

## ORIGINAL ARTICLE

# Heterochromatin density in the course of cell “dedifferentiation” represented by blastic transformation of human mature T lymphocytes

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### Summary

The present study was undertaken to provide more information on the heterochromatin density in central and peripheral nuclear regions during “cell dedifferentiation” represented by blastic transformation of mature T lymphocytes. Heterochromatin was visualized using a simple cytochemical method for the demonstration of DNA followed by computer-assisted densitometry of digitised images. The results indicated that the blastic transformation was accompanied by a marked and significant decrease in the heterochromatin density at the nuclear membrane. Thus, this nuclear peripheral region seems to be important not only for cell differentiation but also dedifferentiation events. It is also interesting that the non-stimulated resting mature cells in the present study were characterized by less condensed heterochromatin at the nuclear membrane than differentiated granulocytic or erythrocytic precursors and apoptotic myeloblasts or leukemic B lymphocytes described in the previous study. However, in contrast to these cells, resting and mature T lymphocytes in the present study are known to revert to cycling blastic cells after PHA treatment. In addition, it is also known that nuclear peripheral regions with heterochromatin represent sites of chromosomal attachments as well as “together crowded replicons” and silent genes.

*Key words:* Heterochromatin density – lymphocytes – blastic transformation

### INTRODUCTION

It is generally known that human mature resting T lymphocytes stimulated with phytohaemagglutinin (PHA) “dedifferentiate” and transform back to the

cycling and proliferating blastic stage (Busch and Smetana 1970, Astaldi and Lisiewicz 1971). Such transformation of small mature to large blastic cells is accompanied by nuclear and nucleolar enlargement which is related to the increased nuclear and nucleolar biosynthetic activities of stimulated cells (Rubin 1970, Schnedl and Schnedl 1972, Carson 1983). Chromatin structures appear to be less condensed and ring shaped nucleoli transform to large nucleoli with a relatively uniform distribution of RNA (Astaldi and Lisiewicz 1971, Smetana 1980). On the other hand, information on the heterochromatin density in various nuclear regions in the course of blastic transformation is

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very limited although it seems to be likely that it might be different. Recent studies on differentiating leukaemic lymphocytic, granulocytic and erythrocytic precursors have indicated that in the early stages of both lineages the heterochromatin density in perinucleolar regions and chromocenters is significantly larger than that at the nuclear membrane (Smetana et al. 2007). However, the heterochromatin density at the nuclear membrane apparently increased in the non-proliferating, late maturation stages to values noted in other nuclear

regions. Such an observation might be of interest because of different chromosomal territories and functions located in the nuclear regions mentioned (Pederson 2004, Cremer and Cremer 2006, Comings et al. 1980).

The present study was undertaken to provide more information on the heterochromatin density in central and peripheral nuclear regions during “cell dedifferentiation” represented by the blastic transformation of mature T lymphocytes. Heterochromatin was visualized using a simple

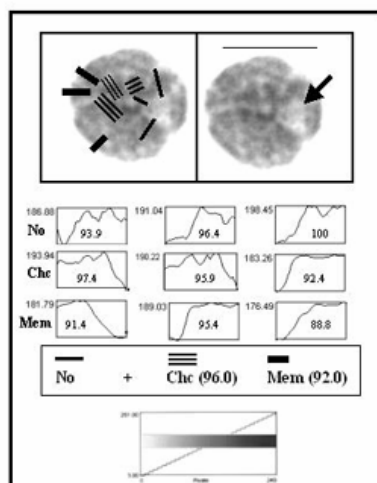


Fig. 1

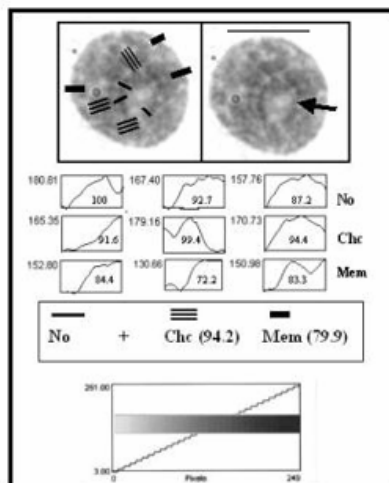


Fig. 2

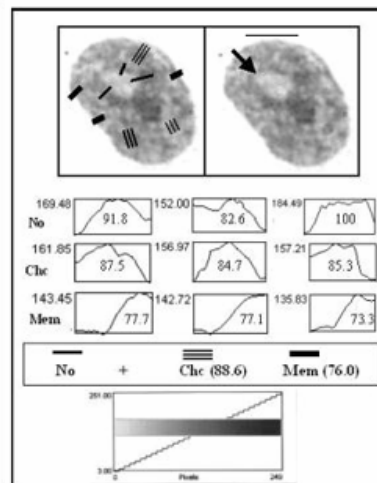


Fig. 3

Fig. 1. **Heterochromatin density in a non-stimulated mature lymphocyte stained for DNA.** Magnification approximately 4000x. The thin black line represents 5µm. Various black lines indicate sites of measurements (see the Figure). No – perinucleolar chromatin, Chc – chromocenters, Mem – heterochromatin at the nuclear membrane. Arrows – nucleolus. Numbers at densitograms – measured density values, numbers within densitograms – calculated relative density values, numbers within rectangles – total relative heterochromatin density values calculated for nuclear regions in the cell image. The density scale is in the bottom of the Figure.

Fig. 2. **Heterochromatin density in a stimulated lymphocyte after 24 hours of cultivation with PHA.** Magnification approximately 3000x. For staining and other legend see Fig. 1.

Fig. 3. **Heterochromatin density in a stimulated and transformed lymphoblast after 48 hours of cultivation with PHA.** Magnification approximately 2200x. For staining and other legend see Fig. 1.

cytochemical method for the demonstration of DNA followed by computer-assisted densitometry of digitised images. The results indicated that the blastic transformation was accompanied by a marked and significant decrease in the heterochromatin density, i.e. by the loosening of heterochromatin at the nuclear membrane.

## MATERIAL AND METHODS

Human lymphocytes were isolated from the peripheral blood of healthy volunteers using a discontinuous density gradient (Böyum 1968) of

Histopaque (Sigma, St. Louis, MO USA). Cells,  $1 \times 10^6/\text{ml}$ , were suspended in medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and stimulated with 10 µg/ml PHA-P from Sigma at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for time frames of up to 48 hrs (Nowell 1960, Kalousek and Křižková 2000). The harvested cells were then prepared for light microscopic observation using a Shandon Cytospin 2 cytocentrifuge (Shandon Southern Products, UK), 800 rpm for 10 min.

The nuclear chromatin structures in methanol fixed cytopsins were visualised by DNA staining with acidified methylene blue after 1N HCl hydrolysis at 60 °C under conditions extracting

histones and RNA (Smetana et al. 1967, Busch and Smetana 1970). Nucleoli in unfixed cytopins were visualised by RNA staining with acidified methylene blue at pH 5.3 (Smetana et al. 1969, Ochs 1998).

Micrographs were captured with a Camedia digital photcamera C4040 ZOOM (Olympus, Japan) placed on a Jenalumar microscope (Zeiss, Germany) equipped with the double adapter to provide a larger magnification of resulting images. These images were then processed and nuclear as well as nucleolar size were measured with Quick Photoprogram (Olympus, Japan). The mean nuclear and nucleolar diameters in specimens stained for DNA or RNA were determined on the screen at magnification 4300x and were calculated from two measurements of the long and minor axis for each nucleus and nucleolus (Setala et al. 1977, Monge et al. 1999, Politi et al. 2003).

The heterochromatin density was measured using the NIH Image Program – Scion for Windows (Scion Corp., USA) after image conversion to the grey scale. At least 3 density measurements were carried out for 3 generally recognised nuclear heterochromatin regions such as perinucleolar regions, chromocenters and at the nuclear membrane (Comings 1980) in each measured cell (Figs. 1–3).

The density was expressed in relatively arbitrary units, which were standardised and calculated according to the following formula: Measured heterochromatin density in the investigated nuclear region divided by the largest – maximal – density of the perinucleolar chromatin. It should be also mentioned that the heterochromatin of the perinucleolar chromatin regions was always very prominent (Busch and Smetana 1970). In mature cells without distinct nucleoli, the maximal heterochromatin density in

the perinucleolar region was replaced by the maximal density of heterochromatin in a chromocenter. Such calculation and standardisation of arbitrary density units facilitated the comparison of results in various portions of cytopins, which occasionally exhibited various artificial densities due to preparation techniques. This approach decreased artificial measurement values and thus provided better results expressed by smaller variation and standard deviations of mean than the background adjustment to zero, which depended on the investigator.

## RESULTS

### *Nuclear and nucleolar size used as control of the PHA effect*

As was expected, as in previous studies (Schnedl and Schnedl 1972) the nuclear and nucleolar size during blastic transformation gradually increased. On this occasion it should be mentioned that the increasing nuclear and nucleolar size also reflected the return of the stimulated cells to the cell cycle. The maximal values of the mean diameter of both nuclei and nucleoli in stimulated cells were noted after 48 hrs of cultivation when they were in the S phase of the cell cycle (Table 1). However, in contrast to nuclear size, differences in the increased nucleolar mean diameter between 24 (end of the G1 phase) and 48 hrs (S phase) of cultivation with PHA were not significant. Such observations (see also Schnedl and Schnedl 1972) seem to reflect the preceding nucleolar RNA transcription in the G1 before nuclear DNA replication in the S phase (Carson 1983). It should be added that the transformation of “resting“ to fully activated nucleoli with a more or less uniform distribution of

Table 1. Mean nuclear and nucleolar diameters during lymphocyte blastic transformation #

Time of cultivation (hrs)	Mean nuclear	Mean nucleolar
	diameter (μm)	
0	6.3 ± 0.6 <sup>a</sup>	1.0 ± 0.2
24	7.2 ± 0.7*	2.1 ± 0.4*
48	10.1 ± 1.5*	2.8 ± 0.8*

#### Legend

# - based on more than 100 measurements for each group

\* - statistically different from 0 hr using t-test (p<0.03)

a - mean and standard deviation

Table 2. Heterochromatin density in central nuclear regions and at the nuclear membrane#

Time of cultivation (hrs)	PNo + Chcs	Membrane
	(Relative Density Units)	
0	96.8 ± 8.5 <sup>a</sup>	93.8 ± 3.7 <sup>□</sup>
24	96.5 ± 3.8	88.4 ± 6.0 <sup>*⊕</sup>
48	92.5 ± 5.9	74.3 ± 13.6 <sup>*⊕</sup>

## Legend

# - based on more than 40 measurements for each group

□ - statistically marginally different from perinucleolar regions and chromocenters using t-test (p=0.057)

\* - statistically different from perinucleolar region and chromocenters using t-test (p&lt;0.001)

⊕ - statistically different from 0 hr using t-test (p&lt;0.001)

a - mean and standard deviation

PNo - perinucleolar region

Chcs - chromocenters

Membrane - heterochromatin at the nuclear membrane

RNA was observed already after 24 hours of cultivation, i.e. at the end of the G1 phase (Smetana 1980).

*Heterochromatin density*

In control mature non-stimulated cells, the heterochromatin density in the central nuclear regions such as the perinucleolar region or chromocenters and at the nuclear periphery, appeared to be almost similar (Fig.1). However, the mean values of the heterochromatin density at the nucleolar membrane were slightly smaller (Tab.2). On the other hand, in comparison with heterochromatin in central nuclear regions, a markedly and significantly smaller heterochromatin density at the nuclear membrane was noted in stimulated cells after cultivation in the presence of PHA (Tab.2, Fig.2). The smallest values of the heterochromatin density at the nuclear membrane were noted in the transformed “blastic cells“ after 48 hrs of cultivation (Tab.2, Fig.3). Such blastic cells possessed the largest nuclei (see above) and were in the S phase of the cell cycle (Schnedl and Schnedl 1972).

**DISCUSSION**

Based on the present observations, it seemed to be obvious that the heterochromatin density at the nuclear membrane decreased during dedifferentiation, i.e. blastic transformation, when transformed cells re-entered the cell cycle. Such a

phenomenon indicates a heterochromatin loosening in this nuclear region that might be connected with its activation. On this occasion it must be mentioned that an opposite observation was noted in differentiating, and maturing or apoptotic cells (Smetana et al. 2007). In such cells the heterochromatin density at the nuclear membrane significantly increased and might be connected with gene silencing (Frenster 1974, Zhimulev and Beliaeva 2003, Grygoryev et al. 2006). In addition, some earlier studies have also indicated that this nuclear region represents sites of the chromosomal attachment to the nuclear membrane and contains “together crowded replicons“ (Cremer and Cremer 2006). Thus the nuclear peripheral region seems to be important not only for cell differentiation but also for dedifferentiation events as suggested by the present study.

It seems to be also interesting that non-stimulated resting mature cells in the present study were characterized by less condensed heterochromatin at the nuclear membrane than differentiated granulocytic or erythrocytic precursors and apoptotic myeloblasts or leukemic B lymphocytes (Smetana et al. 2007). However, in contrast to these cells, resting and mature cells in the present study were represented by isolated lymphocytes from the peripheral blood of healthy volunteers which mainly belong to the T lineage (Hoffbrand and Pettit 1988). As is generally known, peripheral mature resting T lymphocytes are in a resting mature state and revert to cycling blastic cells after PHA treatment (Cline 1975, Carson 1983).

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