Heterochromatin condensation in central and peripheral nuclear regions of maturing lymphocytes in the peripheral blood of patients suffering from B chronic lymphocytic leukemia – a cytochemical study

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The present study was undertaken to provide complementary information on heterochromatin condensation in central and peripheral nuclear regions during maturation of human leukemic lymphocytes using simple image processing and DNA image densitometry at the single cell level. Such approach indicated that the heterochromatin condensation in perinucleolar and extranucleolar "gene rich" central nucleolar regions preceded that in the "gene poor" nuclear periphery at the nuclear membrane. Thus, the maturation of lymphocytes was accompanied by a marked increase of the heterochromatin condensation at the nuclear membrane that reflected the maturity of these cells. In addition, in contrary to the nuclear size, no substantial differences of the heterochromatin condensation in central and peripheral nuclear regions were noted between untreated and treated patients with cytostatic therapy at the time of taking samples for the present study. On the other hand, the larger heterochromatin condensation in central nuclear regions occasionally persisted in small mature lymphocytes of all studied patients. Such phenomenon might represent the return to the cell cycle or a further type of maturation asynchrony that in leukemic cells is not exceptional.

Keywords: heterochromatin, central and peripheral nuclear regions, maturing human leukemic lymphocytes

It is generally known that the maturation of blood cells is characterized by an increasing chromatin condensation and a decreasing nuclear size [1]. Moreover, according to numerous studies it seems to be apparent that condensed chromatin – heterochromatin – is a morphological manifestation of genetic silencing and terminal differentiation [2, 3, 4]. However, the information on the heterochromatin condensation state in central and peripheral nuclear regions in maturing cells is very limited or missing [5 6]. It should be mentioned that central nuclear regions possess gene rich chromosomal territories and gene poor chromosomal territories are in the nuclear periphery at the nuclear membrane [2, 7, 8, 9]. The present study was undertaken to provide complementary information on the heterochromatin in various nuclear regions of human leukemic lymphocytes during their maturation. These cells represent a very convenient model for such study because they are present in a satisfactory number in the peripheral blood of patients suffering from B-cell chronic lymphocytic leukemia. In addition, the morphology of lymphocytes is well known and immature proliferating and mature resting or terminal stages of these cells are easily identified [1, 10, 11]. The chromatin structure was visualized using a simple cytochemical procedure for DNA and the state of the heterochromatin condensation was determined by computer assisted image densitometry *in situ* at the single cell level.

The results clearly indicated that the maturation of lymphocytes was accompanied by a marked increase of the heterochromatin density – condensation – in nuclear peripheral regions at the nuclear membrane. Thus, the increased heterochromatin chromatin condensation in nuclear peripheral regions reflected the cell maturity and preceded the pre-apoptotic state. It should be also mentioned that no substantial differences were noted in the heterochromatin condensation in maturing lymphocytes of leukemic patients who were untreated and treated by the cytostatic therapy at the time of taking samples for the present study. On the contrary, the mean nuclear diameter of both immature and mature lymphocytes was apparently smaller in patients treated with cytostatic therapy.

Materials and methods

Nuclear heterochromatin was studied in immature and mature lymphocytes of the peripheral venous blood of 8 patients suffering from B – chronic lymphocytic leukemia without any respect to the clinical state and stage of the disease. 4 patients were untreated and 4 patients were treated with current cytostatic therapy [12] at the time of taking peripheral blood samples for the present study. The median of the lymphocytic count in studied patients was 32.3×10^9/L including 12 to 28 per cent of immature lymphocytes and provided a satisfactory number of both immature and mature cells for heterochromatin density and diameter measurements. Peripheral venous blood samples of studied patients were taken for diagnostic purposes and the ethics committee of the Institute approved the protocols for the present study.

Nuclear heterochromatin in lymphocytes was visualized in fixed peripheral blood smears by a simple but sensitive method for DNA using methylene blue buffered with McIlvain´s buffer to pH 5.3 after hydrolysis with 1N HCl [13, 14].

Micrographs were captured with a Camedia digital photo camera C.4040 ZOOM (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany). The double adapter on the microscope increased the magnification of captured images on the computer screen. Increased contrast of stained nuclear bodies by image processing facilitated easy measurements of the nucleolar diameter using Quick Photoprogram (Olympus, Japan) to provide basic information on the nuclear size. The nuclear mean diameter was determined by measurement of large and short axes [see 15, 16] in each of both immature and mature lymphocytes in peripheral blood smears of all studied leukemic patients and 8 not-leukemic persons.

The heterochromatin image density was measured after the conversion of captured colored images (predominantly blue signals) to gray scale using the red channel (NIH Image Program, Scion for Windows, Scion Corp., USA). The heterochromatin image density reflecting its concentration in central and peripheral nuclear regions was expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from the heterochromatin density of central and peripheral nuclear regions. Central nuclear heterochromatin regions were represented by both perinucleolar and extranucleolar heterochromatin. The nuclear heterochromatin and background mean densities were calculated from 2 – 4 values measured in each of nuclear regions and extracellular background around each cell. Such calculations and standardization of arbitrary density units facilitated the comparison of results in monolayers of peripheral blood smears, which usually exhibited different artificial background densities due to smear preparations from the peripheral blood of various patients. This approach provided better and more reliable results than previous ones [6] based on the measured maximal heterochromatin perinucleolar density or background adjustments to zero by the investigator. Central to peripheral heterochromatin image density ratio

for each studied cell was determined according to following formula: [(PeriNoMHDn + ExtraNoMHDn) : 2] : PrphMHDn. *Abbreviations in the formula are: PeriNo – perinucleolar; ExtraNo – extranucleolar; M – mean; H – heterochromatin; Dn – density; prph – peripheral.* For control of heterochromatin image density measurements the image density was increased by a simple high contrast and bleaching image processing of nuclear bodies that clearly exhibited a larger heterochromatin density in central nuclear regions in most of immature lymphocytes (see Results).

The results of measurements 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).

Results

Nuclear mean diameter. From the methodical point of view it should be mentioned that the nuclear size of lymphocytes in thin and thick portion of the smeared peripheral blood was significantly different. In thin monolayer portions of the smear the mean nuclear diameter in mature lymphocytes was 8.96 ± 0.95μm. In thick portion of the same smear, the mean diameter was significantly smaller, i.e. 6.99 ± 0.55 μm using the t-test (p<0.001). Thus all measurements and evaluation of the nuclear morphology in the present study were carried only in thin monolayer portions of smears. On the other hand, damaged cells were not evaluated.

The nuclear diameter in immature lymphocytes such as lymphoblasts and prolymphocytes usually ranged between 9 and 13 μm. In patients untreated with the cytostatic therapy the nuclear mean diameter of immature lymphocytes was slightly larger than that in patients with cytostatic therapy. As it was expected, the nuclear diameter in mature lymphocytes was smaller than in immature cells. It should be also added that the nuclear diameter in mature lymphocytes of treated patients was also smaller in comparison with patients without the cytostatic therapy. It seems to be interesting that the nuclear mean diameter in mature lymphocytes of not-leukemic persons (8.5 ± 0.9 μm) was similar to that in leukemic patients treated with cytostatics (8,6 ± 0.7 μm). Similarly, the larger nuclear mean diameter in immature lymphocytes of not-leukemic persons (10.2 ± 0.1 μm) was close to that in leukemic patients treated with cytostatics (10.3 ± 0.5 μm). However it must be mentioned that lymphocytes in not-leukemic persons mostly belong to the T- and in leukemic patients to the B- lymphocytic lineage [17].

The chromatin structure. The chromatin structure of highly immature and less differentiated cells consists of fibrils and heterochromatin regions, which appear as chromocenters or perinucleolar chromatin. In immature lymphocytes small heterochromatin regions at the nucleolar membrane were usually less dense in comparison with those in the nuclear central regions including the perinucleolar chromatin. Such difference was more apparent when the density
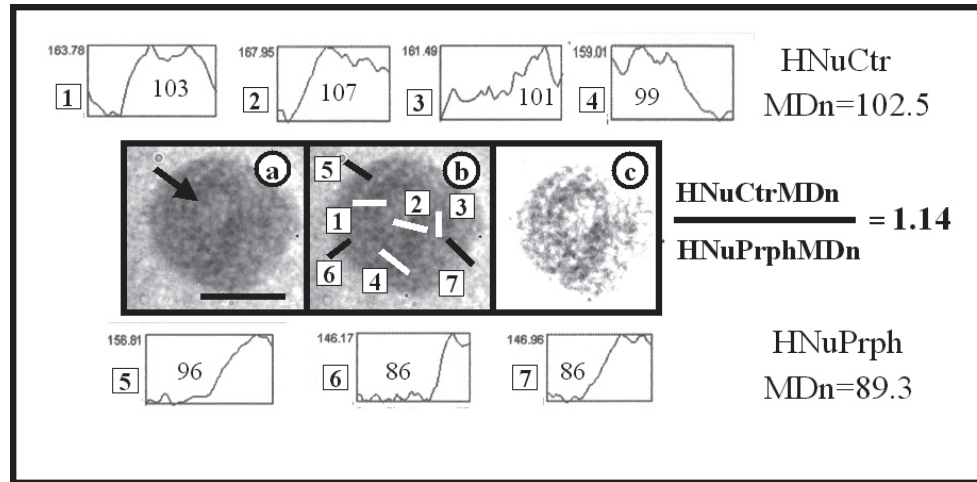
Fig 1. An immature lymphocyte (lymphoblast) without processing the captured image (*Fig a, b*). Large and distinct nucleolus in the *Figure a* (arrow). Bold lines in the *Figure b* within and at the nucleus indicate density measurements. The numbers in this micrograph and at density graphs correspond to measured lines. The numbers within density graphs express calculated density values in arbitrary units (see Material and Methods). Perinucleolar heterochromatin – 1, 2; extranucleolar heterochromatin – 3, 4, peripheral heterochromatin at the nuclear membrane – 5, 6, 7. *Figure c* reflects the nuclear density after increased contrast and bleaching processing. HNuCtr (heterochromatin in nuclear central regions including perinucleolar region); HNuPrph (heterochromatin in nuclear peripheral regions at the nuclear membrane); MDn (mean density). Thick bar in *this and following Figures a* represent 10 μm.
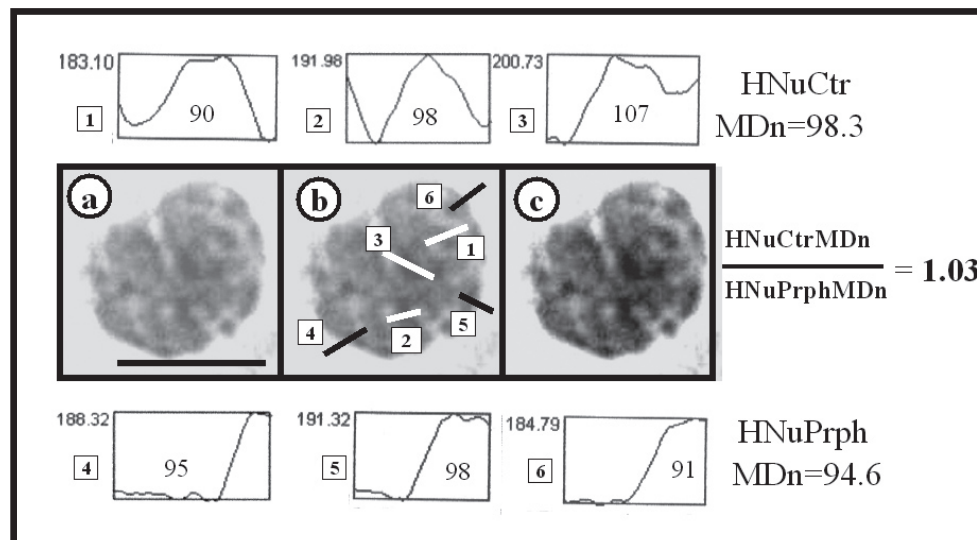

Fig 2. A mature lymphocyte. Measured heterochromatin density in central – 1, 2, 3 – and peripheral – 4, 5, 6 – nuclear regions. For other Legend see Figure 1.

of digitalized images was increased and bleached using the computer processing (Fig. 1). In mature small lymphocytes the heterochromatin structures did not show such distinct differences between peripheral and central nuclear regions including the perinucleolar chromatin (Fig 2). At this occasion it should be mentioned that large heterochromatin chromatin blocks were frequently interconnected or connected with the nuclear periphery by distinct chromatin fibrils. In addition, in some mature lymphocytes it was not

possible to evaluate heterochromatin perinucleolar regions because nucleoli and "larger" interchromatin areas were similar and surrounded by nuclear heterochromatin structures (Fig. 2), The large clustering of the highly condensed heterochromatin in the nuclear periphery might indicate the pre-apoptotic state [6, 18 – 20]. On the other hand, some nuclei of a decreased size in mature lymphocytes still exhibited a larger heterochromatin density in central nuclear regions including the perinucleolar chromatin (Fig. 3).
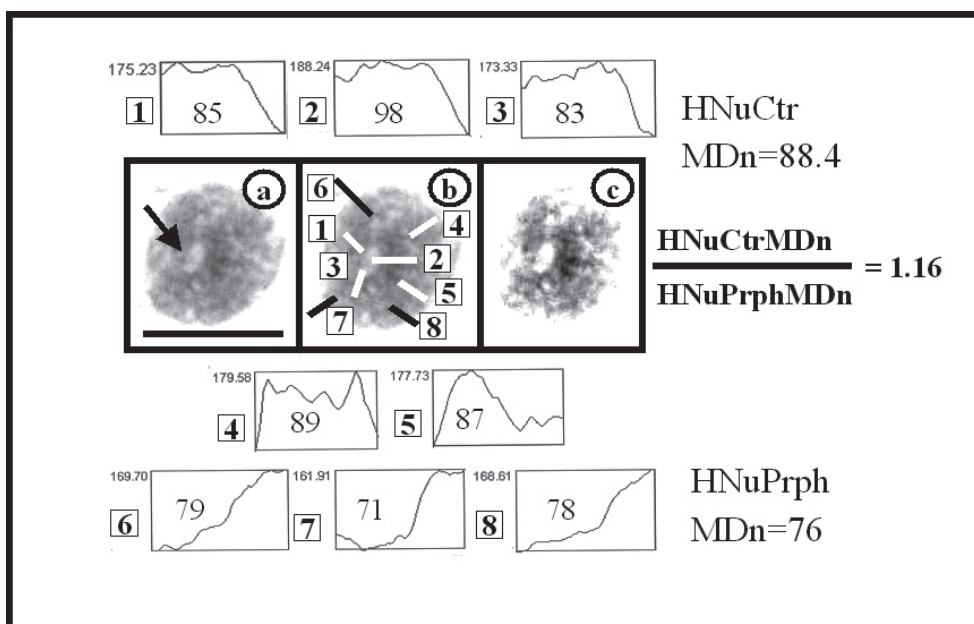
Fig. 3. A mature lymphocyte with the persistent high heterochromatin density in nuclear central perinucleolar and exranucleolar regions. Large and distinct nucleolus in the Figure a (arrow). Measured heterochromatin density in central perinucleolar – 1, 2, 3 -, extranucleolar – 4, 5 – and peripheral – 6, 7, 8 – nuclear regions. For other Legend see Figure 1.

Image densitometry at the single cell level. For quantitative data of the heterochromatin image density in central and peripheral nuclear regions see the Table 1. The image density of heterochromatin regions – chromocenters and perinucleolar chromatin – within nuclei of immature cells was usually larger than at the nuclear membrane (Fig.1). Therefore, the density ratio of the central to peripheral heterochromatin was larger than 1.1. In contrast, in mature lymphocytes, the heterochromatin image density at the nuclear membrane increased (Fig. 2) and reached values similar to those in the "central" heterochromatin regions. Thus the density ratio of the central to peripheral heterochromatin was smaller than 1.1. That ratio was similar in mature lymphocytes in the peripheral

blood of not-leukemic persons (1.07±0.09) although these cells mostly belonged to the T-lineage. In addition, it should be also mentioned that central nuclear regions of few small mature leukemic lymphocytes still exhibited a persistent larger heterochromatin density (Fig. 3).

Discussion

The results of the present study provided complementary information on heterochromatin condensation in central and peripheral nuclear regions of maturing lymphocytes. In immature lymphocytes the heterochromatin density in nuclear central regions was larger in comparison with the nuclear

Table 1. Mean nuclear diameter and heterochromatin density in immature and mature lymphocytes[+]

Lymphocytes	Th	Nu diameter	Heterochromatin density			Ratio
			periNo	extraNo	prph	ctr/prph
immature	0	11.35 ± 1.21	99.6 ± 13.5	97.2 ± 13.4	89.6 ± 17.4[§]	1.13 ± 0.10
mature		9.82 ± 0.76*	104.0 ± 12.2	107.3 ± 11.2	104.4 ± 15.0*	1.01 ± 0.04*
immature	+	10.37 ± 0.52[#]	100.1 ± 16.5	99.5 ± 18.6	85.6 ± 11.4[§]	1.13 ± 0.03
mature		8.68 ± 0.74*[#]	104.4 ± 19.6	100.8 ± 16.9	104.5 ± 20.2*	0.98 ± 0.03*

Legend

[+] – based on 60 measurements in each group of cells

* – significantly different from immature patients using the t- test (p<0.001)

[#] – significantly different from patients without chemotherapy using the t- test (p<0.001)

[§] – significantly different from central perinucleolar and extranucleolar heterochromatin regions using the t-test (p<0.001)

periNo – central perinucleolar region; extraNo – central extranucleolar regions (chromocenters);

prph – peripheral nuclear regions; ctr/prph – central [(periNo + extraNo): 2] to peripheral heterochromatin regions

periphery. Therefore, the heterochromatin condensation in the central nuclear regions, including the perinucleolar chromatin, apparently preceded that at the nuclear periphery. It was evident that the maturation of lymphocytes was mainly accompanied by the increased heterochromatin condensation in the nuclear periphery at the nuclear membrane.

The increasing condensation of heterochromatin at the nuclear periphery apparently reflected the progressing cell maturation including terminal differentiation before the onset of the apoptotic process [5]. At this occasion it should be mentioned that the nuclear periphery at the nuclear membrane is believed to be "gene poor" [7, 9], but still important in respect of the gene replication or transcription activities [9, 21 – 24]. In addition, some genes at the nuclear periphery might play an important role during cell development and differentiation [23 – 25]. The recruitment of genes to the highly condensed heterochromatin adjacent to the nuclear membrane apparently might also contribute to their transcriptional repression [26].

It would be difficult to speculate about the different gene silencing in central and peripheral nuclear regions on the base of heterochromatin condensation studied in the present study. On the other hand, numerous reports suggested that the gene activity is suppressed within but expressed at the condensed heterochromatin regions [9, 23, 27 – 30]. The heavy heterochromatin condensation might be related to the cessation of both DNA replication and RNA transcription. It might prevent both small DNA segment loosening and loop formation at heterochromatin periphery for the replication or transcription process [9, 28, 29]. Thus, the less condensed heterochromatin regions at the nuclear membrane in immature lymphocytes might play an important role in the cell maturation. In mature cells the increased heavy heterochromatin condensation in nuclear periphery might just reflect the final steps and termination of the maturation process. From this point of view it seems to be interesting that the opposite process, i.e. dedifferentiation and blast transformation of mature lymphocytes was accompanied by a marked reduction of the heterochromatin condensation in the nuclear periphery as demonstrated on stimulated T-lymphocytes [31].

The present study also demonstrated that the heterochromatin condensation in lymphocytes was not substantially influenced by the cytostatic treatment. In contrary, the nuclear size of both immature and mature lymphocytes appeared to be slightly, but significantly, reduced in patients treated with cytostatic at the time of taking samples for the present study. However, no satisfactory explanation of that observation is possible on the base of the present study because of a relatively small number of studied patients. On the other hand, numerous reports suggested that lymphocytes "are somehow larger" in patients suffering from B-CLL [17]. The large incidence of cells with the increased nuclear size and intranucleolar distribution of silver stained nucleolar proteins might indicate that such cells are in the early G1 phase of the cell cycle, i.e. in the ready state for activation and proliferation [31, 32]. Thus, the

reduced nuclear size of lymphocytes after the therapy might reflect its effect and increased incidence of resting cells in the G0 of the cell cycle that is characterized by a small size. Actually, the nuclear size of leukemic lymphocytes in patients treated by cytostatic therapy was not substantially different from that in not-leukemic persons. However, most of lymphocytes in the peripheral blood of leukemic patients suffering from chronic lymphocytic leukemia are B-lymphocytes in comparison with T- lymphocytes in not-leukemic persons [17].

The persistence of a smaller heterochromatin condensation in nuclear peripheral regions of some mature leukemic lymphocytes seems to be a maturation abnormality that might be considered as a further maturation asynchrony. It is known that both nuclear and cytoplasmic maturation asynchronies are not unusual in blood cells including leukemic B-lymphocytes [1, 33]. On the other hand, these cells also might represent a subset of cells returning to the cell cycle and proliferation. Such lymphocytes in chronic lymphocytic leukemia were also observed previously. However, that observation was not based on the heterochromatin features, but on the nucleolar size and RNA distribution [34]. In addition, the decreasing heterochromatin condensation in the nuclear periphery was noted in stimulated T lymphocytes during experimental blastic transformation induced by phytohemaglutinin [31].

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