus in early committed cell differentiation and maturation stages might reflect the morphological expression of „silencing of some genes“ in these interphase „gene-rich“ nuclear regions (Frenster, 1974; Cerda et al., 1999; Alcobia et al., 2000; Cremer et al., 2000; Zhimulev and Beliaeva, 2003; Bolzer et al., 2005; Picaard and Pontes, 2007).

The increased heterochromatin density at the nuclear membrane in advanced differentiation stages appeared to be characteristic and might be ontogenetically determined similarly as it was suggested previously for intranuclear chromocentres during haematopoiesis (Alcobia et al., 2000). Thus, heterochromatin in this “gene-poor” region (Tanabe et al., 2002; Bolzer et al., 2005) is apparently more condensed later, i.e. in more advanced maturation stages in contrast to other intranuclear heterochromatin regions. In addition, the chromatin condensation at the nuclear periphery of apoptotic cells is also generally known as an accompanying phenomenon of the apoptotic process (see e.g. Saraste, 1999; Martelli et al., 2000; Smetana et al., 2000).

Nevertheless, regardless of the possible interpretation of presented observations, it should be stressed that a larger heterochromatin density in the perinucleolar chromatin and centrally located chromocentres is characteristic for early differentiation and maturation stages of all studied cell lineages. In advanced differentiation and maturation stages and in apoptotic cells with a loss of proliferation ability, the heterochromatin density at the nuclear membrane increased and was similar to that in other nuclear regions.

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