Histopathological study comparing upstream binding factor expression and AgNOR staining

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Abstract

AgNOR staining has been in past years, the subject of numerous publications, which have failed to reach agreement regarding its usefulness as a proliferation marker. This silver staining method does not react with NORs (actual chromosome regions containing rRNA (ribosomal RNA) genes), but with proteins associated with them, whose quantity increases in parallel with ribosome biogenesis. The transcription factor UBF (upstream binding factor) is associated with NORs and has an important regulatory role in rRNA synthesis as cofactor of RNA polymerase I. Recent research has revealed an additional cytoarchitectural function of UBF in decondensing r-chromatin (ribosomal-chromatin). Immune detection of UBF expression and AgNOR counts are closely correlated as both techniques identify substrates in or closely adjacent to NORs. However, contrary to AgNOR dots, the UBF signal disappears in cells which undergo apoptosis or terminal differentiation. These features imply that UBF evaluation would reflect tumour cell proliferation (growth fraction) more accurately than AgNOR counts. Here we also show that immunohistochemical staining of UBF may reveal distinct active NORs with open, decondensed chromatin and we hypothesize that the large stretches of decondensed r-chromatin revealed by UBF staining may correspond to clusters seen after silver staining and, conversely, shorter areas of decondensed r-chromatin should match the small AgNOR grains typically found in some tumour types. The length of decondensed r-chromatin may be a reflection of the ratio of active to silent r-RNA genes.

Introduction

Nucleolar enlargement is a cytomorphological feature commonly seen in cells of malignant tumours. However, attempts to quantitatively evaluate nucleolar size as an aid to histopathological diagnosis of malignancy have met with limited success. An important step in this direction was the introduction by Plotton *et al.* (1) of a silver staining technique (AgNOR), which labels proteins associated with chromosomal regions containing ribosomal-DNA genes, so called nucleolar organizer regions. Quantities of these proteins increases in connection with ribosome biogenesis and when stained with silver, are visualized as well-defined black dots whose number and surface area are a measure of size and activity of the nucleolus (2).

Numerous studies performed with the help of digital imaging techniques have demonstrated correlation between increased AgNOR counts and malignancy as well as quantitative differences in mean AgNOR value between cells of benign and malignant lesions. However, frequent overlapping precludes application of the method to diagnosis of single cases (3,4). Some studies have shown that numbers of AgNORs correlated with cell cycle doubling time and therefore evaluation should depend on the growth fraction of a tumour cell population (2). Other studies have shown correlations with histological malignant grade (5–7).

The theoretical principle underlying the value of this method as a proliferation marker is that ribosome biogenesis and cell proliferation are closely intertwined, the reason for this being that a very high fraction of a cell's metabolic activity is required for production of the vast amounts of proteins necessary for DNA synthesis and cell doubling. Increased ribosome biogenesis, albeit usually at lower level than that of cells of malignant tumours of cells of malignant tumours, may also occur in hyperplastic states and benign neoplasias, as well as in a variety of conditions characterized by high

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metabolic activity. This accounts for overlapping of AgNOR values in benign and malignant lesions, which undermines the usefulness of the technique (3,4).

On the other hand, some silver stainable proteins remain associated with chromatin when nucleoli disappear in mitosis and/or might have long half-lives and be carried over from one cell cycle to the next. This has led to the concept that AgNOR values tell the cell's replicative history (8). There are also claims that even total block of rDNA transcription is not necessarily accompanied by loss of silver stainability (9).

To overcome these drawbacks of AgNOR staining, we have resorted to immunohistochemical detection of a single NOR-associated protein UBF (10), which, due to its crucial and well-established regulatory function in ribosome biogenesis, could replace AgNOR staining and offer a more precise interpretation of data. rDNA transcription by RNA pol I needs cooperation by cofactors SL1 and UBF, but the latter may increase the rate of transcription by orders of magnitude and appears to be central to adjusting transcription to sudden demands of ribosome production (11–13). Here, we report some features unveiled by the comparison between AgNOR and UBF staining.

Materials and methods

Experiments carried out here took place in two different laboratories. Earlier procedures were performed in the Department of Histopathology, Hospital de Puerto Real, Spain. Pathological samples, anti-UBF staining and silver staining were as reported in (10). Silver staining of gels and western blotting were as in (14). The second set of experiments was performed at the Department of Histopathology, Queen's Hospital, and involved only anti-UBF staining of cytological preparations. This antibody was the same as used in former experiments and was kindly donated to us by Prof. Valdivia (Department of Biochemistry and Molecular Biology, Facultad de Ciencias, Cadiz, Spain). The recombinant antibody against truncated polypeptides 220–764 polypeptide of UBF has been described previously (15). Specimens were from cases diagnosed from cytology and later confirmed by biopsy of breast cancer or chronic leukaemia, as well as cases operated on for low-grade meningioma. Approval from the Ethical Commission and patients' approval were obtained in all cases. For immunohistochemical detection of UBF, an ABC vectastain kit was used with biotinylated anti-rabbit secondary antibody. Development was carried out using Vector Nova Red substrate. Anti-UBF antibody produced no results using tissue embedded in paraffin wax.

Results

In earlier experiments previously reported (10), AgNOR staining and UBF expression were compared in an ample variety of biopsy and cytological specimens. Similar to AgNOR proteins, UBF was detected as compact, well-defined silver dots whose number closely correlated with that of similar specks revealed by silver staining of the same specimens (it must be stated, however, that, in our hands, UBF is not a silver stainable protein). Using the method of D. Hernandez Verdun for AgNOR staining of nitrocellulose filters transferred with cell extracts after gel electrophoresis, and comparing it with western blotting of the same cell extracts, revealed by anti-UBF antibody, we could not detect silver stained bands corresponding to the molecular weight of UBF, whereas western blots clearly revealed the two bands corresponding to UBF1 and UBF2. This is in contrast to results reported by Valdez *et al.* (16) who performed silver staining of recombinant UBF (not shown).

An exception to this parallelism between AgNOR and UBF expression was observed for the first time in several cases of Hodgkin lymphoma where an inverse pattern was seen with diminished or absent UBF and increased AgNOR counts (14). This was demonstrated to be related to apoptosis of cells in which UBF was selectively degraded while AgNOR proteins were either unaffected or increased (in keeping with malignancy).

Further assays not reported to date have revealed the same pattern in lymph node metastasis of one case of malignant melanoma. The majority of tumour cells exhibited this inverse pattern with low or absence of UBF and high AgNOR levels, suggesting that most tumour cells were undergoing apoptosis in the hostile lymph node environment (not shown).

We also examined cytological samples of lymph node metastasis in four cases of small cell lung carcinoma. UBF was absent or barely detectable and AgNOR dots were few and small. In some cases, however, AgNOR dots were small, but relatively numerous (Figs 1–3); UBF was absent (Fig. 4). In accordance with our previous findings in Hodgkin lymphoma and other lymphoid cells, we interpreted this pattern as an indication of apoptosis.

Subsequently, we sought to discover what changes could be detected by these staining procedures in relation to normal cell differentiation. To this end, immunohistochemical detection of UBF and silver staining were simultaneously performed in frozen sections of normal uterine cervix. UBF was expressed in basal and intermediate layers, but became undetectable in superficial epithelial layers (Fig. 5). AgNOR dots could be seen across the entire section and were not lost in superficial

Figure 1. Lymph node metastasis of small cell lung carcinoma. AgNOR staining.

Figure 4. Lymph node metastasis of small cell lung carcinoma. UBF immunodetection.

Figure 2. Lymph node metastasis of small cell lung carcinoma. AgNOR staining.

Figure 5. UBF expression in normal uterine cervix.

Figure 3. Lymph node metastasis of small cell lung carcinoma. AgNOR staining.

Figure 6. AgNOR staining of normal uterine cervix.

layers (Fig. 6). The same pattern was seen in the skin. In addition, shed cells from urine and upper respiratory tract were negative for UBF staining and positive with

AgNOR staining (not shown). This suggests that UBF expression is turned off when cells stop dividing and undergo terminal differentiation.

The next set of experiments was separated from the former by a long temporal gap, as they were carried out

after one of us (A.T.-M.) moved to a new laboratory in another country (UK). However, we continued using the same rabbit polyclonal anti-UBF antibody as earlier. The biological material was comprised of cytological samples of breast cancer, meningiomas and chronic leukaemias, which were assayed for UBF expression. Silver staining was not performed.

To our surprise, the staining pattern of UBF had changed after this time. Instead of round, compact dots, the anti-UBF antibody decorated the periphery of the dots, apparently corresponding to the dense component (DC) seen by electron microscopy (EM), encircling a central lighter area, which corresponds to the fibrillar centre (FC) of (EM.) In this manner, typical structure of interphase NORs as seen by EM could also be identified by light microscopy. Even different forms of FC and associated DC (hereafter referred to as active NORs), described in some EM studies (17) as spherical, cup-

Figure 7. Breast cancer smears (C5) stained with anti-UBF antibodies.

Figure 8. Breast cancer cytology (C5). Anti-UBF staining.

shaped and elongated, could be clearly recognized (Figs 7,8). Some compact dots were also present.

Numbers of active NORs in cells varied between the different cancer types and also, but much less, within different cases of a given tumour histological type. Dramatically enhanced expression of UBF was present in ductal breast cancer cytological specimens. In chronic leukaemia cases, expression was lower than in breast cancer cases, but higher than in meningiomas. The large NOR area encircled by UBF was consistent with large AgNOR-stained clusters seen in ductal breast carcinomas. Small AgNOR grains have been described in pediatric small cell tumours. Our cases of SCLC also had relatively small AgNOR dots. Unfortunately, UBF staining of our small cell lung cancer cases was not feasible for the aforementioned reasons.

Discussion

As expected from their common dependence on early steps of ribosome biogenesis developing in nucleolar organizer regions, AgNOR counts and UBF expression correlated very closely. However, even if our anti-UBF antibody did not work on paraffin wax embedded tissue, it offered some important theoretical advantages over silver staining. Persistence of AgNOR dots in superficial and desquamated cells of epithelia was consistent with the concept that AgNORs reflect a cell's replicative history, but this feature detracts from the usefulness of the stain in assessment of cell proliferation. In contrast, UBF expression is turned on and off very rapidly in relation to cell biosynthetic activity, as shown by its disappearance from terminally differentiated and apoptotic cells.

Data obtained from studies comparing AgNOR counts to a proliferation marker such as Ki-67 can be safely extrapolated to UBF expression, given its parallelism to AgNOR staining. Ki-67 stains an antigen selectively expressed in cycling cells, while AgNORs and UBF expression are only indirectly related to cell proliferation. Both stains detect a small central dot in resting cells and several dots that increase in number and/or area as a result of cell doubling or enhanced metabolic activity.

In agreement with this notion, correlation of Ki-67 and AgNOR counts has varied in different reports and diverse experimental settings. In a report from Kidegawa *et al.* (18) using combined staining by AgNORs and Ki-67, on breast cancer cells, they demonstrated higher AgNOR counts in Ki-67-positive cells compared to Ki-67-negative cells. Neither of the single stains correlated with clinical features, but high levels of AgNORs in Ki-67-positive cells correlated significantly with

tumour size, lymph node status and patient survival. Our results suggest that the Ki-67-negative-AgNOR-positive cells in their study represent non-cycling, terminally differentiated or apoptotic tumour cells, (therefore negative for UBF) which have no consequence in tumour growth. On the other hand, the high predictive value on tumour aggressiveness of Ki-67-positive cells with high AgNOR counts compared to Ki-67-positive cells, indicates a contribution to malignant behaviour resulting from increased biosynthetic activity on top of that related to on high proliferation index.

To our surprise, a change in staining pattern of UBF was noticeable in all assays performed in the last period of this research. The new pattern underscored peripheries of stained dots, suggesting that UBF was located at the periphery of FCs (however, upon revising our old preparations, this pattern was seen occasionally). As our anti-UBF antibody is a polyclonal, it is possible that over the length of time elapsed since the beginning of the work, a population of cross-reacting antibodies could have lost affinity for some cross-reacting determinants, increasing in this way specificity of the polyclonal serum (or higher specificity might simply be due to better quality of secondary reagents in the new kit used). On the other hand, this staining pattern should be expected if (as many investigators believe), rDNA transcription takes place in the DC and/or in the boundary between DC and FC (8). However, other researchers propose that FC is the site of r-DNA transcription. The question remains unresolved (19) and UBF has been found in both locations (9,20). A way to reconcile these findings could be that the two forms of UBF (UBF1 and UBF2) were differentially located in FC and DC, with different antibodies reacting preferentially with one or the other. However, our antibody was produced against a peptide present in both splicing forms of UBF, and it recognized both forms in western blots.

A further possibility is that our anti-UBF antibody had become more selective against epitopes related to sites of acetylation/deacetylation and phosphorylation/dephosphorylation, as these changes can direct UBF to areas of rDNA transcription (21), resulting in changes in specific localization of UBF in relation to r-DNA. Differences in selectivity against these epitopes between anti-UBF antibodies would explain the conflicting results reported by different groups concerning, UBF localization in NORs.

In addition to its role in rDNA transcription as cofactor of RNA pol I, recent research has revealed a structural function of UBF in maintaining r-chromatin in the euchromatic open state (22). Studies with the cross-linking agent psoralen indicate that r-chromatin exists in two forms: undercondensed (euchromatic) and con-

densed (heterochromatic), which correspond to active and silent rRNA genes respectively. Decondensation of r-chromatin is brought about by the ability of UBF to bind across rDNA and intergenic spacers. In this process active NORs are formed, active NORs are formed (22). It is apparent that our UBF antibody outlines active NORs (probably because UBF is more accessible to the antibody when underpinning the open structure of NORs) and can be used to measure their number in a cancer cell.

The observation in some systems that active NORs are stable through the cell cycle is intriguing because rDNA transcription is shut down during mitosis (22). This implies that euchromatic r-chromatin is not forcibly linked to transcription. NORs present in mitosis have been referred to as potentially active and their chromatin as competent but non-functional. The exceedingly high number of active and large NORs detected in our cases of breast cancer seems disproportionate to the demands of ribosome production and suggests that in these malignant cells, NORs may be competent but non-functional or else, be the consequence of the cancer cell's inability to shut down ribosome biogenesis. As suggested above, biosynthetic activity in excess of cell proliferation needs, might be associated with production of some component important for cancer cell survival and resistance to chemotherapy. On the other hand, these large NORs, which very probably contain increased numbers of decondensed rRNA genes, should be found whenever upregulation of RNA I transcription is obtained, by increasing the ratio of active *versus* silent r-RNA genes.

It might be asked whether presence of numerous scattered euchromatic regions may not retard chromosome condensation and mitosis, which could lead to a more protracted clinical course and perhaps masking of the true proliferation index. This would explain discrepancies between grade of malignancy and the proliferation index of some tumours.

The finding of low AgNOR value and absence of UBF expression in lymph node metastasis of SCLC was somewhat unexpected as this is a malignant tumour with a high proliferation index. Some of the tumour cells, however, still contained few to relatively numerous small AgNOR granules. We have previously shown (14) that UBF disappears during apoptosis before other NOR proteins. It is also reasonable to assume that high proliferative activity unsupported by ribosome biogenesis may be incompatible with cell survival. Light microscopic appearance of SCLC cells is suggestive of apoptosis, and Feulgen-positive material derived from nuclei of degenerate cells deposited in the walls of blood vessels, is a usual finding in lymph node metastasis of this tumour (23). A study of AgNORs in lung cancer found

lower AgNORs in the primary site of SCLC compared to NSCLC (24). This result is intriguing as SCLC is a tumour with a high proliferation index (25) which, according to conventional thinking, should be accompanied by increased AgNOR counts. Furthermore, Rb is often mutated or deleted in SCLC. Current knowledge indicates that phosphorylated Rb triggers initiation of the cell cycle and at the same time, liberates bound UBF so that cell proliferation and ribosome biogenesis are coordinated. (26) Likewise, when Rb is deleted, unbound UBF should lead to enhanced rDNA transcription. It has been shown that recruitment of pol I to rDNA to initiate transcription is mediated by acetylated UBF (21). This is prevented by pRb. Conversely, deacetylation of UBF by HDAC requires pRb. Similar to acetylation, phosphorylation of UBF in growing cells also increases its rDNA transcription activity. This molecular modification concurs with higher UBF levels to supply enhanced cell energy demands and conversely, arrest cell proliferation, for instance by nutrient exhaustion, leads to dephosphorylation and decreased level of UBF amounts (21). In summary, it seems that known stimuli for cell proliferation are always coupled to increased UBF activity and therefore our present understanding of cell physiology precludes coexistence of absent-to-low UBF expression and high-to-normal AgNOR counts in a cell, unless it is undergoing apoptosis.

In the above study of lung cancer, it was pointed out that lower numbers of AgNOR counts in SCLC compared to non-small lung cancers might be due to problems in discriminating clusters of stained AgNORs into individual dots. Egan *et al.* (27) reported that small cell tumours of infancy (neuroblastoma and Ewing's sarcoma, which characteristically have high proliferation index) contain uniformly small AgNOR grains. AgNOR dots were also relatively small in our SCLC cases.

As rDNA transcription can be regulated either by speeding up transcription rate of active genes or by increasing ratio of active *versus* silent rDNA genes, we are tempted to speculate that small AgNOR grains of small cell tumours contain short segments of rDNA with few active genes where increased transcription is accomplished through improved efficiency of the initiation complex, while large AgNOR clusters must contain larger stretches of extended r-chromatin where upregulation of rDNA transcription would mainly be due to increased ratio of active *versus* inactive genes. Predominance of one of the two functions of UBF, that is, promoting initiation as cofactor of RNA pol I or decondensing r-chromatin, would underlie each of these regulatory options.

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