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Nucleolar organizer regions of megakaryocytes in chronic myeloproliferative disorders

András Matolcsy, Roland Nádor, Edit Wéber, and Tibor Kónya

Department of Pathology, University Medical School of Pécs, Szigeti ut 12, H-7643 Pécs, Hungary

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Summary. To study megakaryocyte activation, the argyrophilic staining method of nucleolar organizer regions (AgNOR) has been applied to decalcified bone marrow biopsies of 16 individuals with no haematopoietic disorders and 59 patients with chronic myeloproliferative disease. Of the 59 patients, 18 had chronic myeloid leukaemia (CML), 21 chronic megakaryocytic granulocytic myelosis (CMGM), 13 polycythaemia vera (PV) and 7 essential thrombocythaemia (ET). The AgNOR number of megakaryocytes in CML was significantly lower, and in CMGM, PV and ET significantly higher than in healthy individuals. The high number and the clusters of fine-grained AgNORs of megakaryocytes in CMGM, PV and ET are suggestive of active, proliferating cells. The AgNOR number of megakaryocytes and the platelet counts of the patients did not show a convincing correlation. In CMGM, PV and ET the pyknotic, heterochromatinized megakaryocytes with narrow rims of cytoplasm called bare (nude) nuclei, possessed few, large AgNOR granules. The AgNOR staining of bare nuclei and the roughly identical number of granules found in CMGM, PV and ET indicate a common, active mechanism of apoptosis.

Key words: Chronic myeloproliferative disease – Megakaryocyte – Nucleolar organizer region

Introduction

Chronic myeloproliferative disorders (CMPD), including chronic myeloid leukaemia (CML), chronic megakaryocytic granulocytic myelosis (CMGM) or agnogenic myeloid metaplasia, polycythaemia vera (PV) and essential thrombocythaemia (ET), are haematopoietic stem cell disorders characterized by clonal, neoplastic proliferation of erythroid, myeloid and megakaryocytic lineages (Burkhardt et al. 1984, 1986; Georgii et al. 1980, 1990). Hyperplasia and abnormalities of the megakaryocytes are one of the most conspicuous histological features of CMPDs. Morphometric and immunohistological studies of bone marrow biopsies have revealed different densities, distributions and a wide variety of megakaryocytes with dysplastic morphology such as giant, pleiomorphic, immature, micro or dwarf megakaryocytes, pro-megakaryoblasts and bare (naked) nuclei (Thiele et al. 1983, 1987, 1988, 1990a, b). Although the striking morphological heterogeneity of the dysplastic megakaryocytes is documented their biology, activity and function are less well understood. The in vivo observation of megakaryocytes is imperfect, and the in vivo models currently in use do not come close enough to nature (Grossman and Levine 1986).

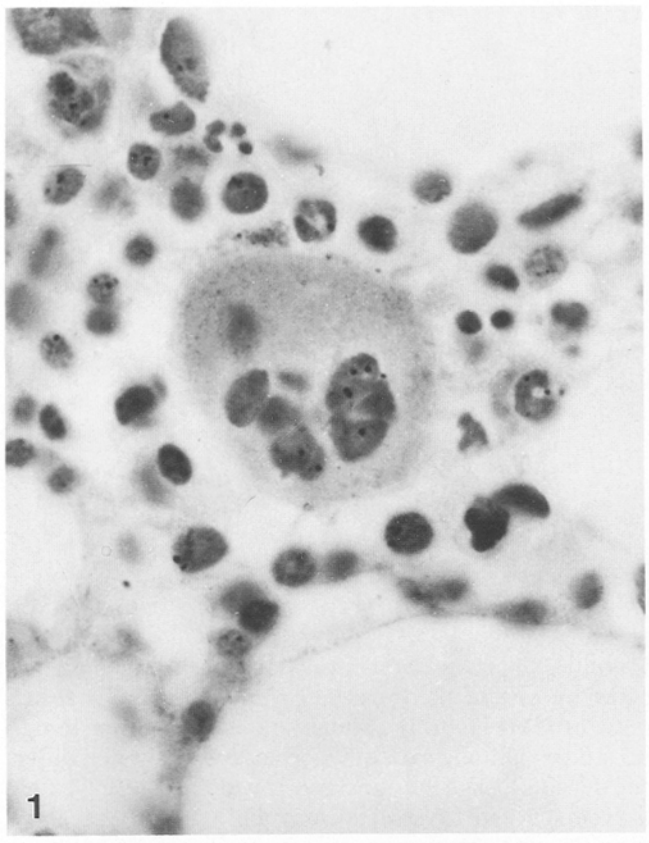
The argyrophilic staining method of nucleolar organizer regions (AgNOR) (Ploton et al. 1986), which reveals loops of DNA encoding for ribosomal RNA, gives information about the synthesis of ribosomes and hereby about the protein synthesis of the cells (Trere et al. 1989). Using this simple staining method on bone marrow biopsies an insight might be gained into the pathophysiology of the different megakaryocytes in CMPD sub-categories as compared with normal megakaryocytes.

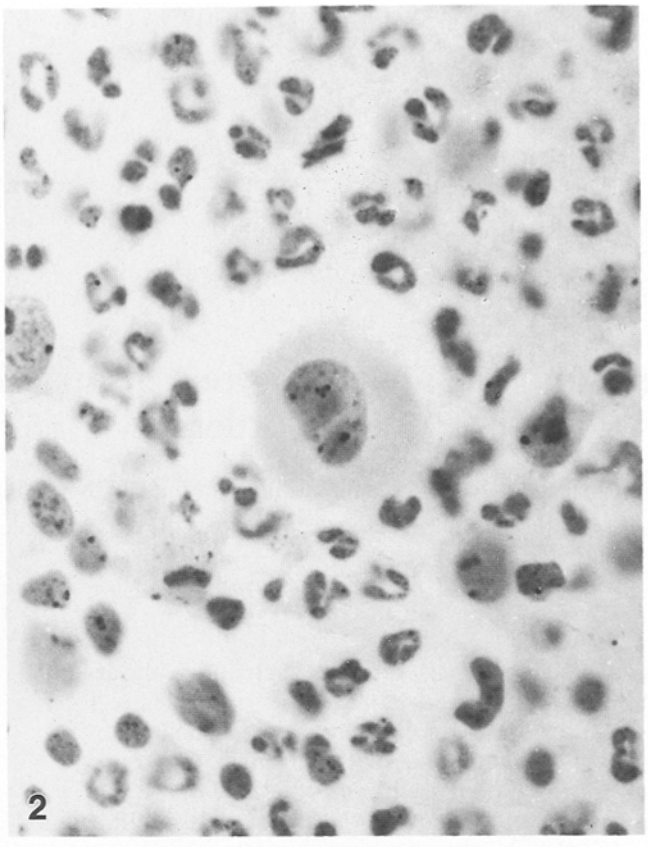
Materials and methods

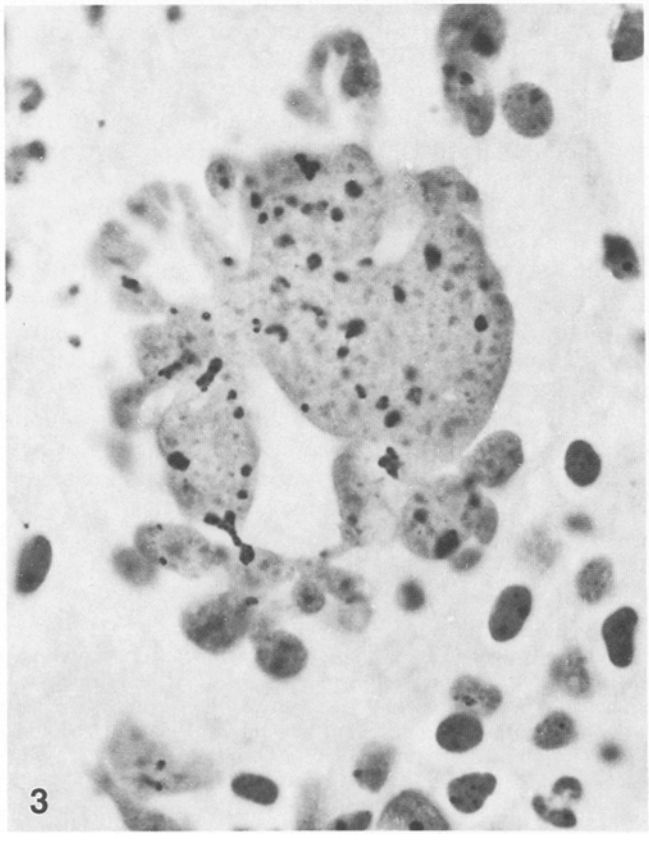
For this study bone marrow biopsies were selected from 59 untreated CMPD patients and 16 individuals who at first were suspected of haematopoietic diseases, but later proved to be healthy. Decalcification, paraffin embedding and a number of staining methods (Giemsa, Gömöri's silver impregnation and the periodic-acid-Schiff reaction) were included in the processing of bone marrow biopsies (Schaefer 1984). The classification of CMPD sub-categories was based on accepted clinical and histological criteria (Georgii et al. 1990). The CMPD cases comprised 18 patients with CML in the chronic phase, 21 patients with CMGM, 13 patients with PV and 7 patients with ET.

Paraffin sections (3 μm) were cut and dewaxed in xylene and hydrated through graded ethanols. The AgNOR staining solution, prepared as described by Crocker and Nar (1987), was poured over the sections and left for 40 min at room temperature in the

Offprint requests to: A. Matolcsy







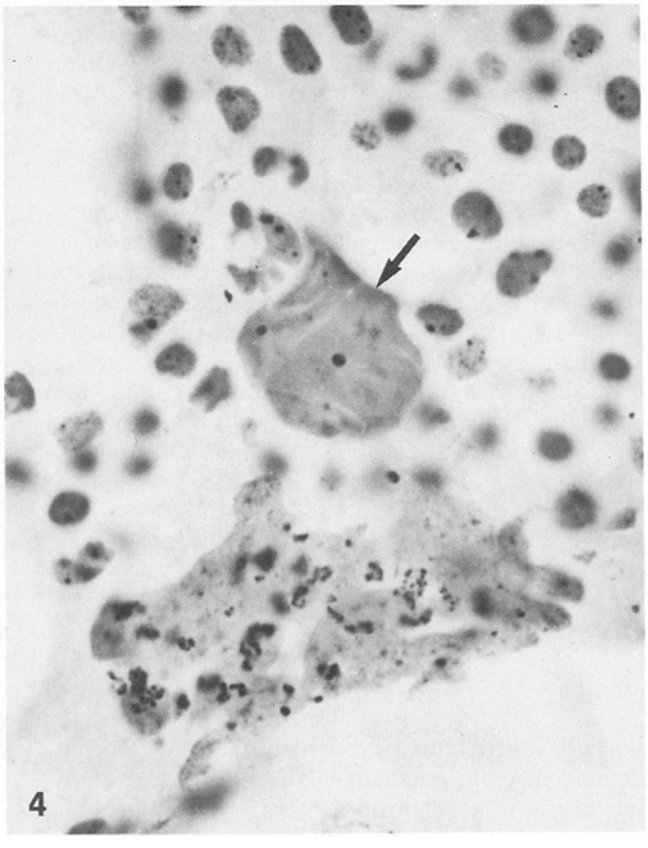


Fig. 1. Megakaryocyte of healthy individual. Single AgNOR granules are present. ×100

Fig. 2. Megakaryocyte of patient with chronic myeloid leukaemia (CML). The small mononuclear megakaryocyte contains few AgNOR granules. ×100

Fig. 3. Megakaryocyte of patient with essential thrombocythaemia (ET). The giant, hypersegmented nucleus contains clusters of small AgNOR granules. Clusters may contain up to 20 small, fine granules. ×100

Fig. 4. Megakaryocyte and bare (nude) nucleus in chronic megakaryocytic granulocytic myelosis (CMGM). The dysplastic, giant megakaryocyte reveals several clusters of AgNOR granules (*bottom*). The bare nucleus shows single, large AgNOR granules (*arrow*). ×100

dark. Then the staining solution was washed off and the sections were first counterstained with neutral red and subsequently dehydrated to xylene and mounted in synthetic medium.

The stained sections were examined by two observers without knowledge of the histological diagnosis. The number of AgNORs in a minimum of 50 megakaryocytes and 50 bare nuclei, selected by use of a random number table, were counted. In cases of small pieces of core biopsies several series of slides were cut and stained to enumerate the AgNORs of 50 megakaryocytes and 50 bare nuclei. Sections were examined using a $\times 100$ oil immersion objective equipped with a green colour filter to reduce chromatic aberration and increase the clarity of NOR perimeters.

The data were analysed by means of Student's paired t-test.

Results

Using the argyrophilic staining method to detect AgNORs, clearly defined "dots" of varying number and size were seen in all nuclei. Single AgNOR granules were found in the megakaryocytes of healthy individuals and CML patients as well as in the bare nuclei of CMGM, ET and PV patients (Figs. 1, 2, 4). In the large megakaryocytes of CMGM, ET and PV patients, AgNOR staining revealed small and fine granules in clusters. A cluster was composed of up to 20–25 granules (Figs. 3, 4).

In Fig. 5 the mean AgNOR scores of megakaryocytes and bare nuclei, their ranges, means and standard deviations were plotted against the histological diagnoses. The AgNOR counts of megakaryocytes of normal and CMPD sub-categories did not overlap. The AgNOR counts of megakaryocytes in CML were lower, and the AgNOR counts of megakaryocytes in CMGM, ET and PV were higher compared with the AgNOR counts of normal megakaryocytes. In CMPD sub-categories only CML was separated from CMGM, ET and PV without overlap. The counts in CMGM, ET and PV showed a considerable overlap. The differences between the AgNOR counts of normal megakaryocytes and the megakaryocytes of CMPD sub-categories were significant ($P<0.001$). In the class of CMPD sub-categories only CML megakaryocytes showed significant differences from CMGM, ET and PV ($P<0.001$). No bare nuclei were seen in healthy subjects and CML patients. Bare nuclei in the group of megakaryocytes of CMGM, PV and ET populations varied between 4% and 20%. In the CMPD sub-categories the mean AgNOR score of bare nuclei showed no significant differences.

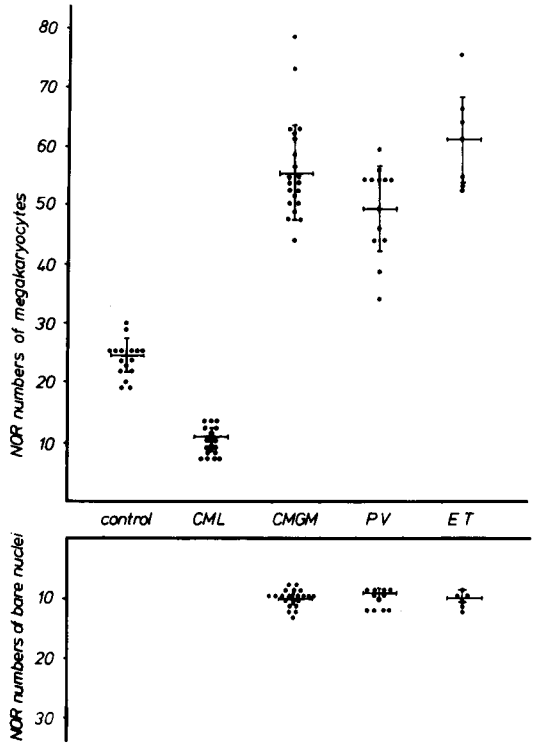


Fig. 5. Mean AgNOR counts of megakaryocytes in chronic myeloproliferative disease (CMPD) sub-categories (CML, CMGM, PV and ET) and healthy individuals

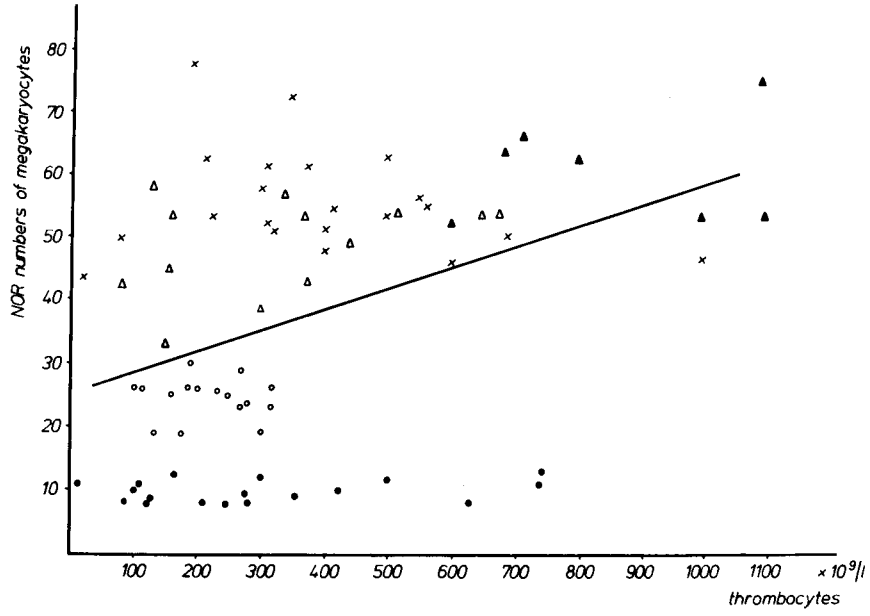


Fig. 6. Linear regression plotted against mean AgNORs of megakaryocytes and platelet counts of CMPD patients and healthy individuals (○, healthy individual; ●, CML; ×, CMGM; △, PV; ▲, ET)

A correlation coefficient was calculated by computer analysis for the relationship between mean numbers of AgNORs and platelet counts. There was a poor correlation between the AgNOR numbers and platelet counts ($r=0.347$) ($P=0.001$). The equation for the regression line was $y=0.0273+26.815x$ (Fig. 6).

Discussion

Having been shown to be useful in detecting transcriptionally active ribosomal RNA genes, the AgNOR technique has been widely used in diagnostic pathology to gain information about cell proliferation, activity and malignancy (Rüschoff et al. 1989; Underwood and Giri 1988). Application of this method to bone marrow cells has been restricted to cytogenetic studies: AgNOR staining has been studied on chromosomes of cells from the bone marrow of healthy individuals (Arden et al. 1989), patients with acute lymphoblastic leukaemia (Mamaev et al. 1987), acute myelogenous leukaemia (Arden et al. 1989), CML (Mamaev et al. 1985; Sato et al. 1986) and promyelocytic leukaemia (Reeves et al. 1984). However, the cells of myeloid, erythroid and megakaryocytic cell lines have not been investigated separately. In this study, the method of Crocker and Nar (1987) was applied to decalcified bone marrow biopsies to examine the AgNOR staining pattern and the number of different haematopoietic cells. The method enables the evaluation of transcriptionally active RNA genes of megakaryocytes in their own microenviroment.

The AgNOR staining of megakaryocytes revealed considerable differences in the group of CMPD. The AgNOR granules of megakaryocytes in CML were single, large dots and significantly fewer than in healthy individuals and other CMPD sub-categories. The decreased number of aggregated large AgNORs reflects transcriptionally inactive or reduced activity RNA genes (Fakan and Hernandez-Verdun 1986). Arden et al. (1989) also found significantly lower AgNOR numbers in the chromosomes of CML patients than in the control group. The reduced number of AgNOR granules in both myeloid and megakaryocytic lineage of CML is suggestive of a common biological defect in ribosomal RNA processing. Cell kinetic studies as well as AgNOR studies of chronic phase CML indicate that the hyperplasia of bone marrow cells is the result of accumulation of leukaemic cells rather than that of increased proliferation (Goto et al. 1982; Andreeff 1986). The significant differences between AgNOR counts of normal and CML megakaryocytes suggest that AgNOR technique might be a differential diagnostic tool in the diagnosis of early CML and leukaemoid reaction.

The AgNOR numbers in CMGM, PV and ET were significantly higher than the normal count, although the AgNOR numbers in CMGM, PV and ET were found to overlap considerably. In these CMPD types, the small AgNORs were scattered all over the nuclei of megakaryocytes, suggesting active proliferation. Underwood and Giri (1988) reported that the high number of AgNORs and their small size indicated an elevated transcriptional activity. Furthermore, Hall et al. (1988) produced reasonable evidence that the increased mean AgNOR numbers reflected active cell proliferation: nuclear Ki-67 immunoreactivity, used as a marker of cell proliferation, and the number of AgNORs showed a strong correlation in non-Hodgkin's malignant lymphomas. High levels of platelet-derived growth factor messenger RNA and protein were detected in the megakaryocytes of ET and PV patients (Katoh et al. 1990), which also supports our finding that megakaryocytes display more enhanced activity in these CMPD sub-groups than they do in CML or in health.

In clinical practice, CMGM, PV and ET are frequently accompanied by thrombocythaemia (Thiele et al. 1983, 1987, Iland et al. 1987) and we observed a high number of AgNORs in these CMPD sub-categories. A statistical analysis of AgNOR numbers and platelet count suggests that platelet production by megakaryocytes is reflected in the AgNOR numbers of megakaryocytes. A loose correlation between AgNOR numbers and platelet counts was detected in CMPD patients without distinguishing sub-categories, but no similar correlation was found in the single CMPD groups (CML, CMGM, PV, ET) or in the group of healthy individuals. The different correlations between AgNOR numbers and platelet counts in unseparated CMPD and CMPD subcategories suggest that other factors or the pathophysiological properties themselves of CMPD sub-categories may influence the actual platelet and AgNOR counts.

Dense, pyknotic and heterochromatinized nuclei of megakaryocytes with narrow cytoplasm, called bare (naked) nuclei, have frequently been observed in CMGM, PV and ET (Thiele et al. 1983; Matolcsy and Majdic 1990). According to their morphology these bare nuclei are believed to be apoptotic, terminal stages of the life cycle of megakaryocytes (Chott et al. 1990). The AgNOR numbers of bare nuclei are similar in all three forms of CMPD sub-categories, in which morphologically small numbers of large granules are present. The relatively low number and large size of AgNORs suggest less active cells in the group of megakaryocytes, but the presence of AgNOR granules supports the idea of an active phenomenon of apoptosis which requires RNA activity and proteins.

The altered AgNOR number of megakaryocytes in CMPD and the significant deviation from normal refer to a different role and cellular activation of megakaryocytes. Further study of gene activation and rearrangement of megakaryocytes may contribute to an understanding of the developing and progression of CMPD.

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