

## To the nucleolar density and size in apoptotic human leukemic myeloblasts produced *in vitro* by Trichostatin A

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The present study was designed to provide more information on nucleoli in apoptotic cells, which were represented in the present study by cultured leukemic myeloblasts (Kasumi-1 cells). The apoptotic process in these cells was produced by trichostatin A (TSA) that is a histone deacetylase inhibitor with strong cytostatic effects. The selected TSA concentration added to cultures facilitated to study apoptotic and not-apoptotic cells in one and the same specimen. The nucleolar diameter and density were determined using computer assisted measurement and densitometry in specimens stained for RNA. In comparison with not-apoptotic cells, in apoptotic cells, nucleolar mean diameter did not change significantly and nucleolar RNA density was also not apparently different. On the other hand, the cytoplasmic RNA density in apoptotic cells was markedly reduced. Thus it seemed to be possible that the transcribed RNA remained "frozen" within the nucleolus but its transport to the cytoplasm decreased or stopped. However, the possibility of the RNA degradation in the cytoplasm of apoptotic cells based on the present study cannot be eliminated. At this occasion it should be added that AgNORs reflecting nucleolar biosynthetic and cell proliferation activity in apoptotic cells decreased in number or disappeared. The presented results also indicated that large nucleoli intensely stained for RNA need not be necessarily related to the high nucleolar biosynthetic or cell proliferation activity and may be also present in apoptotic cells responding to the cytostatic treatment.

**Key words:** apoptotic cells, nucleolar density, nucleolar diameter.

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**N**ucleolar changes in apoptotic cells and apoptotic bodies were less studied although nucleolar morphology is a very useful tool and marker for the evaluation of various cell states at the single cell level (Busch and Smetana 1970; Wachtler and Stahl, 1993). According to previous studies various cells in pre-apoptotic or apoptotic state may contain nucleoli with altered structure and size, which, together with other RNP components may be even expelled out from the nucleus (Biggiogera *et al.*, 2004; Martelli *et al.*, 2001; Pellicciari *et al.*, 2000; Smetana, 2002). On the other hand, only few reports are dealing with the nucleolar size and RNA density when the apoptotic process was induced by a cytostatic treatment without preceding terminal maturation (Smetana, 2004). Thus the present study was undertaken to provide more information on the density of nucleolar bodies in apoptotic leukemic myeloblasts in which the apoptotic process was induced by a histone deacetylase inhibitor represented by trichostatin A (TSA). The cytostatic and apoptotic effect of that drug on some malignant cells is known and represents a subject of a great interest and discussions concerning the pathway of action (Choi, 2005; Smetana *et al.*, 2007b; Wu *et al.*, 2007; Yamashita *et al.*, 2003). Kasumi-1 cells originated from acute myeloid leukaemia (type M2 according to FAB classification) and represented leukemic myeloblasts in the present study. These myeloblasts are a very convenient model for AML *in vitro* and do not lose an ability to differentiate (Asou *et al.*, 1999; Larizza *et al.*, 2005). In addition, the selected concentration of TSA added to cultures of these cells facilitated to study not-apoptotic and apoptotic myeloblasts in one and the same specimen (Smetana *et al.*, 2007a).

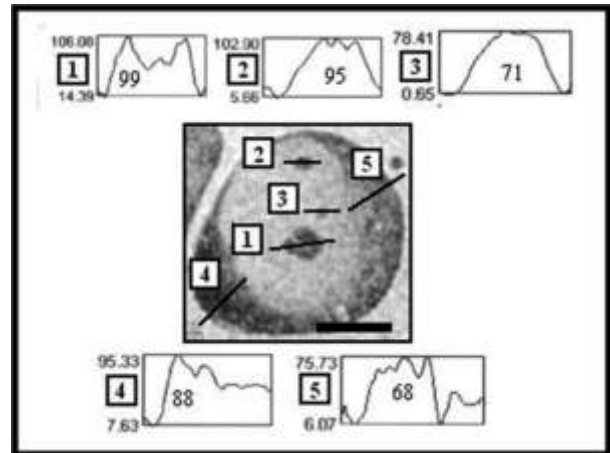
## Materials and Methods

Kasumi-1 cell line originating from human acute myeloid leukaemia – type M2 according to FAB classification (see DSMZ, 2004; LGC Promochem, 2006) was received as a generous gift from Dr. O. Krejčí (Massachusetts General Hospital Cancer Center, Boston, USA). Leukemic myeloblasts were cultured in RPMI 1640 medium with 20% foetal bovine serum (Gibco, USA) at 37°C in an atmosphere containing 5% carbon dioxide (see LGC Promochem, 2006). Aliquots were cultured without or with TSA (Sigma Aldrich, USA) at concentration 120 nm for 24h. Under these conditions, mitotic divisions in TSA treated cells disappeared and the number of apoptotic cells significantly increased (see Smetana *et al.*, 2007a,b). Cytospins of cultured leukemic myeloblasts were prepared by centrifugation at 800r/min for 5 min using a Universal 16 R centrifuge, rotor 1624 (Hettich, Germany).

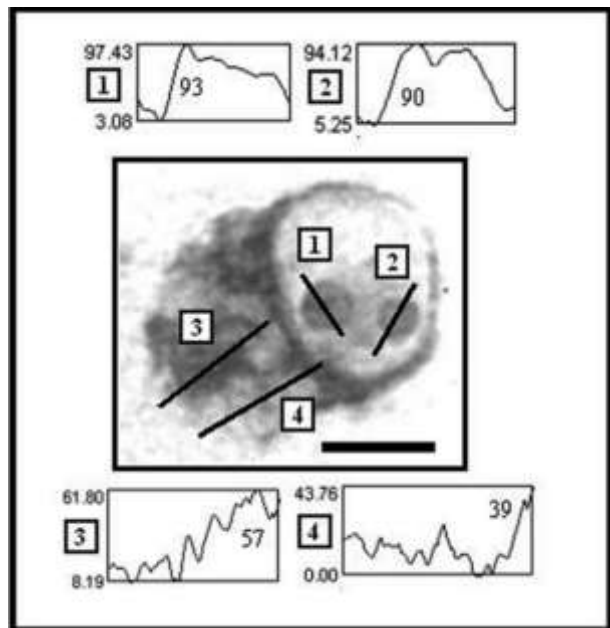
Dry cytopins not older than 24 h were stained without fixation for RNA by 0.05% acidified methylene blue at pH 5.3 (Smetana *et al.*, 1969; Ochs 1998). Silver stained proteins of AgNORs were visualised by silver reaction on dry unfixed cytopins older than 6 days. Then cytopins were incubated in silver reagent for 5-10 min at 37°C in the dark. The silver reagent consisted of 2 parts of 50% silver nitrate and 1 part of colloidal developer containing 2% gelatine in 1% formic acid (Howell and Black, 1980; Ploton *et al.*, 1986). The incidence of apoptotic cells and bodies was evaluated in methanol fixed cytopins stained for DNA by acidified methylene blue for 10 min after hydrolysis with 1N HCl at 60°C for 10 min (Busch and Smetana, 1970; Smetana *et al.*, 2000).

Micrographs were taken with a Camedia digital photo camera C40C40 ZOOM (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany). The captured images were processed with Quick Photoprogram (Olympus, Japan) in combination with Infanview (Irfan Skiljan, Austria) and Power Point Microsoft (USA) programs. Mean nucleolar diameters of each nucleolar body without perinucleolar chromatin were measured at magnification 4300x on the screen using Quick Photoprogram. Then these measured diameters were used for calculation of mean nucleolar diameter for each evaluated cell (see e.g. Smetana *et al.*, 2007a). The nucleolar and cytoplasmic density was measured on specimens stained for RNA using National

Institutes of Health Image Program – Scion for Windows (Scion Corp., USA) after image conversion of captured colour images to grey scale. The measured density was expressed in arbitrary units calculated by subtracting measured nucleolar and cytoplasmic density from the background surrounding each measured cell (Figures 1 and 2). Such calculation eliminated artificial density due to the background density produced by the specimen preparation for microscopy.



**Figure 1.** A TCA untreated cell. Numbers in the Figure correspond to densitographs. Numbers within densitographs represent arbitrary density units. Mean nucleolar density/cell =  $88.3 \pm 10.7$ , mean cytoplasmic density/cell =  $78.0 \pm 7.0$ . Black lines indicate measurement lines. The black thick bar in this and following figures represents 5  $\mu$ m.



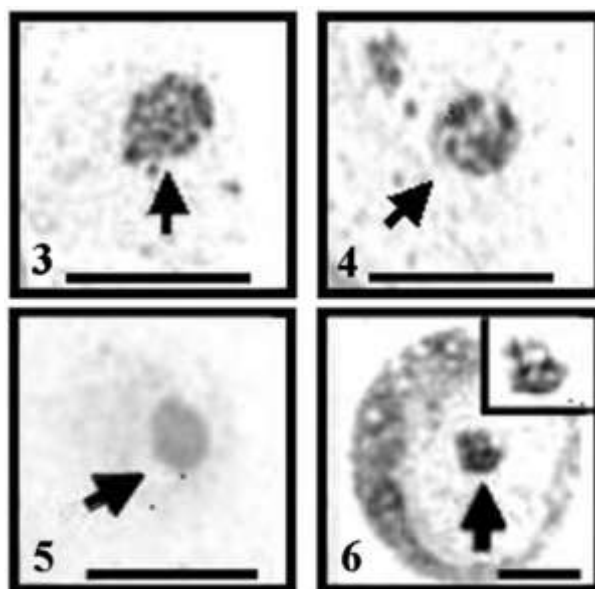
**Figure 2.** A TCA treated apoptotic cell. Nucleolar density =  $91.5 \pm 1.2$ , cytoplasmic density =  $48.0 \pm 7.3$ . For legend see Figure 1.

## Results

In untreated control cultures the number of apoptotic cells was very small and practically did not reach 5%. In contrast, in TSA treated cultures the percentage of apoptotic cells or bodies with a characteristic appearance was large enough ( $51\pm 6$  per cent) and facilitated to study nucleoli in both apoptotic cells and bodies and less sensitive or resistant cells, i.e. in not-apoptotic cells (see also Smetana *et al.*, 2007b). In addition, no signs of cell differentiation were noted in both TSA untreated and treated cultures.

In untreated control cells, nucleoli were characterized by numerous AgNORs representing fibrillar centres in the electron microscope (Figure 3, ref. Smetana, 2002; Wachtler and Stahl, 1993). In treated but not-apoptotic cells, nucleoli appeared similar. However, in some of them, enlarged AgNORs were apparently reduced in number and occasionally translocated to the nucleolar periphery (Figure 4). Such decrease and translocation of AgNORs indicated a reduced nucleolar biosynthetic activities and/or premature ageing (Derenzini, 2000; Smetana *et al.*, 2006). Apoptotic cells and bodies also possessed a reduced number of AgNORs, which occasionally disappeared (Figure 5).

After staining for RNA, nucleoli in untreated cells appeared as relatively uniform bodies, which, however, possessed less stained small areas corresponding to fibrillar centres. Such small areas were more apparent in not-apoptotic cells cultured with TSA (Figure 6). The mean density of nucleoli was similar in both TSA untreated or treated not-apoptotic and apoptotic cells (Figures 1 and 2, Table 1). However, the variability of measured values in individual treated cells was large and the variability coefficient was about 25 per cent for not-apoptotic and 15 per cent for apoptotic cells (see also the



**Figure 3.** Numerous AgNORs (arrow) in a TCA untreated cell. **Figure 4.** In comparison with untreated cells (see Figure 3), the TCA treated cell contains a reduced number of enlarged AgNORs in the larger nucleolus (thick arrow). **Figure 5.** The absence of AgNORs (arrow) in an apoptotic body. **Figure 6.** A TCA treated not-apoptotic cell containing a nucleolus (arrow) with light areas, which appeared more distinct in the insert after enlargement and high contrast printing.

standard deviation, Table 1). In contrast, the cytoplasmic density in apoptotic cells or bodies was significantly smaller than in TSA untreated or treated but not-apoptotic cells (Figures 1 and 2, Table 1). Such decrease of the cytoplasmic density in apoptotic TSA treated cells was reflected by the significantly increased of nucleolar to cytoplasmic density ratio of apoptotic cells and bodies (Table 1). At this occasion it should be noted that the variability of measured values of the cytoplasmic RNA density was also large in both treated not-apoptotic and apoptotic cells or bodies. In addition, the RNA density within the cytoplasm of apoptotic cells exhibited a large variability due to less or more stained areas (Figure 2). The variability coefficient calculated for mean cytoplasmic RNA density was much higher in both groups treated not-apoptotic (about 30%) and apoptotic cells (about 25%) in comparison with untreated ones (about 8%).

The nucleolar mean diameter in specimens stained for RNA appeared to be only slightly decreased in TCA treated not-apoptotic and apoptotic cells or bodies. However, in comparison with TCA untreated cells, the slightly decreased nucleolar size in not-apoptotic and apoptotic cells or bodies was not significantly different (Table 1).

**Table 1.** The nucleolar size, nucleolar and cytoplasmic RNA density.<sup>§</sup>

Cells	RNA density		No/Cy RNA density	Dia ( $\mu$ m)
	No	Cy		
TSA untreated	98.3 $\pm$ 8.1*	69.3 $\pm$ 7.3	1.3 $\pm$ 0.1	2.5 $\pm$ 0.4
TSA treated not-apo	102.8 $\pm$ 26.3	76.4 $\pm$ 30.0	1.3 $\pm$ 0.5	2.3 $\pm$ 0.4
apo	97.9 $\pm$ 15.9	55.2 $\pm$ 25.9*	1.9 $\pm$ 0.6*	2.2 $\pm$ 0.3

<sup>§</sup>based on 300 measurements; \*mean and standard deviation; \*significantly different from untreated and treated not-apoptotic cells using t-test ( $p < 0.02$ )  
No, Nucleoli; Cy, Cytoplasm; Dia, mean nucleolar diameter per cell; not-apo, not-apoptotic cells; apo, apoptotic cells.

## Discussion

The present study demonstrated that the nucleolar RNA density in not-apoptotic and apoptotic cells was similar in contrast to the cytoplasmic one. On the other hand, the nucleolar biosynthetic or cell proliferation activities of apoptotic cells or bodies were apparently reduced and abolished demonstrated by the decreased number of AgNORs (Derenzini, 2000; Giuffrè *et al.*, 2006; Morimoto *et al.*, 2001; Smetana *et al.*, 2004). Thus it seems to be possible that the transcribed RNA remained *frozen* within the nucleolus but its transport to the cytoplasm might decrease or stop. Such explanation is also supported by the markedly decreased cytoplasmic RNA density, which was reflected by the increased nucleolar to cytoplasmic RNA density ratio. However, the previously reported degradation of cytoplasmic RNA in altered or apoptotic cells (Delic *et al.*, 1993) on the base of the present study also cannot be eliminated and the variability of the RNA density within the cytoplasm does not contradict such possibility. In contrast, the release of RNA at the surface of apoptotic cells (Biggiogera *et al.*, 1998) was not observed because of the absence of an increased RNA density at the cell periphery.

According to presented results in apoptotic cells or bodies without decreased nucleolar RNA density, the nucleolar mean diameter was also not substantially influenced. Thus in contrast to naturally occurring apoptosis or other morphological expression of the apoptotic process (see Biggiogera *et al.*, 1998; 2004; Martelli *et al.*, 2001; Pellicciari *et al.*, 2000; Smetana, 2002), the apoptotic process induced by a strong cytostatic agent in the present study did not influence both the nucleolar RNA density and size. The similar nucleolar RNA density in both not-apoptotic and apoptotic cells was also noted after photodynamic treatment but the cytoplasmic RNA density was not measured at that time (Smetana *et al.*, 2004). Therefore, large nucleoli intensely stained for RNA need not be necessarily related to the nucleolar large biosynthetic or cell proliferation activity and may be also present in apoptotic cells responding to the cytostatic treatment. At this occasion it should be mentioned that AgNORs were reduced in number and/or absent after TSA treatment. Moreover, mitotic cells in TSA treated cultures were not present and the incidence of mitotic cells was generally considered as a tool for the evaluation of anti-tumour agents (Ikeda *et al.*, 2000).

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