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ORIGINAL ARTICLE

A morphological and cytochemical note to the reversible intranucleolar translocation of AgNORs (silver stained nucleolus organizer regions) in early leukemic granulocytic progenitors represented by cultured K 562 cells

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Summary
The present study was undertaken to provide more information on the nucleolar size, number and distribution of AgNORs (silver stained nucleolus organizer regions) in nucleolar bodies of aging leukemic granulocytic progenitor cells represented by cultured K562 cells. In these aging and starving cells without further differentiation and with translocated AgNORs to the nucleolar periphery, the diameter of the nucleolar size was reduced together with a reduction in the number of these nucleolar compartments. However, after re-feeding cell cultures, the nucleolar size increased again. Similarly, the number of AgNORs also increased and was distributed in the whole nucleolar body as in control cells. Such cells apparently entered into the cycle and proliferation documented by the re-occurrence of mitotic divisions in studied cultures, an increased number of AgNORs and increased nuclear size. This observation suggests that early granulocytic precursors with translocated nucleolar AgNORs might represent a transitionally quiescent population of cells which may return to a fully proliferating state.

Keywords: AgNORs – intranucleolar translocation – nucleolar size – K 562 cells

INTRODUCTION

According to numerous studies a large nucleolar diameter is related to high cell proliferation and nucleolar biosynthetic activities (Derenzini et al. 1992, Keller 1963, Smetana 2005). However, a few studies also indicate that large nucleoli may be present in cells which are in an apoptotic state (Smetana et al., 2000, see Smetana 2005). In addition, it seems to be also clear that large amounts of silver stained nucleolar particles representing AgNORs (silver stained nucleolar organizer regions) are very convenient markers of a high nucleolar biosynthetic and cell proliferation activity (see Derenzini, 2000, Trere et al. 1989, Smetana 2005). On the other hand, the distribution of AgNORs within the nucleolar body of such nucleoli has been less well studied. Recent studies in the authors laboratory noted that AgNORs in large nucleoli of aging cultures of granulocytic progenitors cells, translocated to the nucleolar periphery. At this occasion it should be noted that AgNORs translocated to the nucleolar periphery of granulocytic progenitors were also observed in vivo in patients suffering from myeloid leukemias

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(Smetana et al. 2005a, b) and particularly, in those treated with cytostatic therapy.

The present study was undertaken to provide more information on the nucleolar size, number and distribution of AgNORs in nucleolar bodies in aging leukemic granulocytic progenitor cells. Cultured K562 cells were a very convenient model for such a study because they originate from early leukemic granulocytic precursors and apparently have not lost the differentiation ability and may differentiate not only to granulocytes but also to erythroid cells (Lozzio et al. 1981). The results of the present study demonstrated that in these aging and starving cells without further differentiation and with translocated AgNORs the diameter of the nucleolar size was reduced together with the reduced number of these nucleolar compartments. However, after re-feeding cell cultures, the nucleolar size increased again. Similarly, the number of AgNORs also increased and was distributed in the whole nucleolar body. Such cells apparently entered the cycle and proliferation documented by the re-occurrence of mitotic divisions in studied cultures, increased number of AgNORs and increased nuclear size. This observation suggests that early granulocytic precursors with translocated nucleolar AgNORs might represent transitionally quiescent population of cells which may return to fully proliferating state.

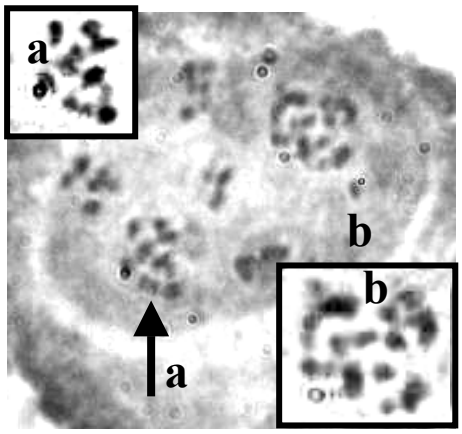

Fig. 1. **Proliferating control cell. Nucleoli consist of numerous small AgNORs which appear as intensely stained particles interconnected by less stained strands.** Nucleoli marked by arrows are magnified and printed with a larger contrast in inserts. Magnification approximately 4 200x, insert a - 5 300, insert b - 6000x.

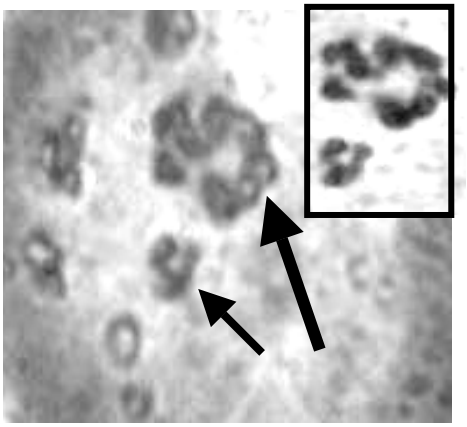

Fig. 2. **Aging cell. AgNORs in a slightly over-focused nucleolus (large arrow) appear as white bodies.** The insert contains the same nucleolus which consists of fusing dense enlarged AgNORs.
Magnification approximately 6 600x.

MATERIAL AND METHODS

Nucleoli and AgNORs were studied in unfixed cytospins of K 562 (see below) which were prepared using Shandon II Cytocentrifuge (Shandon Southern Products, UK) – 6000 RPM for 10 min. Nucleolar bodies were visualized by acidified methylene blue for demonstration of RNA (Smetana et al. 1969). The nuclear chromatin structure and perinucleolar chromatin were visualized in methanol fixed cytospins by acidified methylene blue after hydrolysis with HCl (see Busch and Smetana 1970). AgNORs were visualized by the silver reaction under conditions facilitating clear view of silver particles within nucleolar bodies (Ochs 1998, Smetana et al. 1999).

K 562 cells (European Collection of Animal Cell Cultures, Salisbury, UK) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 50µg streptomycin at 37˚ C in 5% humidified atmosphere. Cultures of these cells were fed by dilution in fresh medium to a density $2x10^5$ per ml three times a week. Aging cells were harvested after 72 hours of cultivation without feeding. Originally aging cells were re-fed 24 hours later (see Smetana et al. 2005b).

Micrographs were taken with a Camedia digital photocamera C-4040 ZOOM (Olympus, Japan) placed on a Jenalumar microscope (Zeiss, Germany) with a special mechanical adapter. The resulting images were processed with Quick Photoprogram (Olympus, Japan) in combination with L-view and Power Point Microsoft programs. (Microsoft, USA). The nuclear and nucleolar diameters were measured using Quick Photoprogram. Phase contrast microscopy was used to facilitate the measurement of the nuclear as well as nucleolar diameter and cells containing nucleoli larger than ring shaped nucleoli or micronucleoli (see Smetana 2005), i.e., larger than 1.3 µm were considered for evaluation.

The data are expressed as the mean ± SD, the two-sided t-test at the significance level $2\alpha=0.05$ was used.

Table 1. **The largest nuclear and nucleolar diameter (µm)[a].** Silver reaction for AgNORs and phase contrast microscopy

Nuclei	Nucleoli	Cells
14.92 (1.01)[b]	2.63 (1.00)*#	aging
12.72 (1.82)*#	2.23 (0.72)*#	aging – AgNORs translocated.
14.78 (2.83)	3.75 (0.91)	aging + re-fed
14.44 (1.36)	3.77 (0.81)	control

*- significant difference in comparison with control cells
- significant difference in comparison with starving and re-fed cells
[a] - based at least on 200 measurements in each group
[b] - mean (standard deviation)

Table 2. **The incidence of mitoses, apoptotic cells and AgNORs (per cell and nucleolus)[a]**

DNA staining			Silver reaction for AgNORs		
Mitoses	Apoptotic Nu	Swollen Nu	AgNORs/Cell	AgNORs/No	Cells
0.5 ((0.7) [b] *#	5.0 (1.6)*	2.5 (0.5)	11.4 (3.0)*#	2.7 (0.9)*#	aging
9.0 (2.4) *	3.0 (1.4)	0.6 (0.9)*	34.7 (12.0)*	5.6 (2.2)*	aging + re-fed
3.0 (0.8)	2.6 (0.4)	3.3 (2.0)	44.0 (6.6)	8.4 (2.4)	control

* - significant difference in comparison with control cells
# - significant difference in comparison with starving and re-fed cells
[a] - based on at least 200 measurements in each group
[b] - mean (standard deviation)

RESULTS

Proliferating control cells

The size of large nucleoli (>1 μm) was dependent on the visualization procedure used. The largest mean values determined for diameter of such nucleoli were noted after staining for DNA (4.67, Standard Deviation 0.35 μm). In contrast, the smallest mean values were noted for such nucleoli after staining for RNA (3.00, S.D. 0.77 μm) which actually visualized the nucleolar body without the perinucleolar chromatin.

After silver reaction and phase contrast microscopy facilitating a clear view of the nuclei and AgNORs within large nucleoli, the mean values of nuclear diameter were ranging from 13 to 16 μm and that of nucleoli from 3 to 5 μm (Table 1). At this occasion it should be noted that the measurements of the nuclear and nucleolar diameter with AgNORs in combination with phase contrast microscopy appeared to be optimal and was used for comparison of all groups of studied cells. The number of AgNORs per cell and nucleolus was very high, i.e. on average 44 silver particles per nucleus and 8.44 per nucleolus (Table 2). AgNORs were distributed in the whole nucleolar body and as described previously (Ploton et al. 1987), they were interconnected by fewer silver stained strands (Fig. 1). The incidence of mitotic divisions, apoptotic cells and swollen nuclei seen in specimens stained for DNA was relatively small (Table 2). However, the incidence of mitotic cells was markedly higher than in aging cells.

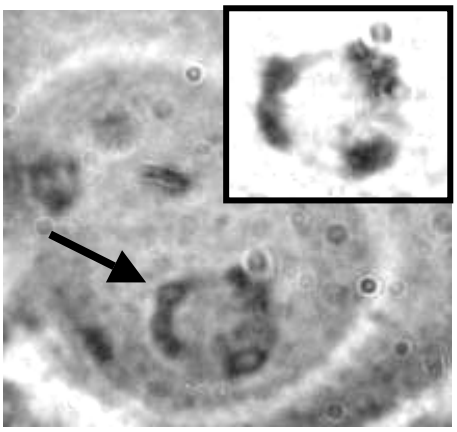

Fig. 3. **Aging cell. Enlarged AgNORs are translocated in the peripheral part of the nucleolus (arrow) which is magnified and printed with a larger contrast in the insert.** Magnification approximately 6 000x, insert 8 600x.

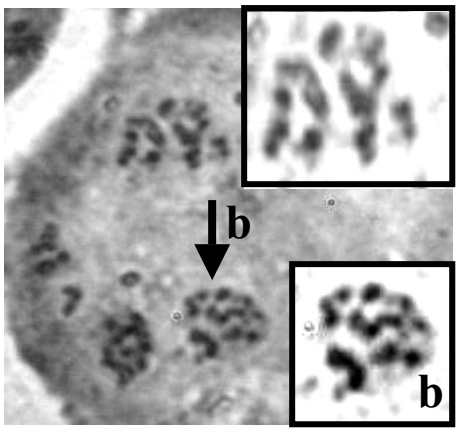

Fig. 4. **Re-fed cell.** Nucleoli possess numerous small AgNORs interconnected by less stained strands. Arrows indicate nucleoli which are magnified and printed with a larger contrast in inserts. Magnification approximately 4500x, insert a - 6 300x, insert b - 5 800x.

Aging not- proliferating cells

In aging cultures mitotic cells almost disappeared and did not reach 1 per cent (Table 2). The number of apoptotic cells and bodies was also small but larger than in controls. Blastic cells did not exhibit any morphological signs of further differentiation. The number of AgNORs per cell as well as nucleolus was markedly reduced (Table 2) and their intranucleolar distribution changed. AgNORs were mostly translocated to the periphery of the nucleoli (Fig. 2) the diameter of which was slightly but significantly decreased (Table 1). The diameter of nucleoli measured in specimens stained for DNA (3.08, S.D. 0.58 μm) and RNA (1.75, S.D. 0.56 μm) was also smaller than in proliferating control cells. In addition, the number of AgNORs is such nucleoli were also significantly smaller (Table 1). AgNORs translocated to the nucleolar periphery were apparently larger and frequently fused (Fig. 2, 3). At this occasion it should be also mentioned that the nuclear diameter in cells with reduced and translocated AgNORs was also slightly reduced in comparison with control or re-fed cells (Table 1).

Re-fed proliferating cells which originally aged

In re-fed cells the number of mitotic cells markedly increased in comparison with aging and control cells (Table 2). The incidence of apoptotic cells and swollen nuclei was very limited (Table 2). The number of AgNORs per nucleus as well as per nucleolus was large again in comparison with aging cells but did not reach the values in control cells (Table 2). It must be mentioned that AgNORs were mostly distributed in the whole nucleolar body and interconnected by fewer silver stained strands (Fig. 4) as in the control cells (Fig. 1). The nucleolar and nuclear diameter increased again and practically returned to values determined for control cells (Table 1). It should be also noted that the nucleolar diameter in specimens stained for DNA (3.93, S.D. 0.93) and RNA (3.78, S.D. 0.97) was also increased in comparison with aging not-proliferating cells (see above).

DISCUSSION

The presented study demonstrated that the reversible intranucleolar translocation of AgNORs due to the cell aging produced by starvation is accompanied by changes of both nucleolar and nuclear diameter. On the other hand, the slightly but significantly reduced nucleolar diameter in aging cells was still larger, i.e. above 1.3 μm, than that of resting ring shaped nucleoli and especially micronucleoli. The latter are known to reflect the terminal differentiation and maturation which represents the pre-apoptotic cell state (Smetana 2005). Such a state, however, was not noted in aging cultures although studied cultured cells are known to retain differentiation ability (Lozzio et al. 1981). Moreover, the incidence of apoptotic cells in studied aging cultures was very small.

On the other hand, the decreased number of translocated and enlarged AgNORs in aging cells apparently indicated the reduced nucleolar biosynthetic and cell proliferation activity. The reduced number of enlarged AgNORs in a variety of cells, similarly as demonstrated by previous studies, reflects both these events (see Smetana 2005). In addition, the simultaneous reduction of mitotic divisions in aging cultures observed in the present study is in agreement with such conclusions. The reversibility of described changes seems to be not only interesting but might be important from the clinical point of view since translocated AgNORs were also frequently noted in leukemic granulocytic progenitors of patients receiving cytostatic therapy (Smetana et al. 2005a).

The decrease of both the nucleolar and nuclear diameter in aging and starving cells might be related to the reversible cell arrest possibly in the G0/1 phase of the cell cycle as noted in the previous study (Smetana et al. 2005b). The larger nuclear and nucleolar diameter of proliferating cells in control or re-fed cultures supports such a conclusion. It is generally known that in the course of the cell cycle both the nucleolar and nuclear size increases in S phase reaching the maximum in the G2 phase due to the DNA replication and nucleolar fusion (Gonzalez and Nardone 1968, Schnedel and Schnedel 1972).

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