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Age related decrease of NOR activity in bone marrow metaphase chromosomes from healthy individuals

Estela Pedrazzini, Nikolai Mamaev, Irma Slavutsky

Abstract

***Aims*—To present data obtained from human bone marrow preparations from healthy individuals showing that the proportion of metaphases with silver stained nucleolar organiser region (AgNOR) chromosomes is associated with the age of the donor.**

***Methods*—Bone marrow preparations from eight Russian and 10 Argentinian healthy individuals donating bone marrow for heterologous transplantation were studied by silver staining. The Russian bone marrow preparations were used directly, while the bone marrow specimens from Argentinian donors were incubated for 24 hours at 37°C in F-10 medium with 15% fetal bovine serum. The slides were silver stained by the one step method of Howell and Black with slight modifications. Thirty metaphases with clearly defined D and G group chromosomes were scored for the numbers of AgNORs. All metaphases that were adjacent to silver stained interphase nuclei were analysed to assess the percentage of AgNOR positive mitoses. The Kruskal Wallis test and Kendall's rank correlation coefficient (rK) were used to assess the relation between age and the percentage of AgNOR positive cells.**

***Results*—The mean numbers (SE) of AgNORs per metaphase were 5.06 (0.17) and 5.56 (0.23) for the Russian and Argentinian groups, respectively, with no significant differences between the two groups. The common percentage of AgNOR positive cells decreased significantly as a function of age, with an rK = −0.57 (p < 0.0012).**

***Conclusions*—The percentages of AgNOR negative metaphases in bone marrow from healthy individuals is strongly associated with age and this may be related to age related telomere loss.**

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Keywords: nucleolar organiser regions; silver staining; healthy human beings; aging

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A cytochemical silver nitrate technique can be used effectively to monitor the activity of ribosomal RNA cistrons because a subset of nucleolar argentophilic proteins are associated with transcriptionally active rDNA.[1] According to recent findings, the proteins responsible for nucleolar organiser regions silver staining (AgNORs) on metaphase chromosomes include RNA polymerase I, topoisomerase IIα, transcription factor UBF, and probably nucleolin.[2] Studies on the AgNOR staining pattern in bone marrow cells from donors and patients with haematological malignancies have revealed significant decreases of modal AgNOR classes as well as a high percentage of AgNOR negative mitoses (mean, 48%; range, 34–70%) compared with PHA stimulated lymphocytes from the same subjects.[3–13] On the basis of these data, a hypothesis was put forward which related the AgNOR negative mitoses in bone marrows from healthy individuals and patients with leukaemias to polychromatic normoblasts and myelocytes, whose activity with regard to the synthesis of rRNA was clearly decreased.[4–6 12 14] This hypothesis has been verified by analysing the pattern of AgNOR staining in interphase nuclei of bone marrow erythroid and granulocytic elements of various maturities obtained from healthy donors and patients with leukaemias.[2 5–7 12 14–18]

Recently, Pedrazzini and Slavutsky noted that the AgNOR pattern of normal bone marrow cells is age related, but that this phenomenon is not seen in patients with mycosis fungoides[13] and acute lymphoblastic leukaemia (unpublished data). Additional analysis of our previously published data[5] also supported this conclusion.

Materials and methods

Bone marrow samples were obtained from eight Russian[5] and 10 Argentinian healthy individuals donating bone marrow for heterologous transplantation (table 1).

The Russian bone marrow preparations were made from aspirates of the sternum, fixed in 3:1 methanol:acetic acid for 10 minutes, and air dried smeared.[5] Bone marrow samples from Argentinian donors were incubated for 24 hours at 37°C in F-10 medium with 15% fetal bovine serum. Colcemid (final concentration 0.1 µg/ml) was added 40 minutes before harvesting. Cells were incubated in 0.075 M KCl for 20 minutes at 37°C, pelleted, and fixed in methanol:glacial acetic acid (3:1). After the cells had been washed three times in the fixative, air dried slides were prepared. In both cases, the slides were stained with the one step method of Howell and Black[19] that uses gelatine as the silver colloidal protector and pretreatment in 2 M formic acid for 10 minutes to improve the evaluation of AgNORs in bone marrow cells. A total of 30 metaphases with clearly defined D and G group chromosomes were scored for the mean number of AgNORs. All metaphases that were adjacent to

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Table 1 Frequency of AgNORs in bone marrow cells

Sample	Age/sex	Mean number of AgNORs	Cells scored (n)	AgNOR+ cells (%)
*1	8/F	7.37	230	58.7
†2	14/M	4.30	136	62.0
†3	16/M	4.80	34	89.5
†4	17/M	5.40	43	60.0
†5	18/M	5.40	82	78.8
†6	18/M	5.40	89	53.7
*7	22/M	5.73	200	75.0
†8	22/F	4.40	70	68.6
†9	22/M	5.40	111	56.0
*10	24/M	5.80	178	64.6
†11	25/M	5.40	131	56.0
*12	28/F	5.30	640	49.6
*13	29/M	4.97	286	49.1
*14	35/M	5.20	548	35.6
*15	38/F	4.77	254	22.0
*16	40/F	5.80	355	20.0
*17	40/M	5.43	307	56.0
*18	53/M	5.20	368	37.1
Mean (SE)	19.0 (1.3)	5.34 (0.15)		55.1 (3.7)

*Argentinean donor; †Russian donor.

silver stained interphase nuclei were analysed to assess the percentages of AgNOR positive mitoses.

Because the data for the bone marrow samples were not distributed normally, we used the Kruskal-Wallis test to compare groups and the Kendall's rank correlation coefficient (rK) for the relation between age and the percentage of AgNOR positive cells.

Results

Results from the silver staining of normal bone marrow cells are summarised in table 1. Among the 10 potentially stainable chromosomes the mean (SE) numbers of AgNORs per metaphase were 5.06 (0.17) and 5.56 (0.23) for the Russian and Argentian groups, respectively. There were no significant differences between the two groups, with a mean (SE) of 5.34 (0.15) AgNOR chromosomes per bone marrow cell when the two groups were analysed together. As regards the proportion of mitoses demonstrating silver staining on at least one chromosome, when the two groups were analysed together the mean (SE) was 55.1% (3.7%); when the two groups were analysed separately, the results were 65.6% (4.5%) and 46.8% (5.7%) for the Russian and Argentinian groups, respectively, with a significant difference between them ($p < 0.04$). Upon analysis, it was apparent that each group had a different mean age ($p < 0.01$); accordingly, we

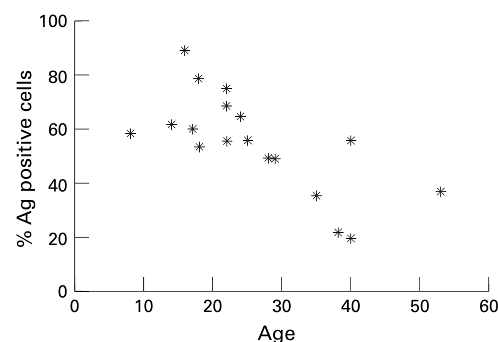

Figure 1 Scatter diagram showing the correlation between the percentage of AgNOR positive mitoses and aging. There is a significant negative correlation, rK = −0.57, p < 0.0012.

examined the relation between the percentage of AgNOR positive mitoses and the age of the bone marrow donors. We found that the percentage of AgNOR positive cells decreased significantly in function with age, with an rK = −0.57 ($p < 0.0012$) (fig 1).

Discussion

The above results show that the percentage of AgNOR negative metaphases in bone marrow from healthy individuals is associated strongly with age. In relation to these findings, it must be remembered that AgNOR negative mitoses in normal bone marrow are mainly present in erythroid and granulocytic elements. The latter finding has been shown by us in patients with multiple myeloma, where all mitoses of diseased cells (with chromosome markers) were AgNOR positive, whereas the majority of AgNOR negative mitoses were in cells with a normal karyotype.[6]

As discussed, the technical approaches used by the two laboratories participating in this study were not identical. Nevertheless, similar data were obtained, although the more active erythroid precursors appear to be the main mitotic cells in the bone marrow preparations from the Russian donors. On the other hand, the great majority of mitoses from the short term bone marrow cultures of the Argentinian controls are of granulocytic origin because cultured erythroid cells cannot grow effectively without the addition of erythropoietin. Accordingly, it is not surprising, that the mean number (SE) of AgNOR positive metaphases in the Russian samples was significantly higher than that found in the Argentinian samples (65.6% (4.5%) and 46.8% (7%), respectively; $p < 0.04$). These findings are in agreement with earlier published data related to age dependent decreases in the numbers of AgNORs in PHA stimulated human lymphocytes.[20–23] The relevant investigators related these findings to either a progressive decrease of lymphocyte proliferative activity,[21 24] or a hypothetical progressive inactivation of rRNA genes with aging.[20 22] Also, there is little evidence of an expected age related decrease in proliferative activity of erythroid and granulocytic precursors.[25 26] Meanwhile, there is increasing evidence that anaemia and leucocyte abnormalities in otherwise healthy elderly patients are not a normal physiological consequence of aging, but rather result from multiple insults and increased haemopoietic demands.[26]

In the light of these controversial ideas it seems that there may be a relation between the number of AgNORs in interphase nuclei and their indices of proliferative activity. This evidence is indirect, despite the fact that nucleolar argentophilic proteins such as RNA polymerase I, topoisomerase IIα, transcription factor UBF, and nucleolin[2] can be good markers of cell proliferation. It is likely that cells need rDNA expression, ribosome formation, and protein synthesis not only for proliferation but also for differentiation, maturation, repair, and so on.[12] Hence, the age related AgNOR positive proportion of cells may decrease in

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haemopoietic precursors to compensate/repair haemopoiesis in relation to insults to the haemopoietic system without a decrease in the proliferative activity of erythroid and granulocytic lineages. This would be in agreement with many clinical findings.[27–32]

A possible factor that could be very closely related to bone marrow failure in the elderly is telomere loss.[33–37] It is known that these highly repeated genetic elements are located at the ends of all eukaryotic chromosomes.[38] They are necessary for the preservation of genome stability and cell viability because they prevent aberrant recombination and degradation of DNA. During aging, telomere numbers decrease significantly because of replication problems.[39] Also, this age related telomere loss in germ cells, tumour cells, and leukaemic cells is actively compensated for by a high level of telomerase activity.[40–45] Theoretically, telomerase activity may be associated with both the level of cellular proliferation and rDNA cistron activity.[46] Therefore, the changes revealed here in the age related decrease in the proportion of AgNOR staining on metaphase chromosomes in bone marrow erythroid and granulocytic elements may be explained partly by telomere alterations. Such a hypothesis explains why the number of AgNOR positive mitoses in bone marrow samples from patients with mycosis fungoides[13] and acute lymphoblastic leukaemia (Pedrazzini and Slavutsky, unpublished data) are not age related. In these diseases, mitotically active cells are represented by more immature haemopoietic elements whose telomerase activity is higher than maturating erythroid and granulocytic progenitors. Future investigations should show whether or not the hypothesis is correct.

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