Original Articles

Intranucleolar Translocation of AgNORs in Early Granulocytic Precursors in Chronic Myeloid Leukaemia and K 562 Cells

(AgNORs / translocation / granulocytic precursors / chronic myeloid leukaemia)

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Abstract. The present study was undertaken to provide missing information on the distribution of AgNORs in large nucleoli of human leukaemic early granulocytic precursors *in vivo* as well as *in vitro*. *In vivo*, the distribution of AgNORs was studied in early granulocytic precursors of patients suffering from chronic myeloid leukaemia who were both untreated and treated with imatinib mesylate. AgNORs were visualized by silver reaction under conditions which facilitated to see their distribution by light microscopy. *In vitro*, the distribution of AgNORs was studied in proliferating and ageing K 562 cells which originated from chronic myeloid leukaemia. *In vitro*, the ageing of K 562 cells produced intranucleolar translocation of AgNORs to the nucleolar periphery. Such translocation was also observed in some leukaemic early granulocytic precursors *in vivo*, e.g. in bone marrow myeloblasts and promyelocytes of leukaemic patients. As was expected, the intranucleolar translocation of AgNORs in early granulocytic precursors was more frequent in patients treated with the cytostatic therapy – imanitib mesylate. The abovementioned findings suggest that myeloblasts and promyelocytes with AgNORs translocated to the periphery of large nucleoli might be in the ageing state, similarly as blastic cells of leukaemic myeloid origin (K 562 cells) in ageing cultures. Thus, the translocation of AgNORs might be a useful marker of premature ageing in the future and might contribute to the evaluation of the single cell state under various experimental as well as clinical conditions. However, more clinically oriented studies are required in this direction.

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Abbreviation: AgNORs – silver-stained nucleolus organizer regions.

As is generally known, AgNORs (silver-stained nucleolus organizer regions) are markers of nucleolar biosynthetic and cell proliferation activities (Grotto et al., 1991; Derenzini, 2000; Smetana, 2002). AgNORs represented by silver-stained particles seen by light microscopy correspond to fibrillar centres with adjacent nucleolar regions in the electron microscope (Wachtler and Stahl, 1993; Smetana, 2002), which are sites of the rRNA transcription and storage of rDNA, as well as proteins participating in this process (Wachtler and Stahl, 1993; Hozák et al., 1994; Raška, 2003).

It has also been reported that some of these proteins responsible for the reaction with silver in leukaemic myeloblasts translocate out of the nucleolus. Such translocation was apparently related to the reduction of nucleolar biosynthetic and cell proliferation activities as well as to the apoptotic process (Busch, 1997; Chan and Chan, 1999). On the other hand, the translocation of AgNORs in leukaemic blastic cells has not been reported. Therefore, the present study was undertaken to provide information on the distribution of AgNORs in these cells *in vitro* as well as *in vivo*.

The results clearly demonstrated that AgNORs in leukaemic granulocytic precursors translocated to the nucleolar periphery, where they formed a ring-like structure. Such translocation of AgNORs was easily produced by ageing in cultures of blastic leukaemic granulocytic precursors (K 562) *in vitro.* Such translocation of AgNORs in some leukaemic early granulocytic precursors was also observed *in vivo* in both untreated as well as treated leukaemic patients with the cytostatic therapy. These observations suggest that leukaemic early granulocytic precursors, i.e., blastic cells and promyelocytes with translocated AgNORs in the periphery of large nucleoli might be in the ageing state, similarly as blastic cells of leukaemic myeloid origin in ageing cultures. Thus, the translocation of AgNORs might be a useful marker in the future and

might contribute to the evaluation of the single cell state under various experimental as well as clinical conditions.

Material and Methods

Nucleoli were visualized by acidified methylene blue for demonstration of RNA and silver reaction for AgNORs in unfixed smears or cytospins under defined conditions as described previously (Smetana et al., 1969; Ochs, 1998; Smetana et al., 1999). The distribution of AgNORs was studied only in nucleoli larger than 1 μm measured using Quick PHOTO Program (Olympus, Tokyo, Japan). Such "large" nucleoli were characterized by the presence of multiple AgNORs in comparison with smaller ring-shaped nucleoli or micronucleoli, which usually possess only one AgNOR (Grotto et al., 1991, 1993; Smetana, 2002).

In vitro, the distribution of AgNORs was evaluated in large nucleoli (>1 μm) of mononuclear leukaemic granulocytic blastic precursors represented by K562 cells originating from leukaemic early granulocytic precursors (European Collection of Animal Cell Cultures, Salisbury, UK). These cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 U/ml penicillin and 50 μg/ml streptomycin at 37° C in 5% humidified atmosphere. Control cultures "were fed" by dilution with fresh medium to a density 2×10^5 per ml three times a week. Ageing cultures without "feeding" were kept for 3 to 7 days in the same unchanged medium. The cell division arrest occurred after 72 h of cultivation. Harvested cells were investigated in cytospins using a Shandon II cytocentrifuge (Shandon Southern Products, UK) – 6000 RPM for 10 min.

In vivo, the distribution of AgNORs in large nucleoli (> 1 μm) of leukaemic granulocytic precursors, i.e. myeloblasts and promyelocytes, was evaluated in smears of diagnostic bone marrow biopsies taken from 10 patients suffering from chronic phase of chronic myeloid leukaemia (5 were untreated and 5 treated with cytostatic therapy – imatinib mesylate). The use of the clinical material was approved by the ethic board of the Institute.

Micrographs were taken with a Camedia digital photocamera C-4040 ZOOM (Olympus, Japan) placed on a Jenalumar microscope (Zeiss, Germany). The resulting images (Quick Photo Program - see above) were further magnified, processed and printed using L-view and Power Point Microsoft programs (Microsoft, Redmond, Ireland). Phase contrast microscopy was used to facilitate the identification of nuclei in cultured cells and early granulocytic precursors such as myeloblasts or promyelocytes in bone marrow smears.

Results

RNA distribution in large nucleoli of early granulocytic precursors

Mononuclear blastic cells and promyelocytes in bone marrow of leukaemic patients as well as in both control and ageing cultures were mostly characterized by the presence of large nucleoli with more or less uniform distribution of RNA as described in numerous previous studies (see Smetana, 2002).

The distribution of AgNORs in large nucleoli of cultured leukaemic mononuclear granulocytic precursors – K562 cells (Fig. 1)

In control cells, similarly as in other proliferating blood cells, AgNORs were distributed in the whole nucleolar body, which appeared as a cluster of small

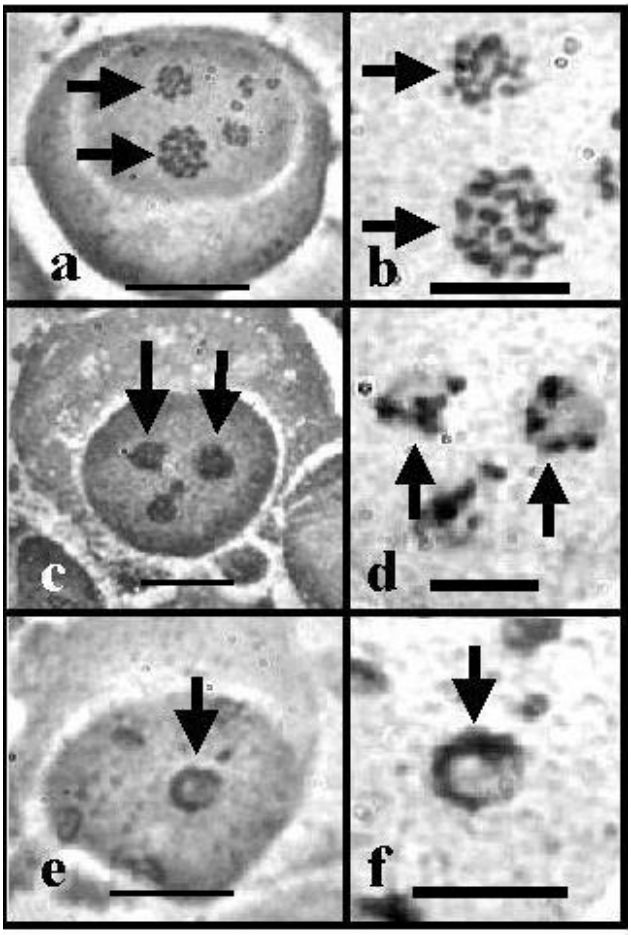

Fig. 1. Blastic (K562) cells before (a, b) and after ageing of the culture (c–f) stained with silver for AgNORs seen by phase contrast (a, c, e) and simple transmission light microscopy (b, d, f). In ageing cells AgNORs translocated to the nucleolar periphery (c, d), where they apparently fused and formed an intensely stained uncomplete ring (e, f). Arrows indicate nucleoli which are magnified with slightly increased contrast in parts b, d, f. Thin bars – 10 μm, thick bars – 5 μm. Magnification approx. 1 200x (a); 500x (b); 1 100x (c); 2 200x (d); 1 200x (e); 3 000x (f).

silver-stained particles (Fig. 1a, b). In ageing cultures, AgNORs in large nucleoli were frequently larger and apparently reduced in number (Table 1). The reduced number of AgNORs translocated to the nucleolar periphery (Fig. 1c, d). Occasionally, AgNORs in the periphery of large nucleoli appeared to fuse and form a silver-stained more or less complete ring (Fig. 1e, f).

The distribution of AgNORs in large nucleoli of early granulocytic precursors in bone marrow of leukaemic patients (Fig. 2)

Large nucleoli in these cells, i.e. in myeloblasts (Fig. 2a) and promyelocytes (Fig. 2c), mostly appeared as clusters of silver particles representing AgNORs which were distributed in the whole nucleolar body (see Grotto et al., 1991, 1993; Smetana et al., 2000; Smetana, 2002). On the other hand, in some myeloblasts and promyelocytes, a reduced number of frequently enlarged AgNORs (Table 1) translocated to the nucleolar periphery (Fig. 2b). In addition, in a few large nucleoli, AgNORs in the nucleolar periphery appeared to be fused and frequently formed a silver-stained ring similarly as in ageing cultured blasts (see above). In patients treated with the cytostatic therapy – imatinib mesylate - the percentage of these cells exhibiting the intranucleolar translocation of AgNORs (Fig. 2d) was larger (41.2, S.D. ± 14.8) than in those who did not receive the cytostatic therapy at the time of taking samples for the present study (22.0, S.D. ± 9.4; $P < 0.05$).

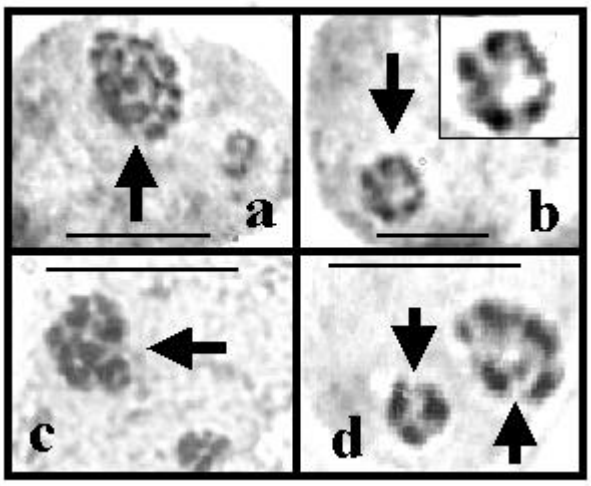

Fig. 2. Nucleoli (arrows) in leukaemic early granulocytic precursors stained with silver for AgNORs seen by phase contrast (a, b) and simple transmission light microscopy (b-insert, c, d). Nucleoli appear as clusters of silver-stained particles (AgNORs) distributed in the whole nucleolar body (a, c). Blastic cell with a nucleolus containing a reduced number of enlarged and translocated AgNORs in the nucleolar periphery (b). Blastic cell with two nucleoli containing translocated enlarged AgNORs in the nucleolar periphery of a patient treated with imatinib. Thin bars – 5 μm. Magnification approx. 2 900x (a); 2 300x, insert 3 300x (b); 3 800x (c, d).

*Table 1. The number of AgNORs in large nucleoli with and without translocation in K562 blastic cells and early granulocytic precursors in bone marrow of leukaemic patients**

Cells	The number of AgNORs in nucleoli	
	without translocation	**with translocation**
K562	**7.8** ± 0.9‡	**3.8** ± 1.2§
Mybl + Promyelo†	**9.2** ± 1.4	**3.8** ± 1.4§

* - based at least on 60 measurements in each group
† - myeloblasts and promyelocytes in bone marrow biopsies
‡ - **mean** and standard deviation
§ - significant difference in comparison with large nucleoli without translocation of AgNORs using t–test ($P < 0.001$)

Discussion

The present observations clearly demonstrated the translocation of AgNORs to the nucleolar periphery in large nucleoli of blastic cells in ageing cultures originating from leukaemic granulocytic precursors. In addition, translocated AgNORs apparently may fuse and form silver-stained ring in the periphery of the nucleolar body. Both these newly observed phenomena were also noted in early granulocytic precursors of leukaemic patients suffering from chronic phase of chronic myeloid leukaemia and especially after cytostatic therapy. All these observations suggest that leukaemic early granulocytic precursors, i.e., blastic cells and promyelocytes with translocated and reduced number of AgNORs in the periphery of large nucleoli, might be in the ageing state similarly as blastic cells of leukaemic myeloid origin in ageing cultures. Thus, the translocation of AgNORs might be a useful and simple marker of premature cell ageing. These discussed present observations are consistent with previous studies which demonstrated that reduced numbers of AgNORs (Smetana et al., 2000), similarly as translations of some silver-stained nucleolar proteins from the nucleolus (see Busch, 1997; Chan and Chan, 1999), are related to the decreased nucleolar biosynthetic and cell proliferation activity as well as to the cell ageing and programmed cell death (see Kitano and Imai, 1998; Comai, 1999; Morimoto et al., 2001; Campisi, 2001; Smetana, 2002). On this occasion it should be noted that the possibility of ageing of some leukaemic blastic cells without further development was also suggested previously. However, such conclusion was based on a completely different approach and data (Gavosto, 1964).

In the present study, the number of early granulocytic precursors such as myeloblasts and promyelocytes exhibiting the translocation of AgNORs was significantly larger in patients suffering from chronic phase of chronic myeloid leukaemia treated with the cytostatic therapy - imatinib mesylate - than in untreated patients.

Thus, this cytostatic therapy apparently increased the number of ageing leukaemic granulocytic precursors since the translocation of AgNORs appeared to be related to this process as demonstrated *in vitro* (see above). On the other hand, more studies should be made on the translocation of AgNORs in large nucleoli of leukaemic cells, which should be related to the clinical state of patients or the leukaemia type and especially to the cytostatic therapy. The only reason for the presented report was to attract attention to the translocation of AgNORs, which might be a useful and simple marker of the cell ageing in the future and might contribute to the evaluation of the single cell state under various experimental as well as clinical conditions.

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