Original Article

Morphometric and Densitometric Analysis of Heterochromatin during Cell Differentiation Using the Leukaemic Granulocytic Lineage as a Convenient Model

(nuclear diameter / heterochromatin condensation / leukaemic granulocytes)

K. SMETANA, D. MIKULENKOVÁ, H. KLAMOVÁ

Institute of Haematology and Blood Transfusion, Prague, Czech Republic

Abstract. Granulocytic early progenitors and terminally differentiated – mature granulocytes with segmented nuclei were studied using computer-assisted diameter and heterochromatin optical image densitometry to provide more information on the nuclear size and heterochromatin condensation state. Bone marrow smears of patients suffering from chronic myeloid leukaemia untreated as well as treated with "specific" anti-leukaemic therapy with imatinib mesylate are a convenient model for such study because they possess a satisfactory number of cells for diameter and optical density measurements. In addition, the identification of developmental stages of granulocytes is very easy and the morphology is not different from that in not-leukaemic persons. As it was expected, the mean diameter of nuclear segments in fully differentiated and mature granulocytes was much smaller than that in non-segmented nuclei of early granulocytic precursors. Therefore, no wonder that the heterochromatin condensation state in nuclear segments of mature granulocytes was much larger than in non-segmented nuclei of granulocytic progenitors. On the other hand, the sum of mean diameters of all nuclear segments per cell was close to the mean nuclear diameter of early granulocytic progenitors. The heterochromatin condensation state in granulocytic progenitors or fully differentiated mature granulocytes exhibited marked stability and did not change after the anti-leukaemic therapy. In addition, Barr bodies of characteristic drumstick appearance bearing inactive X chromosome in interphase nuclei of mature granulocytes in fertile female patients exhibited a heterochromatin condensation state similar to nuclear segments. This heterochromatin condensation state was also stable and constant, and was not apparently influenced by the anti-leukaemic therapy.

Introduction

In clinical cytology and particularly in haematology, heterochromatin is a very useful tool for cell identification, including the differentiation and maturation stages. On this occasion it should be mentioned that heterochromatin represents the site of dormant and inactive genes (Cohen and Jia, 2014, Jost et al., 2015). The structural organization of heterochromatin in both mitotic chromosomes and interphase nuclei apparently prevents association of chromatin and DNA filaments with factors necessary for gene activation (DuPraw, 1966; Smetana et al., 2012; Politz et al., 2013).

It is generally known that during the differentiation and maturation of granulocytes, the nuclear chromatin structure appears to be increased. However, such visible increase of the nuclear heterochromatin structure was apparent by enlargement of heterochromatin regions with the maximum in fully mature – terminally differentiated stages (Bessis, 1973). On the other hand, the heterochromatin condensation state during the cell differentiation has been less studied (Smetana et al, 2011). The nuclear size in differentiated stages of granulocytes is known, but the size of nuclear segments in advanced differentiation or fully mature granulocytes in CML patients has also been less studied and remains mostly unknown. Therefore, the present study was undertaken to provide more information on the nuclear size and heterochromatin condensation state of differentiating and terminally differentiated, i.e. fully mature granulocytes. The nuclear size was expressed by the nuclear mean diameter and the heterochromatin condensation state by the density using computer-assisted measurements. The

Received July 22, 2016. Accepted November 2, 2016.

The present study was supported in part by the Ministry of Health of the Czech Republic, Project for the Research Organization Development.

Corresponding author: Karel Smetana, Institute of Haematology and Blood Transfusion, U nemocnice 1, Praha 2, 128 20, Czech Republic. E-mail: karel.Smetana@uhkt.cz. Phone: (+420) 221 977 271; Fax: (+420) 221 977 249.

Abbreviations: CML – chronic myelocytic leukaemia, HChCS – heterochromatin condensation state, MGGR – May-Grünwald-Giemsa-Romanowsky procedure.

bone marrow smears of patients suffering from chronic myeloid leukaemia provided a satisfactory number of granulocytes in all stages of development for the measurements. It should be mentioned that the morphology of these leukaemic cells is similar to that of non-leukaemic persons (Cline, 1975). The May-Grünwald-Giemsa-Romanowsky (MGGR) procedure was very suitable for such study because of the easy cell identification including the differentiation and maturation stages. In addition, this procedure facilitated heterochromatin visualization as a cytochemical method (Wittekind, 1983; Smetana et al., 2011).

As it was expected, the results demonstrated a decreased nuclear size accompanied by the increased heterochromatin condensation state. It seems to be interesting that the summary of mean diameters of nuclear segments in fully mature granulocytes was close to the mean nuclear diameter of myeloblasts representing early developmental precursors – committed stem cell stages. Therefore, no wonder that the heterochromatin condensation state in nuclear segments of mature cells was markedly increased. These developmental phenomena were not influenced by "specific" anti-leukaemic therapy with imatinib mesylate (Glivec, Novartis Pharmaceuticals, Prague, Czech Republic). The heterochromatin condensation state of the Barr body representing inactive X chromosome in women (Davidson and Smith, 1954; Bessis, 1973; Goto and Monk, 1998) appears to be similar to that in other nuclear regions.

Material and Methods

The heterochromatin condensation state (HChCS) was studied in myeloblasts of bone marrow smears of eight female patients in fertile age with the chronic phase of Ph+ chronic myelocytic leukaemia (CML). Four patients were untreated and four treated with the current "specific" anti-leukaemic therapy with imatinib mesylate (Glivec, Novartis Pharmaceutical). The percentage of myeloblasts in bone marrow smears of patients suffering from CML was smaller than 6 % and the incidence of granulocytic cell lineage was markedly larger than that of other nucleated blood cells. All studied patients exhibited the common characteristics of CML such as clinical state and laboratory markers including cytology, genetics, and FACS phenotyping. The studied bone marrow biopsies were originally taken for diagnostic purposes and were approved by the ethics committee and leading authorities of the Institute of Haematology and Blood Transfusion, Prague, Czech Republic.

Bone marrow smears and cytospins were stained by the MGGR standard procedure (Undritz, 1972) and a simple cytochemical method for the demonstration of DNA (Busch and Smetana, 1970). MGGR was used for both bone marrow cell identification and chromatin visualization (Undritz, 1972; Wittenkind, 1983). Heterochromatin was also visualized by a cytochemical method for the demonstration of DNA. In this method, DNA was visualized by methylene blue at pH 5.3 using the McIlvain buffer after HCl hydrolysis, histone and RNA extraction (Busch and Smetana, 1970). On this occasion it should be added that no differences in heterochromatin density and size measurements were noted after comparison of both these visualization procedures (Smetana et al., 2011).

Micrographs of the studied cells were captured with a Camedia digital camera C4040 ZOOM (Olympus, Tokyo, Japan) placed on a Jenalumar microscope (Zeiss, Jena, Germany) with double adapter. The double adapter increased the magnification for diameter and density measurements at the single cell level on the computer screen. The mean diameter of the cell nucleus and nuclear segments was calculated from the measured large and small axis in each single cell using the Quick Photo Program (Olympus).

The heterochromatin image optical density was measured in original captured images of single cells after conversion of the captured colour signals to grey scale using the red channel of the NIH Image Program – Scion for Windows (Scion Corp., Austin, TX). The heterochromatin density reflecting the condensation state was measured in three central and three peripheral nuclear regions of single myeloblasts and single nuclear segments in fully differentiated mature neutrophil granulocytes. The results of density measurements were expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from the mean heterochromatin density of central and peripheral nuclear regions (Smetana et al., 2011).

The results of all measurements at the single cell level such as the mean, standard deviation and significance were evaluated using Primer of Biostatistic Program, version 1 developed by S.A. Glantz (McGraw-Hill, Canada, 1968).

Results

Quantitative data see in Table 1.

It seems to be clear that the mean nuclear diameter in early differentiation stages of granulocytic precursors myeloblasts is larger than in nuclear segments of terminal differentiation and maturation stages (Figs. 1, 2). As it was expected, the smallest mean diameter was found in nuclear segments of fully mature – terminally differentiated granulocytes (Fig. 2). However, the sum of mean diameters of all nuclear segments present per cell in these cells was close to the mean nuclear diameter in early granulocytic precursors – myeloblasts. On this occasion it should be added that mature – terminally differentiated cells mostly possessed 2–3 nuclear segments similarly as "normal" mature granulocytes. It was also interesting that no substantial differences were noted between untreated patients and patients treated with anti-leukaemic therapy.

In addition to the known enlargement of heterochromatin regions, the mean heterochromatin condensation

*Table 1. Nuclear diameter and heterochromatin condensation state in myeloblasts and terminally differentiated – mature leukaemic granulocytes of untreated patients (Th 0) and patients treated (Th +) with anti-leukaemic therapy**

Developmental cell stage	Nucleus Dm§	Nu Se Dm§	Nu Se Dm sum§	HChCS§§
Myeloblasts				
Th 0	12.4 ± 0.4	–	–	62.4 ± 12.4
Th +	12.5 ± 0.3	–	–	73.6 ± 4.1
Mature cells with Nu Se				
Th 0	–	4.1 ± 0.2	12.3 ± 0.5	99.2 ± 5.5#
Th +	–	4.0 ± 0.1	11.9 ± 0.7	98.2 ± 13.1#

Legend: * – mean and standard deviation based on the measured nuclear diameter and heterochromatin condensation state in myeloblasts (> 400 measurements) and mature segmented granulocytes (> 900 measurements) of untreated patients or patients treated with anti-leukaemic therapy. § – µm, §§ – AU (arbitrary units, see Smetana et al., 2011), # – statistically different from myeloblasts using *t*-test ($2\alpha = 0.05$).
Dm – diameter, Nu Se – nuclear segments, ChCS – heterochromatin condensation state

state during the differentiation of granulocytic precursors was increased. The largest heterochromatin condensation state was noted in nuclear segments in fully differentiated and mature developmental stages of leukaemic granulocytes. Similarly as the nuclear mean diameter of early and advanced or terminal developmental stages of granulocytes, no substantial differences in the heterochromatin condensation state were noted between granulocytes of untreated patients and patients treated with the anti-leukaemic therapy.

As it was expected, the Barr bodies in female fertile patients representing inactive X chromatin were very distinct and in differentiated mature granulocytes appeared as drumsticks (Davidson and Smith, 1954; Bessis, 1973; Goto and Monk, 1998; Fig. 2). The mean diameter of a Barr body (~200 measurements) was similar in both untreated patients (1.12 ± 0.2 µm) and patients treated with the anti-leukaemic therapy (1.18 ± 0.2 µm). The heterochromatin condensation state (~100 measurements) was also similar to that in other nuclear regions, i.e. 105 ± 25 and 110 ± 8.9 AU in untreated and treated

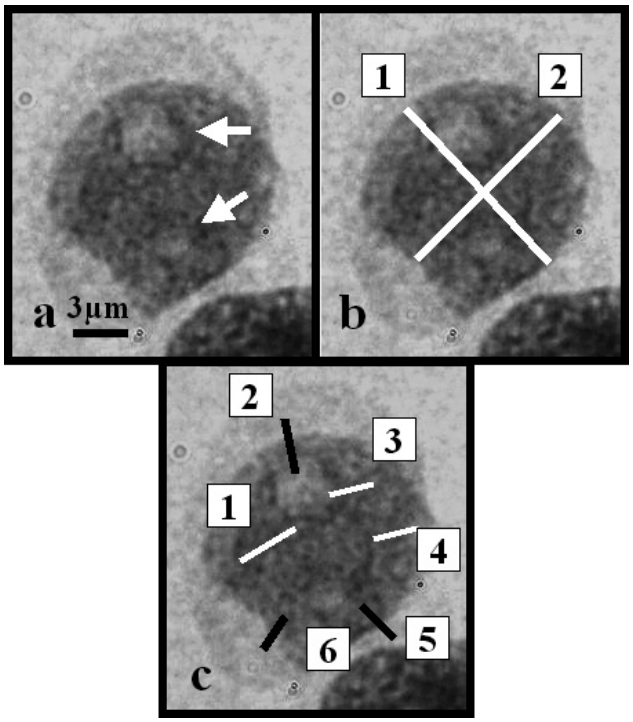

Fig. 1. Myeloblast. **a.** Original captured myeloblast with more or less distinct nucleoli (white pointers). The bold thick line represents 3 µm. **b.** White lines represent the sites of measurements: the calculated mean nuclear diameter in µm: [11.8 (line 1) + 11.5 (line 2)] : 2 = 11.6. **c.** Short white lines indicate the density measurements in central nuclear regions and black lines represent the density measurements in the nuclear periphery. The calculated mean nuclear heterochromatin condensation state in arbitrary density units is as follows: [62.5 (line 1) + 49.8 (line 2) + 63.1 (line 3) + 67.1 (line 4) + 63.0 (line 5) + 57.5 (line 6)] : 6 = 60.5

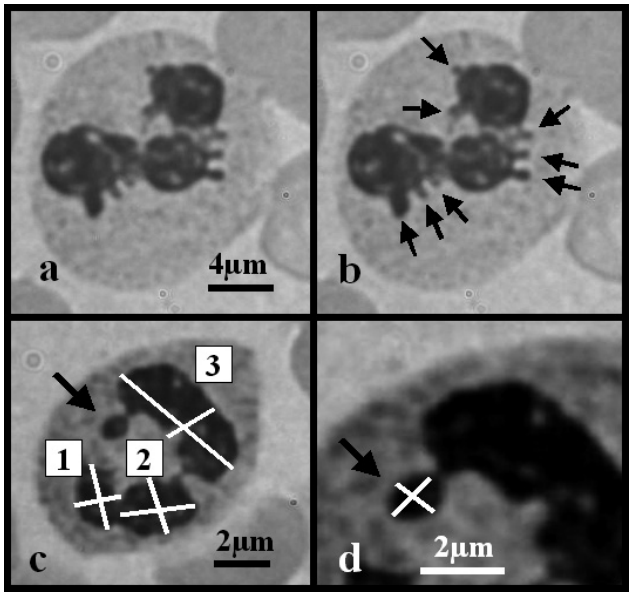

Fig. 2. Differentiated mature granulocytes. **a.** Original captured cell with nuclear segments that exhibit numerous appendages. **b.** Marked appendages – black pointers. **c.** Barr body – black pointer. White lines represent diameter measurements of each nuclear segment. The sum of measured mean diameters of each segment in µm is as follows: 2.7 (segment 1) + 3.4 (segment 2) + 4.9 (segment 3) = 11.0. **d.** Larger magnification of the Barr body represented by the drumstick. The mean diameter of the "body on the stick" was calculated from the measurement of the long and short axes: [1.7 + 1.8] : 2 = 1.75 µm.

patients. On the other hand, it should be noted that some nuclear appendages also resembled the Barr bodies. However, the number of these nuclear appendages in the nucleus was usually larger (Fig. 2). They were considered to be possibly "karyoschizes" and might reflect nuclear degenerative processes (Bessis, 1973).

Discussion

It is generally known that during the cell differentiation and maturation, heterochromatin regions are usually enlarged and the nuclear size is smaller (Bessis, 1973). According to previous reports and present results such changes are accompanied by an increased heterochromatin condensation state in both central and peripheral nuclear regions (Smetana et al., 2011). Since the structural organization of heterochromatin prevents gene activation, the increased heterochromatin condensation state might contribute to the known decreased accessibility of factors necessary for the gene activation process (Politz et al., 2013; Cohen and Jia, 2014). In addition, according to classical cytology, the nuclear size is minimal when most of chromatin is condensed (DeRobertis et al., 1970). Moreover, the terminal differentiated stage is stable (DeRobertis and DeRobertis, 1987). The developmental nuclear changes in the leukaemic granulocyte cell lineage were in perfect harmony with these classica1 conclusive remarks.

Early differentiation granulocyte precursors are characterized by a larger nuclear diameter and smaller heterochromatin condensation state (Smetana et al., 2011). In contrast, the fully differentiated and mature granulocytes are characterized by the presence of small nuclear segments and markedly increased heterochromatin condensation. On the other hand, the mean diameter sum of these small nuclear segments in fully differentiated granulocytes was close to the large nuclear mean diameter in granulocyte precursors (present results). Thus, the enlarged heterochromatin regions accompanied by the increased heterochromatin condensation state just reflect the gene inactivation during the differentiation and maturation process in the studied leukaemic granulocyte lineage (Alcobia et al., 2000; Guillemin and Francastel, 2010).

The similarity of the nuclear mean diameter in granulocyte early progenitors with the mean diameter sum of nuclear segments of differentiated and mature granulocytes might be related to the known chromatin and DNA content stability (Busch, 1974). However, it is accompanied by structural condensation changes during the differentiation and maturation (Smetana et al., 2011). In addition, the increased heterochromatin condensation changes appear to be constant and stable because they did not change even after the "specific" anti-leukaemic therapy with imatinib mesylate. Such conclusion is also in harmony with heterochromatin involvement in gene silencing and maintenance of the cell genome stability (Politz et al., 2016).

The Barr body in nuclear segments of differentiated mature granulocytes is actually the heterochromatin that is characteristic of women carrying the inactive X chromosome (Davidson and Smith, 1954; Bessis, 1973; Goto and Monk, 1998). Thus it seems to be natural that the heterochromatin condensation state of the Barr body in female CML patients was similar to that with silent genes in other nuclear regions. Similarly, the Barr body was also stable and not influenced by the anti-leukaemic therapy.

Acknowledgement

The authors would like to express their gratitude to the physicians and technicians of the Institute for Haematology and Blood Transfusion for their continuous support of the present study.

Discloser of conflict of interest

The authors have declared that no competing interests exist.

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