

## ORIGINAL ARTICLE

# The heterochromatin condensation state in central nuclear regions of individual granulocytes

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### Summary

The knowledge on the heterochromatin condensation state (HChCS) in central nuclear “gene rich” regions is limited although the heterochromatin reflects the gene silencing. Therefore, the HChCS in these regions was studied in proliferating granulocytic progenitors and terminally differentiated (mature) neutrophilic granulocytes. The HChCS was measured using computer assisted image optical densitometry at the single cell level. The bone marrow smears of patients with chronic myeloid leukemia represented a convenient model because of the increased number of progenitor and mature cells for density measurements. In addition, the heterochromatin density of central nuclear regions was also measured in myeloblasts of the K 562 cell lineage originated from the patient with that disease. The HChCS in central nuclear regions of both granulocytic progenitors and mature granulocytes was heterogeneous. The maximal and minimal density values were about 9–10 per cent above and below mean values in both myeloblasts and mature granulocytes. The heavy condensed heterochromatin of central nuclear region might reflect the location of silent genes during the whole granulocytic development. In contrary, the less condensed heterochromatin structures in central nuclear regions of granulocytic progenitors and mature granulocytes might just represent foci with the potential for the gene activation regardless of the differentiation and maturation stage. The HChCS in central nuclear regions was larger in small nuclear segments of mature granulocytes than in large nuclei of their progenitors. The HChCS was also increased in apoptotic K 562 myeloblasts with the decreased nuclear diameter. Such phenomena opened an “old – new” question on mutual relationship between the reduced nuclear size and the HChCS.

*Key words:* heterochromatin quantitative densitometry; leukemic granulocytes; central nuclear regions

## INTRODUCTION

It is generally known that in diagnostic cytology and especially in hematology, the morphology of the heterochromatin is a very useful tool for the cell identification including the differentiation and

maturation stages (Naegeli 1931, Undritz 1972, Bessis 1973, Cline 1975). Recent studies also demonstrated differences of the heterochromatin condensation state in central and peripheral nuclear regions. It should be mentioned that in early progenitors of granulocytic and lymphocytic blood cell lineages such as myeloblasts and lymphoblasts the heterochromatin condensation state in central nuclear regions including the perinucleolar region was larger than at the nuclear periphery. In contrary, the heterochromatin condensation state markedly increased mainly in peripheral nuclear regions of the advanced differentiation and maturation stages of these granulocytic and lymphocytic blood cell lineages (Smetana et al. 2011a, b, 2012).

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Since the central nuclear regions are believed to be gene rich (Boyle et al. 2001, Pederson 2004, Cremer and Cremer 2006) and the heterochromatin condensation state might be related to the gene activity (Jost et al. 2012), the present study was undertaken to provide more information on the heterochromatin condensation state in that region. The heterochromatin condensation state is apparent by easy measurements using computer assisted image optical densitometry at the single cell level (Smetana et al. 2011a, b, 2012). The granulocytic progenitors such as myeloblasts and mature neutrophils with segmented nuclei in the bone marrow of patients suffering from the chronic phase of the chronic myeloid leukemia were a very convenient for such measurements because of their increased number in these patients. The panoptic May-Grünwald – Giemsa-Romanowsky (MGGR) staining provided easy cell identification as well as heterochromatin visualization and was also useful for quantitative heterochromatin density measurement in captured digitalized images of studied cells (Smetana et al. 2011b).

As it was expected, the heterochromatin condensation state in central nuclear regions was larger in segmented nuclei of mature granulocytes. However, the heterochromatin condensation state in central nuclear region of both granulocytic progenitors and mature granulocytes was heterogeneous and the maximal and minimal density values (condensation states) were about 9–10 per cent above or below the mean density values. Thus, a possibility exists that the heavy condensed heterochromatin of central nuclear region represents silent gene locations in both leukemic granulocytic progenitors and mature granulocytes, i.e. through the whole development of the granulocytic lineage. In contrary, the less condensed heterochromatin structures in central nuclear regions of granulocytic progenitors as well as mature granulocytes might just represent foci with the increased potential for the gene activation during the whole granulocytic development. On the other hand, the heterochromatin condensation state in central nuclear regions was larger in small nuclear segments of bone marrow mature granulocytes than in their progenitors. The heterochromatin condensation state was also increased in apoptotic K 562 myeloblasts with the decreased nuclear diameter. Such phenomena opened an “old – new” question on mutual relationship between the reduced nuclear size and the heterochromatin condensation state.

## **MATERIAL AND METHODS**

Leukemic myeloblasts and mature granulocytes were studied in bone marrow smears of 3 selected patients suffering from the chronic phase of chronic

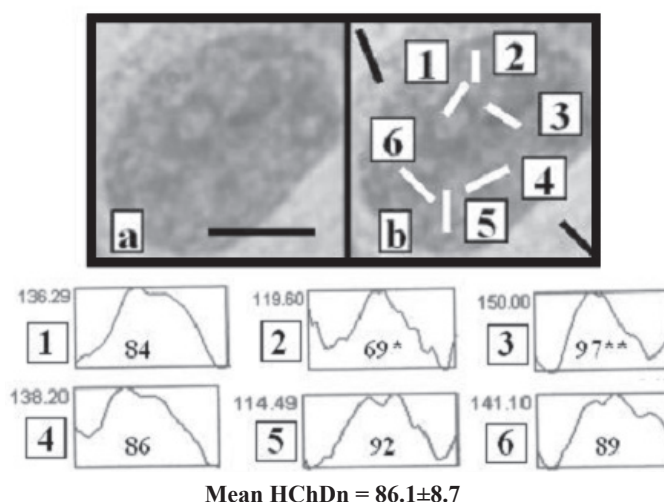
myeloid leukemia without cytostatic treatment at the time of taking samples for the present study. The granulocytic to erythroid ratio in hyperplastic bone marrows of these patients was slightly above that in not-leukemic persons (Rundles 1983). The morphology of studied cells in leukemic patients also did not differ substantially from that in not-leukemic persons (Bessis 1973, Cline 1975). The bone marrow biopsies were taken for diagnostic purposes and the ethics committee of the Institute approved the protocols. Myeloblasts were also studied in proliferating cultured K 562 cell line originated from the same type of myeloid leukemia (see ATCC-LGC, Product informatic sheet, cell lines and hybridomas, London, GB. 2011). Myeloblasts of K 562 cell line were cultured in RPMI 1640 medium with 10% fetal 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 of these cells were prepared using Shandon II cytocentrifuge (UK). Studied cells in bone marrow smears as well as cytopins were visualized by the MGGR panoptic standard staining procedure. That panoptic procedure is very useful for identification of studied cells and may be also used for the chromatin visualization and image optical density measurements as a histo-chemical tool (Naegeli 1931, Undritz 1972, Wittekind 1983, Smetana et al. 2011b).

Micrographs were captured with a Camedia digital photo camera (C4040Z, Olympus, Japan) placed on a Jenalumar microscope (Zeiss, Germany) with a double adapter to increase the magnification of captured images on the computer screen.

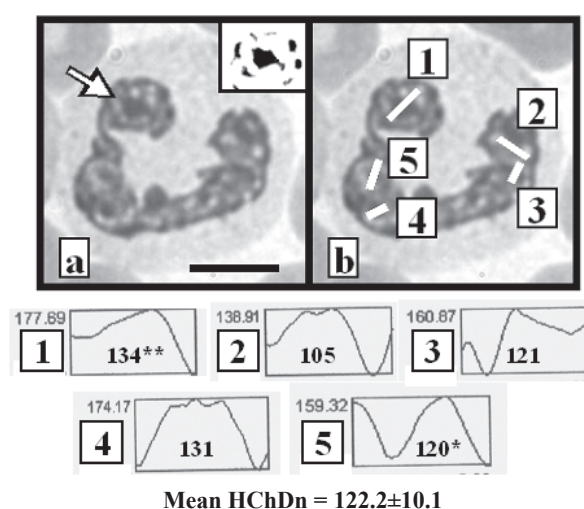
The nuclear image optical density reflecting the heterochromatin condensation state was measured in specimens stained by the MGGR procedure after the conversion of captured colored images to gray scale using the red channel (NIH Image Program, Scion for Windows, Scion Corp., USA; Fig. 1, 2). At this occasion it should be mentioned that the color and density of captured images were not modified by further image processing before optical density measurements. The heterochromatin condensation state was expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from measured heterochromatin density in the central nuclear region. The density values of the background were always determined by measurements of two different locations around the measured cell (not shown). The heterochromatin density was measured in 4–6 different locations of central nuclear regions in each cell (Fig. 1, 2). Such measurements and standardization of arbitrary density units facilitated the comparison of results in monolayers of bone

marrow smears or cytopspins, which frequently exhibited different artificial background densities due to smear or cytopspin preparations. This approach decreased artificial results of density measurements and provided better results than the background adjusted to zero, which depended on the investigator. The basic information on nuclear size and size of nuclear segments was based on their diameter in individual cells. The nuclear diameter in myeloblasts and the diameter of individual segments in mature segmented neutrophilic granulocytes were calculated

from measured long and short axes (diameters) in each cell (Setälä et al. 1997, Tseleni et al. 1997, Politi et al. 2003). At this occasion it should be mentioned that the diameter of inter-segmental bridges were not included to the measurements. The measurements were carried out on captured digital cell images on the computer screen using Quick Photoprogram (Olympus, Japan). The number of nuclear segments per cell was calculated by dividing the number of segments by number of mature neutrophilic granulocytes in which they were counted.



**Fig. 1. Heterochromatin condensation state in a myeloblast.** The black bold bar represents 5 µm (a). White lines and numbers within the micrograph (b) indicate sites of intranuclear measurements. The black lines indicate sites of cytoplasmic and surrounding extracellular measurements (the values are not shown). Numbers at density graphs correspond to numbers of measurement sites within the nucleus. Numbers within density graphs represent arbitrary density units calculated by subtraction of background density around the cell from measured heterochromatin density. Within density graphs at the density numbers one star indicates the minimal and two stars represent the maximal density value. The mean heterochromatin density value with standard deviation calculated from all measurements in the central nuclear region is in the frame at the bottom of the Figure.



**Fig 2. Heterochromatin condensation state in mature segmented granulocyte.** The highly condensed heterochromatin chromocenter marked by a pointer is very distinct after high contrast image processing in the insert (a) and corresponds to largest measured density of the heterochromatin (1) in the Fig. b. For other legend see the previous Figure.

The results of measurements such as mean, standard deviation and t-test (at the significance level  $2\alpha=0.05$ ) were evaluated using “Primer of Biostatistic Program, version 1” developed by S. A. Glantz (McGraw-Hill, Canada, 1968). The mean, maximal or minimal heterochromatin density of central nuclear regions in myeloblasts and mature granulocytes were calculated from mean values of each measured single cell.

## RESULTS

The density of heterochromatin structures in the nuclear central region of myeloblasts appeared to be heterogeneous. However, the heterogeneity of the heterochromatin density in the nuclear central regions was better evident after computer assisted image optical density measurements (Fig. 1, 2, Table 1). In both bone marrow myeloblasts and cultured K 562 myeloblasts the heterogeneity of the heterochromatin condensation state in central regions of myeloblasts was evident by a large difference between maximal and minimal density values. The maximal or minimal density reached about 11 per cent above and below the mean density values.

As it was expected, the heterochromatin condensation state within nuclear segments of leukemic mature granulocytes was significantly

larger than in their progenitors, i.e. myeloblasts and was similar to that in the nuclear periphery (Smetana et al. 2011b). However, similarly as in nuclear central regions of granulocytic progenitors, the heterochromatin heterogeneity within nuclear segments was reflected by the wide range of the heterochromatin density, i.e. between 10 per cent above and 9 per cent below the mean values. At this occasion it should be mentioned that in bone marrow mature granulocytes the heterochromatin with the increased heterochromatin density is present in nuclear segments, which are characterized by much smaller size than that in nuclei of bone marrow granulocytic progenitors – myeloblasts. It should be also noted that the central heterochromatin regions in nuclear segments of mature granulocytes were frequently connected with the heterochromatin at the nuclear periphery that also exhibited a large condensation state (Smetana et al. 2011b).

The heterogeneity of the heterochromatin condensation state in cultured leukemic myeloblasts of the K 562 cell line was similar to that of bone marrow myeloblasts. The heterochromatin density data such as mean, maximal and minimal density values did not show any substantial differences from bone marrow myeloblasts. It should be added that the heterochromatin condensation state increased in apoptotic myeloblasts ( $139.0\pm24.9$  AU) with the reduced nuclear diameter ( $12.3\pm2.3$   $\mu\text{m}$ ) in comparison with not-apoptotic cells (see the Table 1).

Table 1. The heterochromatin image density (condensation state) in nuclear central regions and nuclear diameter of leukemic progenitors and mature segmented granulocytes\*.

| Cells     | Mean HCh density          | Maximal HCh density        | Minimal HCh density       | Nu diameter ( $\mu\text{m}$ )** | NuSg                       |
|-----------|---------------------------|----------------------------|---------------------------|---------------------------------|----------------------------|
| BM Mbl    | $81.6\pm12.5^{\#}$        | $92.0\pm9.8$ (+11%)        | $71.5\pm7.3$ (–11%)       | $11.6\pm2.5$                    | –                          |
| BM S      | $117.7\pm17.2^{\S\Delta}$ | $129.2\pm17.6^{\S}$ (+11%) | $106.8\pm21.7^{\S}$ (–9%) | –                               | $4.3\pm0.5^{\blacksquare}$ |
| K 562 Mbl | $85.5\pm12.8$             | $94.1\pm15.9$ (+11%)       | $78.3\pm14.1$ (–9%)       | $16.5\pm1.6$                    | –                          |
| Apo Mbl   | $139.0\pm24.9$            | $144.4\pm22.0$ (+10%)      | $134.0\pm28.8$ (–10%)     | $12.3\pm2.3$                    | –                          |

\* Based on 200–300 measurements in each group of cells.

\*\* Based on at least 100 measurements in each group of cells.

<sup>#</sup> Mean  $\pm$  S.D.

<sup>§</sup> Statistically significant as compared with BM myeloblasts.

<sup>■</sup> Sum of mean diameters of nuclear segments per cell =  $12.0\pm2.6$   $\mu\text{m}$ . The mean number of nuclear segments per cell was  $2.8\pm0.87$ .

<sup>Δ</sup> This density reached that in the peripheral nuclear region at the nuclear membrane –  $118\pm12.3$  (Smetana et al. 2011b).

BM, bone marrow; Mbl, myeloblasts; S, mature neutrophilic granulocytes; Nu, nuclear; NuSg, nuclear segments; HCh, heterochromatin; Apo, apoptotic.

Numbers in brackets are the percentage difference of maximal and minimal heterochromatin density (condensation state from mean heterochromatin density values, which represent 100 per cent).



## DISCUSSION

The present study provided complementary data on the heterochromatin condensation state in “gene rich” nuclear central regions. It seems to be clear that within the nucleus the heterochromatin condensation state is heterogeneous. However, it should be added that the differences in the heterochromatin condensation state are more apparent using the computer assisted image optical density measurements after visualization by MGGR method.

The computer assisted density measurements indicated that some of heterochromatin structures within central nuclear regions of proliferating leukemic granulocytic progenitors as well as mature granulocytes are in a highly condensed state regardless of the differentiation and maturation stage. Thus a possibility exists that such heterochromatin structures in nuclear central gene rich region carry silencing genes through the whole granulocytic development. Such speculation is supported by previously published studies according to which the increasing chromatin condensation and heterochromatin formation reflect the gene silencing (Frenster 1974, Spector 1993, Zhimulev and Beliaeva 2003, Fakan 2004, Janicki et al. 2004, Grigoryev et al. 2006, Pikaard and Pontes 2007, Finlan et al. 2008, Jost et al. 2012). The periphery of the heavy condensed heterochromatin may prevent the small DNA segment loosening with loop formation and its association with necessary factors for the gene activation process (Pederson 1972, Frenster 1974, Fakan and Puvion 1980, Spector 1993, Fakan 2004, Janicki et al. 2004, Cremer and Cremer 2006, Guillemin et al. 2009). At this occasion it should be also noted that a heavy heterochromatin condensation occurs in the nuclear peripheral regions at the nuclear membrane during the granulocytic differentiation and maturation (Smetana et al. 2011b).

The increased heterochromatin condensation within small nuclear segments of mature granulocytes also deserves a small note. It was interesting that the sum of the diameter of nuclear segments per cell in mature granulocytes was close to the nuclear diameter in granulocytic progenitors – myeloblasts. In addition, the increased heterochromatin condensation was accompanied by a decreased nuclear size in apoptotic cultured K 562 myeloblasts. Such phenomena reopened a question on the mutual participation of the reduced nuclear size and the heterochromatin condensation. They are in harmony with the classical cytology as well as molecular biology according to which “the nuclear size is minimal when most of chromatin is condensed” and “most of non-coding genome in the nucleus is tightly packed in the nucleus” (De Robertis et al. 1970, De Robertis and

De Robertis, Jr. 1987). In addition, the increased chromatin condensation together with the reduction of the nuclear size represents a regular phenomenon of the blood cell differentiation and maturation (Bessis 1973).

The less condensed heterochromatin structures in central nuclear regions might just represent nuclear foci with the increased potential for the gene activation. Numerous studies indicated that the DNA replication or transcription is located adjacent to heterochromatin periphery (Raška et al. 1990, Kurz et al. 1996, Fakan 2004, Guillemin et al. 2009). However, no information exists on the heterochromatin condensation state at the gene activity foci although the central nuclear regions are considered to be gene rich territories (Boyle et al. 2001, Cremer and Cremer 2006). In addition, the positioning of active genes and heterochromatin is still under discussion (Jost et al. 2012). It should be added that the cell return from the resting state to proliferation is accompanied by a heterochromatin loosening that, however, is more distinct in the nuclear peripheral region (Smetana et al. 2007).

At the end of the discussion on the condensed heterochromatin in central gene rich nuclear regions it should be mentioned that the heterochromatin condensation state is easily detectable using image processing or computer assisted image optical densitometry at the single cell level. On the other hand, such methodical approach does not provide further information on the gene activities present in these studied nuclear regions. Similarly, it is also not possible to distinguish exactly the facultative or constitutive heterochromatin (Arrighi 1974, Spector 1993, Grigoryev et al. 2006, Grewal and Songtao 2007, Pichugin et al. 2011) by image optical density measurements. However, such cytochemical procedure at the single cell level might provide useful and complementary information on the heterochromatin in central nuclear regions for studies by other sophisticated methodical approaches including studies of the topography and distribution chromosomal territories (Mayer et al. 2005, Jost et al. 2012). It should be added that it might be also useful for looking after abnormalities of the individual leukemic cell including various differentiation or maturation asynchronies (Bessis 1973, Cremer et al. 2003).

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