Short Communication

RNA Concentration and Content in the Nucleoli and Cytoplasmic Rim in Differentiating Lymphocytes of Patients Suffering from B Chronic Lymphocytic Leukaemia – a Cytochemical Note

(RNA / leukemic lymphocytes / cytochemistry)

K. SMETANA[1], D. MIKULENKOVÁ[1], J. KARBAN[2], M. TRNĚNÝ[2]

[1]Institute of Haematology and Blood Transfusion, Prague, Czech Republic
[2]1st Department of Medicine – Department of Haematology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic

Abstract. Nucleolar RNA optical density (concentration) measurements at the single cell level indicated that differentiation of lymphocytes is accompanied by a slightly decreased nucleolar RNA concentration in contrast to the cytoplasmic rim around the nucleus. On the other hand, the nucleolar size was markedly reduced and the cytoplasmic rim surrounding the nucleus was reduced only weakly. Concerning the calculated rough estimate of the RNA content, the differentiation induced its larger decrease in the nucleoli than in the cytoplasmic rim. These observations indicated that the nucleolar RNA concentration and RNA content together with the nucleolar morphology are more sensitive markers of the differentiation process than the RNA concentration and content in the cytoplasm. Thus, the nucleolar RNA transfer to the cytoplasm in advanced differentiation steps might still be going on regardless of the decreasing or inhibited nucleolar biosynthetic activity. In addition, the presence of ring-shaped nucleoli and micronucleoli characteristic of mature and terminal lymphocytes in some lymphocytic less differentiated steps, i.e., lymphoblasts and prolymphocytes, might indicate the premature differentiation state of such cells.

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Corresponding author: Karel Smetana, Institute of Haematology and Blood Transfusion, U Nemocnice 1, 128 20 Prague 2, Czech Republic. E-mail: karel.smetana@uhkt.cz

Abbreviations: CLL – chronic lymphocytic leukaemia.

Introduction

Previous cytochemical studies demonstrated a very narrow relationship between the nucleolar and cytoplasmic ribosomal RNA representing about 80 per cent of RNA present in the cell (Caspersson and Schultz, 1940; Kwiatkowska and Maszewski, 1985a, b; Risueno and Medina, 1986; De Robertis and De Robertis, 1987). The differentiation of granulocytes and erythroblasts was accompanied by a gradual decrease of RNA concentration in both nucleoli and cytoplasm. On this occasion, it should be mentioned that the reduction of RNA concentration in the cytoplasm of advanced differentiation steps in granulocytes was larger than in small nucleoli. Such difference might be due to inhibited migration of nucleolar pre-ribosomes to the cytoplasm (Smetana et al., 2010a, 2011, 2019). In contrast, experimentally induced cell dedifferentiation of lymphocytes evoked a gradual increase of RNA concentration in both nucleolar bodies and cytoplasm. The increased RNA concentration in nucleoli of dedifferentiated cells – lymphoblasts – appeared to be slightly larger than in the cytoplasm. This difference possibly resulted from reactivation of the nucleolar – ribosomal RNA transcription (Smetana et al., 2008).

The present study was undertaken to provide more information on the nucleolar and cytoplasmic RNA content in single cells during the differentiation of human leukemic lymphocytes. In comparison with mature granulocytic or erythroid nucleated precursors, the RNA concentration in the nucleolus and cytoplasm during the differentiation of lymphocytes demonstrated a remarkable stability despite the nucleolar size reduction (Smetana et al., 2010a). Lymphocytes of patients suffering from B chronic lymphocytic leukaemia (CLL) represent a very convenient model for the differentiation effect on the nucleolar and cytoplasmic RNA concentration. They are present in the peripheral blood smears of these pa-

tients in a satisfactory number for optical density and size measurements at the single cell level. Moreover, all differentiation steps of lymphocytes are easily recognizable and possess characteristic nucleoli (Smetana et al., 2010a). Different types of nucleolar bodies may be easily classified according to the size and RNA distribution and the number of fibrillar centres (Smetana et al., 2010a, 2020). The cytoplasmic RNA-containing shell around the nucleus is also accessible for optical density and diameter measurements (see Material and Methods below).

The RNA density measurements indicated that the differentiation of CLL lymphocytes was characterized by a slightly decreased nucleolar RNA concentration and by a marked reduction of the nucleolar diameter. On this occasion, it should be noted that some lymphocytic precursor cells were in the state of premature differentiation, because they possessed ring-shaped nucleoli characteristic of differentiated mature lymphocytes (Smetana et al., 2010a).

Material and Methods

Nucleoli and cytoplasm of early and late differentiation steps of the lymphocytic lineage were studied in peripheral blood smears of four selected patients suffering from CLL. These selected patients did not receive any anti-leukemic therapy at the time of taking samples for the present study, because previous studies indicated a possible variation of the lymphocytic nucleologram induced by the anti-leukemic treatment (Kopec et al., 1983; Malacarne et al., 1986; Klobusická et al., 2010). In the studied CLL patients, nucleolar bodies in various differentiation steps exhibited a characteristic morphology (Fig. 1) (Smetana et al., 2020). Mature differentiated lymphocytes (69.3 ± 6.4 %) mostly possessed ring-shaped nucleoli, and terminal lymphocytes (19.3 ± 3.2 %) were characterized by the presence of micronucleoli. Precursor cells such as lymphoblasts and prolymphocytes (11.4 %) usually contained large nucleoli with multiple silver-stained fibrillar centres, which appeared as less visible small light areas in specimens stained for RNA. Some of these precursor cells (13.3 ± 3.8) exceptionally possessed large ring-shaped nucleoli (Fig. 1b), suggesting premature differentiation. The peripheral blood samples of the studied patients were originally taken for diagnostic purposes with the supervision and approval of the Institute authorities.

RNA in the nucleoli and cytoplasm in unfixed smear preparations was visualized by a simple but sensitive procedure using methylene blue at pH 5.3 buffered with McIlvain's buffer (Smetana et al., 1969; Ochs, 1998). The low pH and citric acid of the buffer prevented loss of RNA from unfixed cells in the dry smears, which were not older than 24 hours. Nucleolar fibrillar centres were visualized by the silver reaction that facilitated distinguishing fibrillar centres with the periphery as silver-stained dots (Ochs, 1998; Smetana et al., 1999).

Micrographs were captured with a Camedia digital photo camera (C4040 Z, Olympus, Japan) placed on a Jenalumar microscope (Zeiss, Germany) equipped with a double adapter to provide larger magnification of captured resulting images on the computer screen. The nucleolar, cell and nuclear body diameters were measured on the PC screen using a specific software program (Quick Photoprogram, Olympus, Japan). The cytoplasmic rim around the nucleus was calculated by subtracting nuclear from cell body diameter.

Nucleolar and cytoplasmic density reflecting the RNA concentration was measured after conversion of the captured blue signal to grey scale using the red channel (NIH Image Program, Scion for Windows, Scion Corp., USA). The RNA concentration was expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from the measured mean density of nucleolar bodies or cytoplasmic rim around the nucleus. The cytoplasmic density was measured in two locations of the cytoplasmic rim that exhibited the lowest and highest positivity. Such calculations and standardization of arbitrary density units facilitated comparison of results in peripheral blood smears, which occasionally exhibited various density due to smear preparations. The RNA content estimate was calculated by multiplication of the nucleolar and cytoplasmic rim RNA concentration by the diameter. The results of measurements and calculations such as mean, standard deviation and *t*-test were evaluated using "Primer of Biostatistic Program, version 1" developed by S.A. Glantz (McGraw-Hill, Canada, 1968).

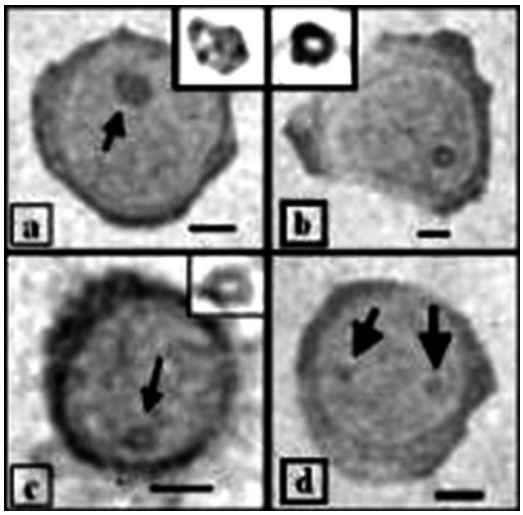

Fig. 1. CLL lymphoblasts (**a**, **b**) and differentiated mature lymphocytes stained for RNA (**c**, **d**). Nucleoli – arrows. Large nucleolus with small light and dense areas corresponding to fibrillar centers with surrounding RNA components characteristic of the nucleolar RNA transcription (arrow and insert in Fig. 1a). Ring-shaped nucleolus with a single large fibrillar centre characteristic of the reduced RNA transcription (arrow and insert in Fig. 1b and c). Micronucleoli reflecting the cessation of the nucleolar RNA transcription (Fig. 1d). Thick bars in Fig. – 2 μm.

*Table 1. Nucleolar and cytoplasmic RNA detection, calculated RNA concentration and diameter measured in single cells of the lymphocytic lineage of patients suffering from CLL**

Cells	No Ctr	Ctpl Ctr	No/Ctpl Ctr	FCs	No Dm	Ctpl Dm	No/Ctpl Dm
Lybl + PC	**84.8** ± 1.5	**65.6** ± 6.8	**1.30**	> 2	**2.1** ± 0.7	**3.6** ± 0.7	**0.58**
	100 %	100 %	100 %	100 %	100 %	100 %	
Lycyt I	**75.6** ± 4.5[a]	**66.0** ± 11.1	**1.17**	< 2	**1.4** ± 0.1[a]	**2.6** ± 0.8[a]	**0.53**
	89.1 %	100.6 %	90.1 %	66.6 %	72.2 %	91.3 %	
Lycyt II	**73.0** ± 7.4[a]	**66.6** ± 9.1	**1.09**	< 2	**0.9** ± 0.1[a]	**2.5** ± 0.4[a]	**0.36**
	86.0 %	101.5	83.8 %	42.8 %	69.4 %	62.0 %	

*Mean and standard deviation based on 160–200 measurements in each group of patients. [a]Significantly different from progenitor and precursor cells (Lybl + PC) using *t*-test ($2\alpha = 0.05$). No – nucleolar, Ctr – calculated concentration, Ctpl – cytoplasmic, FCs – number of fibrillar centres, Dm – diameter, % – percentage of values in Lybl + PC, Lybl – progenitor cells, lymphoblasts, PC – precursor cells, prolymphocytes, Lycyt I – differentiated mature cells (lymphocytes with ring-shaped nucleoli), Lycyt II – differentiated terminal cells (lymphocytes with micronucleoli), see also Smetana et al. (2020).

Results and Discussion

The decrease of the nucleolar RNA concentration during the differentiation of CLL lymphocytes was small but statistically significant. However, the reduction of the RNA concentration was accompanied by a markedly decreased nucleolar size and RNA detection (Table 1). The latter was expressed by a reduced number of fibrillar centres (Table 1), the periphery of which represents sites of nucleolar – ribosomal – RNA transcription (Hozák et al., 1994; Raška et al., 2006; Smetana, 2011; Weipotshammer and Schöfer, 2016; Penzo et al., 2019). On the other hand, the RNA concentration in the cytoplasm apparently did not change. On this occasion, it should also be noted that some lymphocytic precursors such as lymphoblasts and prolymphocytes (13.3 ± 3.8 %) possessed ring-shaped nucleoli characteristic of differentiated mature lymphocytes (Smetana et al., 2010a, 2020). Thus, such progenitor or precursor cells appeared to be in the premature differentiation state (Fig. 1b). This state would correspond to the frequent developmental asynchrony or anarchy of cell organelles in leukemic cells (Bessis, 1973).

The slightly decreased nucleolar RNA concentration together with the markedly reduced size of nucleolar bodies (Smetana et al., 2010b, 2020) during the differentiation of CLL lymphocytes morphologically expressed a marked reduction of the nucleolar RNA content (Table 2). The RNA content in the cytoplasmic rim of differentiated mature or terminal lymphocytes was also reduced, but much less (Table 2). Therefore, during the differentiation of lymphocytes, the RNA content in the nucleoli was decreasing to a greater extent than that in the cytoplasmic rim around the nucleus. On this occasion, it should be mentioned that mature or terminal lymphocytes with micronucleoli still possessed RNA in the cytoplasm. However, the ability of maturing ribosomes for polysome assembly and biosynthetic activity seems to be limited (Billington and Itzhaki, 1974; Sbarrato et al., 2016). Actually, electron microscopy also demonstrated free ribosomes in CLL lymphocytes, which apparently did not participate in the polysome formation (Schumacher et al., 1970).

According to the above-presented observations on the nucleolar or cytoplasmic RNA concentration and content, the differentiation of lymphocytes in CLL patients

Table 2. Calculated nucleolar and cytoplasmic RNA concentration related to the nucleolar and cytoplasmic diameter or volume indicating the approximate content estimate in single cells of the lymphocytic lineage in CLL patients

Cells	No Ctr x Dm	Ctpl Ctr x Dm	No/Ctpl Ctr x Dm	No Ctr x Vo	Ctpl Ctr x Vo	No/Ctpl Ctr x Vo
Lybl + PC	177.6 ± 3.1	235.8 ± 24.9	0.70	144.1 ± 4.5	333.2 ± 7.5	0.43
	100 %	100 %	**100 %**	100 %	100 %	**100 %**
Lycyt I	105.6 ± 6.5[a]	171.2 ± 29.1[a]	0.62	57.4 ± 5.0[a]	171.6 ± 15.4[a]	0.33
	59.4 %	72.5 %	**88.5 %**	39.8 %	51.5 %	**76.4 %**
Lycyt II	65.7 ± 6.7[a]	166.2 ± 22.8[a]	0.39	32.8 ± 8.0[a]	146.0 ± 10.5[a]	0.22
	36.9 %	70.4 %	**55.7 %**	22.7 %	43.8 %	**51.1 %**

Vo – calculated volume based on measured diameter and virtual height 0.5 μm in the calculation formula (Calculator.net). For other legend see Table 1. Bold lettering in Table indicates similar trends using both diameter and volumetric calculations for the rough RNA content estimate in the nucleolus and cytoplasmic rim in progenitor or precursor and differentiated mature or terminal cells.

differs from that of granulocytes or erythroblasts. In granulocytic and erythroid cell lineages, the larger reduction of RNA concentration in the cytoplasm of advanced differentiation steps might be due not only to the decreased nucleolar biosynthetic activity, but also to the "frozen" transport of nucleolar RNA to the cytoplasm (Smetana et al., 2010a, 2010b, 2011). In contrast, the reduction of the RNA concentration and content in the cytoplasm of advanced differentiation steps of CLL lymphocytes was smaller than that in the nucleolus. Such observation might also indicate the continuing transfer of the nucleolar – ribosomal RNA to the cytoplasm despite the decreased polysome assembly in these cells (Billington and Itzhaki, 1974; Olszewska et al., 1984; Kwiatkowska and Maszewski, 1985a, b; Sbarrato et al., 2016). On this occasion, it should also be mentioned that apoptotic lymphocytes and Gumprecht ghosts of lymphocytic origin exhibited a reduction of RNA concentration in the cytoplasm similar to the terminal differentiation steps of erythroid and granulocytic lineages (Smetana et al., 2010a, 2010b, 2011).

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Competing interests

The authors declare that no competing interests exist.

References

Bessis, M. (1973) *Living Blood Cells and Their Ultrastructure.* Springer, Berlin.

Billington, R. W., Itzhaki, R. F. (1974) Ribosomal RNA synthesis in chronic lymphocytic leukaemia. *J. Cancer* **29**, 318-324.

Caspersson, T. D., Schultz, J. (1940) Ribonucleic acid in both nucleus and the function of the nucleolus. *Proc. Natl. Acad. Sci. USA* **26**, 507-515.

De Robertis, E. D. P., De Robertis, E. M. F. Jr. (1987) *Cell and Molecular Biology.* Lea and Febiger, Philadelphia.

Hozák, P., Cook, P. R., Schofer, C., Mosgoller, W., Wachtler, F. (1994) Site of transcription of ribosomal RNA and intranucleolar structure in HeLa cells. *J. Cell Sci.* **107**, 639-648.

Klobusická, M., Kusenda, J., Stevulová, A., Babušíková, O. (2010) Possible prognostic value of nucleolar morphology in pathologic cells of B-chronic lymphocytic leukemia. *Neoplasma* **57**, 429-437.

Kopec, J., Sitarska, E., Leszko, B. (1983) Nucleoli in peripheral blood lymphocytes of patients with chronic lymphocytic leukemia treated with cytostatic drugs. *Arch. Immunol. Ther. Exp.* **31**, 481-487.

Kwiatkowska, M., Maszewski, J. (1985a) Functional and structural heterogeneity of nucleoli: the dependence of the activity of transport of newly synthesized rRNA on nucleolar size and phase of the cell cycle. *Folia Histochem. Cytobiol.* **23**, 117-126.

Kwiatkowska, M., Maszewski, J. (1985b) Changes in the activity of transport of newly synthesized RNA from nucleus and nucleolus into cytoplasm during interphase. *Folia Histochem. Cytobiol.* **23**, 127-134.

Malacarne, P., Marzola, M., Santini, A., Nielsen, I. (1986) Nucleologram variations in peripheral lymphocytes after chemotherapy. *Folia Biol. (Praha)* **32**, 349-353.

Ochs, R. L. (1998) Methods used to study structure and function of the nucleolus. *Methods Cell Biol.* **53**, 303-301.

Olszewska, M. J., Damsz, B., Kuran, H., Marciniak, K. (1984) Enhanced rRNA transport from nucleoli into cytoplasm at G1/S, S/G2, and G2/M transitory points of the cell cycle in root meristem of Helianthus annuus L. *Eur. J. Cell Biol.* **33**, 1-6.

Penzo, M., Montanaro, L., Treré, D., Derenzini, M. (2019) The ribosome biogenesis – cancer connection. *Cells* **8**, 55.

Raška, I., Shaw, P. J., Cmarko, D. (2006) Structure and function of the nucleolus in the spotlight. *Curr. Opin. Cell Biol.* **18**, 325-334.

Risueno, M. C., Medina, F. J. (1986) *The Nucleolar Structure in Plant Cells.* Servicio Editorial Universidad Del Pais Vasco, Bilbao.

Sbarrato, T., Horvilleur, E., Pöyry, T., Hill, K., Chaplin, I. C., Spriggs, R. V., Stoneley, M., Wilson, L., Jayne, S., Vulliamy, T., Beck, D., Dokal, I., Dyer, M. J. S., Yeomans, A. M., Packham, G., Bushell, M., Wagner, S. D., Willis, A. E. (2016) A ribosome-related signature in peripheral blood CLL B cells is linked to reduced survival following treatment. *Cell Death Dis.* **7**, e2249.

Schumacher, H. R., Maugel, T. K., Davis, D. (1970) The lymphocyte of chronic lymphatic leukemia I. Electron microscopy – onset. *Cancer* **26**, 895-903.

Smetana, K. (2011) The nucleolus through the years. *J. Appl. Biomed.* **9**, 119-127.

Smetana, K., Lejnar, J., Potměšil, M. (1969) A further contribution to the demonstration of RNA and nucleoli in smear preparations. *Folia Haematol.* **91**, 381-384.

Smetana, K., Jirásková, I., Perlaky, L., Busch, H. (1999) The silver reaction of nucleolar proteins in the main structural compartments of ring-shaped nucleoli in smear preparations. *Acta Histochem.* **101**, 167-183.

Smetana, K., Otevřelová, P., Jirásková, I., Kalousek, I. (2008) On the nucleolar and cytoplasmic RNA density during "cell dedifferentiation" represented by blastic transformation of human mature T lymphocytes – a cytochemical study. *Folia Histochem. Cytobiol.* **46**, 429-432.

Smetana, K., Klamová, H., Jirásková, I., Mikulenková, D. (2010a) A short cytochemical note on the nucleolar and cytoplasmic RNA concentration in differentiating cells represented by human erythroblasts. *Acta Histochem.* **112**, 407-411.

Smetana, K., Karban, J., Trněný, M. (2010b) To the nucleolar bodies (nucleoli) in cells of the lymphocytic lineage in patients suffering from B – chronic lymphocytic leukemia. *Neoplasma* **57**, 495-500.

Smetana, K., Jirásková, I., Mikulenková, D., Klamová, H. (2011) Nucleolar and cytoplasmic RNA density – concentration in leukemia granulocytic progenitors in human bone marrow biopsies: A short cytochemical note. *Acta Histochem.* **113**, 158-161.

Smetana, K., Klamová, H., Mikulenková, D. (2019) To the approximate size of the nuclear region occupied by nucleolar bodies during cell differentiation and maturation using the human leukemic granulocytic lineage as a convenient model. *Physiol. Res.* **68**, 633-638.

Smetana, K., Mikulenková, D., Otevřelová, P., Karban, J., Trněný, M. (2020) The rough estimate of the nuclear space occupied by nucleolar bodies in lymphocyte of patients suffering from B chronic lymphocytic leukemia and blood donors. *Clin. Oncol. Res.* **3**, 1-5.

Weipotshammer, K., Schöfer, C. (2016) Morphology of the nuclear transcription. *Histochem. Cell Biol.* **145**, 343-358.