Nucleoli and AgNORs in Hodgkin's disease

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Abstract

Aim—To evaluate the morphofunctional characteristics of lymph node cells from patients with Hodgkin's disease by measuring silver stained nucleolar organiser regions (AgNORs).

Methods—Nucleoli in Hodgkin's and Reed-Sternberg (HRS) cells, lymphocytes and prolymphocytes were investigated in cytological smears and histological sections of lymph nodes from 32 patients with Hodgkin's disease, and from 34 patients with reactive lymphadenopathy. According to the Rye histological classification of Hodgkin's disease, three cases were the lymphocyte predominant (LP) type, 14 the nodular sclerosing (NS) type, and 15 the mixed cellularity (MC) type. The investigation was done before treatment, by means of a one step silver staining method. In each case, 50 to 100 HRS cells, lymphocytes, and prolymphocytes were evaluated to determine the mean numbers of nucleoli and AgNORs per nucleus. The nonparametric Wilcoxon Mann-Whitney test was used to compare the groups.

Results—The mean numbers of nucleoli and AgNORs were higher in lymphocytes and prolymphocytes compared with those from reactive lymph nodes used as controls. Numbers of nucleoli and AgNORs were higher (not significant) in the NS type of Hodgkin's disease than in the MC type. There was a significant increase in numbers of nucleoli in HRS cells, and their AgNOR counts were increased. The greatest number of nucleoli in HRS cells was found in the NS type. Furthermore, the nucleolar activity of HRS cells was greater in the NS type compared with the MC type (50.2 (SEM 3.9) *v* 37.7 (2.9) AgNORs per nucleus (*p* = 0.025)). Comparative analysis of cytological and histological samples showed that the AgNOR score was significantly higher in touch imprints than in tissue sections with tumours of the same histological type.

Conclusions—Assessment of cell activity in Hodgkin's disease patients by silver staining is more convenient and informative in lymph node imprints than in histological sections. The highest expression of interphase ribosomal RNA cistrons found in NS HRS cells is probably explained by their high proliferative activity and elevated production of transforming growth factor 1 which is known to be the most

potent cytokine present in the NS subtype of Hodgkin's disease.

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Keywords: nucleoli; nucleolar organiser regions; Hodgkin's disease; silver staining

The quantity of interphase silver stained ribosomal RNA cistrons in most cells has been shown in recent works to reflect directly their proliferative potential,^{1–5} the degree of nucleolar ploidy,^{6–8} and general cellular function.⁹ On this assumption, several investigations were carried out in malignant lymphomas,^{10–17} including Hodgkin's disease.^{18–19} Tsenga *et al*¹⁹ have demonstrated a significant difference in the mean numbers of silver stained nucleolar organiser regions (AgNORs) per neoplastic cell among different histological types. According to these findings the lymphoid predominant (LP) type is characterised by lower AgNOR scores than the other types (*P* < 0.05). The highest AgNOR numbers were found in the lymphocyte depleted type followed by the nodular sclerosing (NS) and mixed cellularity (MC) types. The aim of the present study was to evaluate the morphological characteristics of lymph node cells from Hodgkin's disease patients by measuring AgNORs in tissue sections and lymph node imprints.

Patients and methods

Tissues from 32 untreated Hodgkin's disease patients (20 men and 12 women) aged between 17 and 73 years were investigated. Thirty four patients with reactive lymphadenopathy were used as controls. The diagnosis of Hodgkin's disease was made on the basis of routine histological study; according to the Rye histological classification, three cases were lymphocyte predominant (LP), 14 were nodular sclerosing (NS), and 15 were mixed cellularity (MC) type. The stages, according to the Ann Arbor classification, were four in IIa, seven in IIIA, 17 in IIIB, and four in IVB.

Sixty six lymph node touch imprints and 20 lymph node biopsy specimens were studied. Imprints were dried at room temperature, fixed in methanol/acetic acid (3:1), rinsed thoroughly with distilled water, and air dried repeatedly. These were put into 2N formic acid for 10 minutes to reduce the egress of silver from the nucleoli, and, to facilitate the measurement of nucleoli and AgNORs, rinsed again and impregnated with silver nitrate.

The biopsy specimens were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (5 µm) were taken to

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Table 1 Clinical findings and nucleolar silver staining data from patients with different histological subtypes of Hodgkin's disease. Values are means (SEM)

Histological types	Patients (No)	Patients in different clinical stages	Number of nucleoli (above) Number of AgNORs (below)		p
			Lymphocytes and prolymphocytes	HRS cells	
Lymphocyte predominant	3	IIA-1; IIIA-2	1.07 (0.03) 5.77 (0.30)	3.03 (0.88) 32.39 (5.56)	<0.025*
Nodular sclerosing	14	IIIA-4; IIIB-8; IVB-2	1.32 (0.06) 7.85 (0.55)	7.85 (0.55) 50.2 (3.90)	
Mixed cellularity	15	IIA-3; IIIA-1; IIIB-9; IVB-2	1.21 (0.06) 7.42 (0.30)	3.11 (0.23) 37.7 (2.90)	

HRS = Hodgkin's and Reed-Sternberg cells.
* Statistical significance between the nodular sclerosing and mixed cellularity histological subtypes—Wilcoxon Mann-Whitney test.

Figure 1 Silver-stained Hodgkin's and Reed-Sternberg cells in lymph node imprints from a patient with Hodgkin's disease (original magnification $\times 1000$).

Figure 2 The mean number of nucleoli in Hodgkin's and Reed-Sternberg cells in lymph node imprints from patients with different histological subtypes of Hodgkin's disease.

water via xylene and graded alcohols. These were put into 2N formic acid for 10 minutes, rinsed, air dried and impregnated with a mixture of 50% aqueous solution of silver nitrate with gelatin at 68°C for four minutes (after Howell and Black²⁰ with slight modification⁵). The silver colloid was then washed off with distilled, de-ionized water. The preparations were lightly counterstained with 2% Giemsa for 20 seconds, dehydrated through the graded ethanols to xylene and mounted in synthetic medium.

In each case, the mean numbers of nucleoli and AgNORs per nucleus were counted in 50 to 100 Hodgkin's and Reed-Sternberg (HRS) cells, lymphocytes, and prolymphocytes using an oil immersion lens at a magnification of $\times 1000$ or $\times 1200$. Student's *t* test and nonparametric Wilcoxon Mann-Whitney tests were used to compare the patient and control groups.

Figure 3 The average numbers of AgNORs in prolymphocytes, lymphocytes and Hodgkin's and Reed-Sternberg cells in lymph node imprints from patients with different histological subtypes of Hodgkin's disease.

Results

Clinical findings and the results of nucleolar silver staining of lymph node pathological cells from patients with Hodgkin's disease are presented in table 1 and figures 1–3.

NUMBERS OF NUCLEOLI

The numbers of nucleoli in the lymphocyte and prolymphocyte populations from Hodgkin's disease patients ranged from 1.0 to 1.92 per nucleus. The mean (SEM) number of nucleoli did not differ from those in controls (1.24 (0.03) *v* 1.18 (0.01)). There was a slight difference in the nucleolar score in the NS and LP types (1.32 *v* 1.07, *p* < 0.05). The numbers of nucleoli in HRS cells ranged from 1.56 to 5.33. The mean numbers of nucleoli in HRS

Figure 4 Comparative analysis of AgNORs in Hodgkin's and Reed-Sternberg cells in imprints and sections from patients with different histological subtypes of Hodgkin's disease.

cells were higher than in lymphocytes and prolymphocytes (3.22 (0.17), $p < 0.001$). The numbers of nucleoli in the NS type were slightly higher than that in the MC type (7.8 (0.55) *v* 3.11 (0.23)).

AGNOR SCORES

The AgNOR scores in lymphocytes and prolymphocytes from Hodgkin's disease patients ranged from 5.4 to 11.2. The mean AgNOR score was similar in patients and controls. The highest mean number of AgNORs was found in the NS type. The count was intermediate in the MC type, and least in the LP type.

The AgNOR scores in HRS cells ranged from 21.6 to 74.6 per nucleus; the average number of AgNORs was highest in the NS type. The count was intermediate in the MC type and least in the LP. Analysis of variance showed a statistically significant difference between the NS and the MC types (50.2 (3.9) *v* 37.7 (2.9), $p < 0.025$). The AgNOR counts were independent of the stage or the presence of B symptoms and there was no correlation with many other clinical and laboratory parameters.

HRS CELL AGNORS IN IMPRINTS AND SECTIONS

The results of AgNOR comparative analysis in cytological and histological samples obtained from 16 patients with Hodgkin's disease are presented in figure 4. The AgNOR score was significantly higher in touch imprints than in tissue sections within tumours of the same histological type. Moreover, the concordance of counting between two samples from the same patient was not strong.

Discussion

This study confirmed previously reported findings of nucleolar changes in HRS cells from patients with Hodgkin's disease.^{18 19} First, HRS nucleoli were larger and more polymorphic compared with those in lymph node lymphocytes and prolymphocytes from the same patient. Second, the numbers of AgNORs in HRS cells were higher in the NS type than in the MC and LP varieties. Interestingly, a similar conclusion was made previously in an investigation of histological sections.¹⁹ The changes are clearer, however, in imprints than

in sections. In the previous study low AgNOR indices were seen only in the LP type ($p < 0.05$).¹⁹ On the contrary, the difference between AgNOR counts in the NS and MC types was not significant (3.24 (1.41) *v* 2.63 (0.93)). In general, these and our results show that the AgNOR scores in HRS cells may be related to the histological type of Hodgkin's disease; however, the scores are independent of the stage or the presence of B symptoms, and there is no correlation with most other clinical and laboratory parameters. It is noteworthy that the HRS proliferative rate measured by proliferative cell nuclear antigen, Ki-67, and c-myc p62 oncoprotein expression was comparatively high, while the difference between various histological types was not statistically significant. In addition, there was no correlation with AgNOR counts of HRS cells.¹⁹ However, we did notice that the previous results of HRS proliferative rate agreed with our findings of increased AgNOR counts in the NS type. As shown by Tsenga *et al*¹⁹ the percentage of HRS cells stained by PC-10 and Ki-67 antibody was slightly higher in the NS type compared with the MC type. Furthermore, the expression of c-myc p62 oncoprotein was also higher in the NS type of Hodgkin's disease than in the MC and LP varieties. Conversely, a recent investigation showed that the mean mitotic state and other kinetic indices of HRS cells were higher in the MC than in the NS type of Hodgkin's disease.²¹ According to these findings, these cellular parameters were associated with higher DNA fragmentation (related to apoptosis) and the presence of many abortive mitoses in HRS cells. As a result, the percentage of Ki-67 positive HRS cells in Hodgkin's disease did not correlate significantly with their mitotic indices.

Evidence of relatively high ribosomal cistron activity in HRS cells from patients with Hodgkin's disease may be explained partly by their high proliferative potential as well as increased nuclear ploidy. The higher AgNOR counts in HRS cells in the NS variant of Hodgkin's disease may be explained as follows: HRS cells from patients with Hodgkin's disease can produce several cytokines both in vitro and in vivo including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, GM-colony stimulating factor (CSF), tumour necrosis factor (TNF)- α , TNF- β , and transforming growth factor β (TGF-1).²² Most of these cytokines were activated in all histological subtypes of Hodgkin's disease; however, TNF was more readily detected in the cell line established from a patient with the NS type than that from a patient with the MC type.²³ TGF-1 was found to be greatly activated only in the NS type of Hodgkin's disease.²⁴⁻²⁸ As the main sources of TGF in the lymph nodes of patients with the NS type of Hodgkin's disease were mononuclear Hodgkin's cells, some Reed-Sternberg cells, and activated lymphocytes,²⁸ AgNOR elevation in the NS type HRS cells may be related to increased cytokine production.

In conclusion, comparison of AgNOR scores in lymph node sections and imprints prepared from the same patients supported previously

reported results that the latter were more informative for assessment of Hodgkin’s disease cell activity. In our opinion, this observation may be connected with more rough fixation of histological samples inducing an increased AgNOR aggregation and problems in AgNOR examination; and the inevitable decrease of AgNOR counts in sections cut at a thickness less than the nuclear diameter.

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