Original Article

# Heterochromatin Density (Condensation) During Cell Differentiation and Maturation Using the Human Granulocytic Lineage of Chronic Myeloid Leukaemia as a Convenient Model

(central and peripheral heterochromatin density *in situ* / cell differentiation and maturation / human leukemic granulocytic lineage)

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**Abstract. The present study was undertaken to provide complementary data on the heterochromatin condensation in both central and peripheral nuclear regions during the cell differentiation and maturation using computer-assisted density measurements at the single-cell level. The lineage of neutrophilic granulocytes in the bone marrow of patients suffering from chronic myeloid leukaemia was very convenient for such study because the increased number of granulocytes in all developmental stages was satisfactory for heterochromatin density measurements. The morphology of leukaemic and non-leukaemic neutrophilic granulocytes is similar and each differentiation or maturation stage is easily identified. A markedly increasing heterochromatin density – condensation – in the peripheral nuclear region at the nuclear envelope accompanied both the differentiation and maturation of these cells. Thus, peripheral chromosomal territories at the nuclear envelope are important for both the differentiation and maturation process. In contrast, the heterochromatin density of nuclear central regions was already high in early differentiation stages and exhibited a less distinct increase during the differentiation, but was more apparent in late maturation stages representing the terminal differentiation. A limited number of maturing cells with persisting large heterochromatin density in central nuclear regions without markedly increased heterochromatin condensation at the nuclear periphery might represent a further maturation abnormality – asynchrony – during the granulocytic development. From the methodological point of view, both, the cytochemical method for the DNA demonstration and the panoptic May-Grünwald – Giemsa staining, are convenient for computer-assisted chromatin densitometry at the single-cell level.**

## Introduction

Nuclear heterochromatin regions are very characteristic for each cell type with full respect to its differentiation and maturation state. Similarly as in other blood cell lineages, early differentiation stages of neutrophilic granulocytes are characterized by a very fine nuclear chromatin structure that is more condensed in advanced and terminal steps of the differentiation process (Bessis, 1973). Recent studies also indicated that central nuclear regions appeared to be gene-rich in contrast to the gene-poor nuclear periphery at the nuclear envelope (Boyle et al., 2001). On the other hand, the information on the heterochromatin condensation in central or peripheral nuclear regions in differentiating cells, including neutrophilic granulocytes, is still very limited. Thus, the present study was undertaken to provide complementary data on the heterochromatin condensation in these nuclear regions using computer-assisted heterochromatin density measurements at the single-cell level. The lineage of neutrophilic granulocytes in the bone marrow of patients suffering from the chronic phase of chronic myeloid leukaemia was very convenient for such study because the increased number of granulocytes in all developmental stages was satisfactory for the present study (Bessis, 1973; Cline, 1975). Moreover, it is known that in that disease the morphology of leukaemic and non-leukaemic granulocytes is similar (Cline, 1975). In addition, the May-Grünwald – Giemsa-Romanowsky (MGGR) panoptic staining procedure used for the identification of granulocytic differentiation or maturation

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Abbreviation: MGGR – May-Grünwald – Giemsa-Romanowsky.

stages is also very convenient for the chromatin visualization as a useful histochemical tool (Naegeli, 1931; Undritz, 1972; Wittekind, 1983).

The results of the present study clearly indicated that the differentiation of neutrophilic granulocytes is accompanied by a markedly increasing heterochromatin density, i.e. condensation in the peripheral nuclear region at the nuclear envelope. It should be mentioned that the peripheral heterochromatin at the nuclear envelope reached a similar density to central nuclear regions during terminal differentiation, i.e. in mature stages of granulocytes. A limited incidence of these cells in advanced and terminal differentiation stages without the increased heterochromatin condensation apparently represented a further maturation abnormality – asynchrony. In contrast, the heterochromatin condensation in nuclear central regions was less markedly increased during the differentiation process and was more apparent in advanced maturation stages reflecting the terminal differentiation.

## Material and Methods

The neutrophilic granulopoietic lineage was studied in bone marrow smears of three selected patients suffering from the chronic phase of chronic myeloid leukaemia without cytostatic treatment at the time of taking samples for the present study. The increased incidence of neutrophilic granulocytes provided a sufficient number of all differentiation stages for chromatin and DNA density measurements. The granulocytic to erythroid ratio in hyperplasic bone marrows of these patients was slightly above that in non-leukaemic persons (Rundles, 1983) and the morphology of the studied cells did not differ substantially from that in non-leukaemic persons (Bessis, 1973; Cline, 1975). The bone marrow biopsies were taken for diagnostic purposes and the ethics committee of the Institute approved the protocols.

Chromatin in differentiation or maturation stages of the studied cells was visualized by the MGGR panoptic standard staining procedure (Naegeli,, 1931; Undritz, 1972; Wittekind, 1983) and a simple, but sensitive cytochemical method for DNA demonstration using methylene blue at pH 5.3 after HCl hydrolysis (Smetana et al., 1967; Busch and Smetana, 1970). On this occasion it should be mentioned that the panoptic procedure is very useful for identification of all differentiation and maturation stages of the studied cell lineage (Undritz, 1972).

Micrographs were captured with a Camedia digital photo camera (C4040Z, Olympus, Tokyo, Japan) placed on a Jenalumar microscope (Zeiss, Germany) with a double adapter to increase the magnification of captured images on the computer screen. The gradually increased contrast in specimens stained for DNA was achieved by image processing of captured images to see the differences between heterochromatin condensation in central and peripheral nuclear regions (Figs. 1–6).

The nuclear density reflecting the heterochromatin condensation was measured in specimens stained by the MGGR procedure after the conversion of captured coloured images to gray scale using the red channel (NIH Image Program, Scion for Windows, Scion Corp., Frederick, MD), (Figs. 7–8, Table 1). The control measurements were also carried out on specimens stained for DNA (Table 1). The heterochromatin condensation was expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from measured mean heterochromatin density in the central or peripheral nuclear regions. The density values of the background were always determined by measurements of two different locations around the measured cell. The heterochromatin density was measured in two different locations of central or peripheral nuclear regions in each cell. Such measurements and standardization of arbitrary density units facilitated the comparison of results in monolayers of bone marrow smears, which frequently exhibited different artificial background densities due to smear preparation. This approach decreased artificial results of density measurements and provided better results than the background adjusted to zero, which depended on the investigator. The results of measurements of each cell such as mean, standard deviation and significance were evaluated using “Primer of Biostatistic Program, version 1” developed by S. A. Glantz (McGraw-Hill, Canada, 1968). The mean of central to peripheral density ratios for each granulocytic differentiation stage was calculated from mean values of nuclear central and peripheral heterochromatin densities of single cells.

## Results

### *High contrast and bleaching processing of digital images of neutrophilic granulocytes*

In specimens stained for DNA, the high contrast and bleaching processing of digital images of early differentiation stages demonstrated a larger density of the heterochromatin in central nuclear regions such as perinucleolar and extranucleolar chromatin (Figs. 1, 2). This heterochromatin was still clearly visible in contrast to less dense heterochromatin in the nuclear periphery at the nuclear envelope. Such difference between heterochromatin in central and peripheral nuclear regions gradually disappeared in advanced and terminal differentiation – maturation – stages (Figs. 3, 4). However, some of the advanced differentiation stages, such as metamyelocytes, band and segmented forms, still exhibited more condensed heterochromatin in the central nuclear region (Figs. 5, 6). The diameter of heterochromatin areas in both central and peripheral regions in maturing cells appeared to be slightly larger (1.14 μm) than in early differentiation stages (1.08 μm). However, no substantial differences were noted between central and peripheral nuclear regions. It should also be mentioned that it was frequently difficult to distinguish heterochromatin areas of central and peripheral nuclear re-

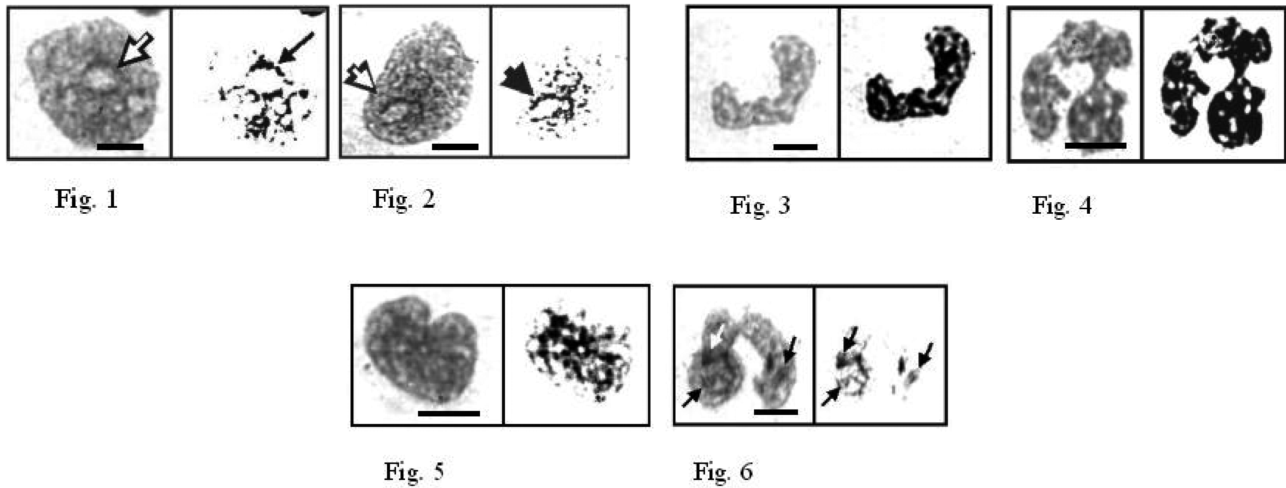


*Fig. 1.* Early differentiation stage – myeloblast. Note that the highly condensed chromatin in the central nuclear region is still visible after high-contrast processing. The perinucleolar chromatin (arrow) facilitates orientation after high-contrast processing.
DNA staining. The thick black bar represents 5 µm.
*Fig. 2.* Early differentiation stage – promyelocyte. The highly condensed chromatin in the central nuclear region is still visible after high-contrast processing. The perinucleolar chromatin (arrow) facilitates orientation after high-contrast processing.
DNA staining. The thick black bar represents 5 µm.
*Fig. 3.* Advanced differentiation (maturation) stage – band form. Note that the heterochromatin within the nucleus and at the nuclear envelope exhibits the same density and is clearly visible after high-contrast processing.
DNA staining. The thick black bar represents 5 µm.
*Fig. 4.* Terminal differentiation and maturation stage – segmented granulocyte. Note that the heterochromatin within the nucleus and at the nuclear envelope exhibits the same density and is clearly visible after high-contrast processing.
DNA staining. The thick black bar represents 5 µm.
*Fig. 5.* Advanced differentiation stage – metamyelocyte. Note that the heterochromatin in the central nuclear region after high-contrast processing is still visible similarly as in early differentiation stages.
The thick black bar represents 5 µm.
*Fig. 6.* Terminal differentiation (maturation) stage – segmented granulocyte. The heterochromatin in the central nuclear region after high-contrast processing is still visible similarly as in early differentiation stages. The highly condensed chromatin (arrows) facilitates orientation after high-contrast processing.
The thick black bar represents 5 µm.

*Table 1. The heterochromatin density in central and peripheral nuclear regions measured in specimens stained with the MGGR procedure⁺*

| Cells | Central Nuclear Regions | Peripheral Nuclear Regions | Central/Peripheral Density Ratio | Central/Peripheral Density Ratio (DNA)⁺⁺ | DIV | ST |
|---|---|---|---|---|---|---|
| Mybl | 70.3 ± 11.5 | 55.3 ± 10.4§ | 1.28 ± 0.04 | 1.19 ± 0.11 | + | D |
| Promyelo | 83.9 ± 18.5* | 69.6 ± 15.9*§ | 1.20 ± 0.01* | 1.13 ± 0.05* | + | D |
| Myelo | 84.5 ± 13.8* | 70.0 ± 12.4*§ | 1.21 ± 0.04* | 1.27 ± 0.15* | + | D+M |
| Meta | 84.7 ± 13.8* | 82.0 ± 12.8** | 1.01 ± 0.02** | 1.02 ± 0.11** | 0 | M |
| Bands | 100.6 ± 16.8□ | 107.6 ±11.0□ | 0.93 ± 0.06□ | 1.04 ± 0.06** | 0 | M |
| Segm | 110.3 ± 11.1□ | 118.0 ± 12.3□ | 0.93 ± 0.14□ | 1.00 ± 0.01** | 0 | M |

⁺ - Mean and standard deviation based on 60 to 100 measurements for each differentiation and maturation stage in 3 patients
⁺⁺ - Based on 20 to 60 control measurements in DNA-stained specimens for each differentiation and maturation stage
* - Significantly different from myeloblasts using *t*-test ($P < 0.001$)
** - Significantly different from myeloblasts, promyelocytes and myelocytes using *t*-test ($P < 0.001$)
□ - Significantly different from myeloblasts, promyelocytes, myelocytes and metamyelocytes using *t*-test ($P < 0.001$)
§ - Significantly different from central nuclear regions using *t*-test ($P < 0.001$)
Mybl – myeloblasts, Promyelo – promyelocytes, Myelo – myelocytes, Meta – metamyelocytes, Bands – mature neutrophils without segmented nucleus, Segm – mature neutrophils with segmented nuclei, Div – division ability, ST – state, D – differentiation, M – maturation

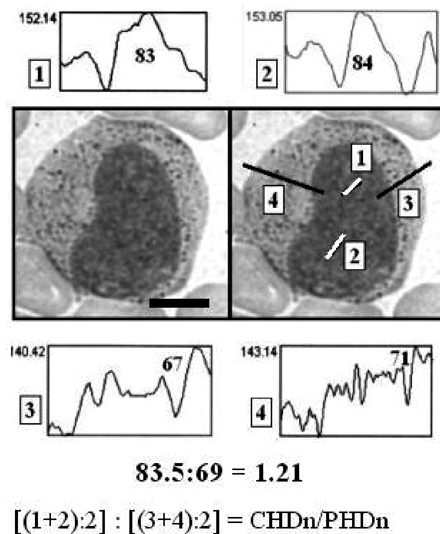


*Fig. 7.* Early differentiation stage – promyelocyte stained by the MGGR procedure. Thin lines and numbers within micrographs indicate sites of measurements. Numbers within density graphs represent calculated arbitrary density units based on measured values at the upper corner and density surrounding the cell. Numbers at the density graphs correspond to numbers of measurement lines. The calculation of mean density values of central to peripheral nuclear heterochromatin ratio is at the bottom of the Figure. The numbers in the calculation formula correspond to the numbers of the measurement lines in micrographs. CHDn – central heterochromatin density, PHDn – peripheral heterochromatin density.
The thick bar represents 0.5 μm.

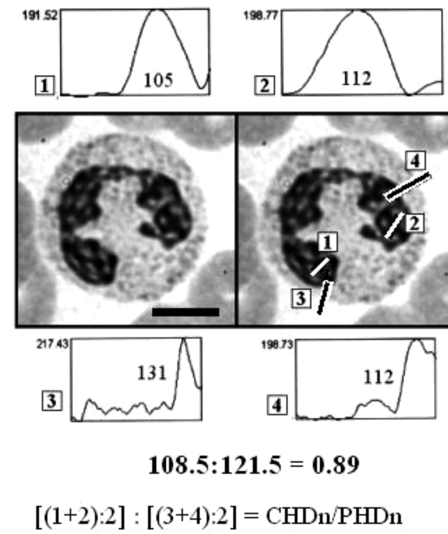


*Fig. 8.* Terminal differentiation (maturation) stage – segmented granulocyte stained by the MGGR procedure. Thin lines and numbers within micrographs indicate sites of measurements. Numbers within density graphs represent calculated arbitrary density units based on measured values at the upper corner and density surrounding the cell. Numbers at the density graphs correspond to numbers of measurement lines. The calculation of mean density values of central to peripheral nuclear heterochromatin ratio is at the bottom of the Figure. The numbers in the calculation formula correspond to the numbers of the measurement lines in micrographs. CHDn – central heterochromatin density, PHDn – peripheral heterochromatin density.
The thick bar represents 0.5 μm.

gions in advanced or terminal differentiation steps of the granulocytic development (Figs. 3, 4).

### *Computer-assisted heterochromatin density measurements in neutrophilic granulocytes*

In specimens stained with the MGGR procedure the heterochromatin density in central nuclear regions of early differentiation stages of the proliferating pool such as myeloblasts, promyelocytes and myelocytes was larger than in nuclear periphery at the nuclear envelope (Fig. 7, Table 1). Such difference usually disappeared in the maturing pool, i.e. during the terminal differentiation of neutrophilic granulocytes such as metamyelocytes, bands and mature cells with segmented nuclei (Fig. 8, Table 1). These cells were characterized by the increased heterochromatin density in the peripheral region of the nucleus at the nuclear envelope and reached similar density values to heterochromatin in nuclear central regions. Such change was reflected by the decreased ratio of the central to peripheral heterochromatin density (Table 1). On the other hand, the heterochromatin density in central nucleolar regions also increased in terminal stages of the studied cell lineage (Table 1).

Similarly decreased density ratio of the central to peripheral heterochromatin density was also noted in control specimens stained for DNA (Table 1). However, the differences in the measured values were less distinct because of the difficulties to precisely distinguish all differentiation and early maturation stages of the measured cells. The variation coefficient ranged between 4 and 11 % in comparison with 2 and 3 % in specimens stained by the MGGR procedure; see also larger standard deviations after DNA staining (Table 1). Nevertheless, the difference between early differentiation and more advanced stages was highly significant. Such difference reflected again the markedly increased heterochromatin density in nuclear peripheral regions similarly as in specimens stained with the MGGR procedure (Table 1). On the other hand, on this occasion it should be mentioned that in some maturing cells (20.5 ± 6.2) the chromatin density was still larger in central nuclear regions and possibly reflected a further nuclear maturation asynchrony.

## Discussion

According to the present study it seems to be clear that the differentiation and maturation process of granulocytes is accompanied by a markedly increased heterochromatin condensation in the peripheral nuclear regions, as documented by both computer-assisted density

measurements and image processing. It is interesting that heavy heterochromatin condensation in central nuclear regions was observed already in early differentiation stages of the granulocytic lineage in contrast to the nuclear periphery. The interpretation of such observations is very difficult from the point of view of gene-rich chromosomal territories in central nuclear regions (Boyle et al., 2001; Cremer and Cremer, 2006). However, the replication or transcription activity is mostly located in the perichromatin region at the heterochromatin periphery (Fakan and Puvion, 1980; Raška et al., 1990; Kurz et al., 1996; Fakan, 2004) and is expressed earlier in the central nuclear regions than in the nuclear periphery (Cremer and Cremer, 2006). On the other hand, there is also a possibility that active genes involved in the differentiation process are present rather in peripheral chromosomal territories represented by less condensed heterochromatin at the nuclear envelope in early differentiation stages. On this occasion it should be mentioned that the peripheral nuclear regions – chromosomal territories – are considered to be gene-poor, but might still be important with respect to the gene activity such as for DNA replication as well as RNA transcription (Bouteille et al., 1974; Cremer and Cremer, 2006; Finlan et al., 2008). In addition, the opposite phenomenon, i.e. the decreased heterochromatin density in the nuclear peripheral region, was observed after the transformation of resting to proliferating cells which returned to the cell cycle (Smetana et al., 2007). Moreover, the replication activity has also been noted in the nuclear periphery at the nuclear envelope in proliferating cells (Bouteille et al., 1974).

The peripheral nuclear heterochromatin reached similar density to that in central nuclear regions during the terminal differentiation process, i.e. in maturing or mature cells that lost the ability to divide. Thus, the increasing heterochromatin condensation in both nuclear central and peripheral regions might be related to the terminal differentiation of the granulocytic cell lineage. Such chromatin condensation in granulocytic progenitors is also produced by the induced apoptotic process (Smetana et al., 2000). In addition, the increased heterochromatin condensation in the nuclear central and peripheral regions during the terminal differentiation of the studied maturing cells might be related to gene silencing. This possibility is supported by the classical haematological cytology according to which the chromatin condensation is characteristic for the cell maturation, i.e. terminal differentiation, and loss of the division ability (Bessis, 1973). In addition, the increased heterochromatin condensation seems to be related to the cessation of both DNA replication and RNA transcription (Frenster, 1974; Fakan, 2004; Janicki et al., 2004). The heavy heterochromatin condensation might prevent both small DNA segment loosening or loop formation at its periphery for the replication or transcription process (Fakan, and Puvion, 1980; Fakan, 2004; Cremer and Cremer, 2006).

The present study also demonstrated that the heterochromatin condensation in the nuclear periphery of a limited percentage of advanced terminal maturation stages was still smaller than in nuclear central regions similarly as in less differentiated cells. Such phenomenon apparently indicated a delay in the peripheral heterochromatin condensation and might represent a further maturation abnormality that was not reported in previous studies. It should be added that various terminal differentiation and maturation abnormalities in leukaemic granulocytes are generally known as maturation asynchrony (Bessis, 1973).

From the methodological point of view it seems to be interesting that the calculated central to peripheral heterochromatin density ratio based on heterochromatin density measurements of specimens stained with panoptic and cytochemical procedures did not show substantial differences. On the other hand, MGGR staining facilitated more precise determination of the differentiation or maturation stage of the evaluated cells. In addition, the MGGR staining seems to be also very useful from the cytochemical point of view (Undritz, 1972; Wittekind, 1983).

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