REVIEW

Nucleolar organiser regions in pathology

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Traditional approaches to pathological diagnosis include macroscopic examination and formalin fixation with the preparation of paraffin sections stained with a variety of histochemical techniques. These routine approaches however may be disappointing in the assessment of many conditions and occasionally fail to render a precise diagnosis or provide adequate prognostic information.

Consequently there has been growing interest within the histopathological community in the state of the nucleus, DNA and proliferation markers. One of the most recent produces of this interst is the study of Nucleolar Organiser Regions (NORs) using a simple silver reduction technique (AgNOR method).

Nucleolar organiser regions are an essential part of the machinery of the nucleolus (Howell, 1982; Derenzini *et al.*, 1983). They can be seen as electron lucent areas at the ultrastructural level (fibrillar centres) which are the interphase equivalent of the condensed chromosomal NORs (Jordan & McGovern, 1981; Hernandez-Verdun, 1986). These structures can be seen at the light microscope level using a variety of techniques including the AgNOR method (Hsu *et al.*, 1975; Ploton *et al.*, 1986). They are involved in ribosome production and potentially qualitative or quantitative changes in interphase NORs may be visible in relation to proliferative activity or transformation and could aid diagnosis or prognostication.

Physiology and theoretical basis

Mammalian nucleoli have three substructures named according to their ultrastructural appearances (Scheer & Benevente, 1990). These comprise (1) the fibrillar centre, (2) the dense fibrillar component, (3) the granular component. The fibrillar centre is probably the site where the primary r-RNA transcript is generated and contains ribosomal DNA, RNA polymerase I and topoisomerase I(a). It is the equivalent of the interphase NOR seen at the light microscopic level. The dense fibrillar component is the proposed site of early processing of the rRNA precursor and stains with antibodies to fibrillarin, a protein associated with the U3 small nuclear ribonucleoprotein (SnRNP) (Scheer & Benevente, 1990). The granular component is composed of ribosome precursor particles (Scheer & Benevente, 1990). Experiments in *Drosophila* (Karpen *et al.*, 1988) have shown that the nucleolus can form anywhere an active ribosomal gene is located and the central element upon which nucleolar structures assemble is the tandemly-repeated ribosomal DNA that codes for the large rRNA precursor. Evidence is accumulating that vertebrates and possibly many other eukaryotes have a common arrangement of regulatory elements that are arranged as two tran-

scription units per tandem repeat unit (Reeder, 1989). Both of these are transcribed by RNA polymerase I. The second polymerase I transcription unit is thought to influence enhancer function (Reeder, 1989). RNA polymerase I transcription is regulated by at least three polypeptide *trans*-acting factors: UBF and a member of the SL1 class which probably act as components of a step where polymerase recognises a stable complex and initiates transcription, and an initiation factor that associates tightly with the polymerase but which can be separated from it (Cavanaugh & Thompson, 1985). Termination of polymerase I transcription requires specific interaction between the polymerase and a terminator protein (Kuhn *et al.*, 1990). The large RNAs of ribosomes are initially transcribed as single precursor molecules and then processed in a complex series of steps to the mature 18S, 58S and 28S RNAs of functional and ribosomes. This process appears to be directed by SnRNPs. The most abundant nucleolar SnRNP contains RNA, U3, fibrillarin (B36) and at least other six proteins (Parker & Steitz, 1987).

As the rRNA is transcribed and processed it assembles with a large number of specific proteins, the function of most of which is structural. Other proteins do not finally appear in mature ribosomes and have roles in maturation, packaging and transport. One such protein is nucleolin (C23 protein) which is localised in the fibrillar centre and dense fibrillar component. Nucleolin binds to nucleic acids in general, shuttles between cytoplasm and nucleus and has been implicated in the regulation of RNA polymerase I (Borer & Lehner, 1989). Other proteins such as NO38 (B23 protein, numatrin) are also associated with the fibrillar centre (NOR) and may have similar transport functions.

NORs vary in size and shape according to nucleolar transcription. They are intimately related to the cell cycle (Goessens *et al.*, 1987), and may be related to proliferation or ploidy in some circumstances (Suresh *et al.*, 1990; Derenzini *et al.*, 1989; Leek *et al.*, 1990). During prophase the components of the fibrillar centre disperse and in metaphase these structures exist in constant positions on the short arms of the five acrocentric chromosomes (namely 13, 14, 15, 21 and 22). In telophase, tiny granules associate with these NOR bearing chromosomes and these are ultimately rearranged into nucleolar structures. The precise arrangement of chromosomes within these nucleolar structures is not known but it is evident that no clear relationship exists between ploidy and the numbers of NORs at the light and electron microscope levels (Mirre & Knibiehler, 1982).

Since NORs occur in pairs (one on each daughter chromatid) on the five acrocentric chromosomes potentially 20 could be seen at metaphase. However this is a very transient phenomenon and ten can generally be regarded as a full complement in diploid cells. Furthermore the acrocentric chromosomes tend to associate through the satellite regions and this tendancy has a major influence on the number of NORs/fibrillar centres observed. Rearrangements and amplification of the NOR sites have been demonstrated in certain cell lines (Crossen & Godwin, 1985), and regulatory effects of hormones on transcription have been observed (DeCapoa *et*

al., 1985). There is good evidence that differentiation *per se* affects the numbers of NORs observed (Reeves *et al.*, 1984; Edward *et al.*, 1991) but in general the level of cell proliferation as measured by several methods, including DNA flow, cytometry and immunocytochemistry appears to have the major influence (Derenzine *et al.*, 1989; Trere *et al.*, 1989). Ploidy, as assessed by DNA flow cytometry or karyotypic analysis, has little or no effect on interphase NOR scores.

It is clear therefore that ribosomal RNA production is complex and requires numerous enzymes, transcription factors, nuclear RNA, and regulatory factors. These combine to form substructures visible within the nucleolus. The sites of ribosomal transcription can be visualised by a variety of techniques directed against the associated proteins (NORAPS) (Table I). These latter are related specifically to the transcriptionally active sites (Ferraro & Lavia, 1983), although the true NOR, that is, rDNA, was originally identified using radiolabelled rRNA. Some NORAPs notably nucleolin have been shown to stain preferentially with silver as a result of their high electron charge density (Williams *et al.*, 1982), and by virtue of their phosphate (Satoh & Busch, 1981) carboxyl (Olert *et al.*, 1979) and -SH and -s-s-sulphur moeities (Buys & Osinga, 1980).

The silver reduction technique described below has found acceptance as a simple and precise method of NOR demonstration ideally suited to the investigation of NORs at both light and electron microscope levels.

The argyrophil (AgNOR) method

In brief, the one step method consists of mixing silver nitrate and formic acid in optimal proportions with gelatin as a colloid stabliser. Paraffin or frozen sections are incubated in this mixture for variable periods (up to 1 h) and then washed and mounted (Ploton *et al.*, 1986). Ultrastructural and light microscope studies have indicated that this method is remarkably specific as a means for the detection of metaphase and interphase NORs by virtue of their associated proteins. Indeed, sequential staining with radiolabelled rRNA and rDNA has shown correspondence between the binding sites and silver stained NORs on chromosomes and in interphase nucleoli (Hsu *et al.*, 1975).

The silver reaction product is seen as discrete black dots at the light microscope level and these can be enumerated using a × 100 oil immersion lens. Counts in 100 cells are usually made and the results expressed as the mean number of NORs per nucleus. The counts are, of course, not absolute since the NORs themselves are small compared with section thickness. Staining may have to be adjusted slightly to the individual silver binding characteristics of each tissue block and internal controls such as lymphocytes (which have one interphase NOR) are usually employed. With minor modification the technique can be used with success with automatic and semiautomatic image analysis hardware, the total amount of argyrophil material per nucleus being measured, rather than the number of sites counted (Ruschoff *et al.*, 1989; Ruschoff *et al.*, 1990). The one step method (Howell & Black, 1980), is an ingenious modification of the more time consuming original three step technique and has itself undergone modification including preincubation with glycine to reduce incubation time (Cromie *et al.*, 1988), the use of polyethylene glyco as a protective colloidal developer (in place of gelatin), to reduce non-specific deposit (Rowlands *et al.*, 1990), and the inclusion of a celloidin film, also to reduce nonspecific

deposit (Chiu *et al.*, 1989). Furthermore, minor adaptations have been employed to allow application to cell imprints (Boldy *et al.*, 1989; Ruschoff *et al.*, 1989), cytospin preparations (Ruschoff *et al.*, 1989), and semithin methacrylate sections (Moreno *et al.*, 1989).

Technical aspects

The one step silver method appears to give reproducible and specific staining results (Hernandez-Verdun *et al.*, 1980; Buys & Osinga, 1980; Ochs & Busch, 1984). From the early stages however, it was clear that uniformity with respect to fixation, technique and enumeration were required to optimise consistent discrimination of silver stained NORs (AgNORs).

In general, prompt fixation in 10% formol saline is perfectly satisfactory (Crocker *et al.*, 1989; Smith *et al.*, 1988). Alcoholic fixatives may give stronger staining, but picric acid- and mercury-containing fiixatives are highly deleterious (Smith *et al.*, 1988). The technique is readily applicable to microwave fixed tissue (Leong & Raymond, 1988), and frozen sections (Murray *et al.*, 1989), furthermore sequential staining with immunohistochemical reagents has no adverse effects (Murray *et al.*, 1989). The latter may be most useful where neoplasms are highly heterogeneous, the immunohistochemical method being used to pinpoint (or exclude) the cells in which AgNOR enumeration is required.

Early studies counted all separate silver stained structures after a constant incubation time but it is now clear that minor alterations according to internal controls are advisable to allow counting of subsiduary dots within nucleolar associations (Crocker *et al.*, 1989; Ruschoff & Plate, 1990). This recent drive to uniformity has obviated certain early work and further studies are currently underway.

Application

The potential of the one step silver colloid technique is histopathology was first investigated by Ploton *et al.* (1986) who stained an unspecified number of specimens of prostate and concluded that the method might prove useful in tumour diagnosis.

Numerous studies have subsequently appeared in the literature investigating malignancy, borderline malignancy and a variety of non neoplastic conditions. These are discussed below and conditions in which AgNORs may be of diagnostic or prognostic use are summarised in Table II.

Malignant conditions – lymphoma

The potential of AgNOR identification and quantification has been investigated in the greatest detail in lymphomas. The assessment and grading of non Hodgkin's lymphoma (NHL) is a clinically crucial procedure and accurate evalua-

Table IIa Situations in which histological evaluation of AgNORs provide information additional to traditional histopathological methods

Lymphoma grading	Islet cell neoplasms
Pleural malignancy	Small cell tumours of childhood
Small cell carcinoma of lung	Infantile fibrosarcoma
Cholesteatoma	Gliomas
In situ carcinoma of the testis	Hepatocellular carcinomas
Borderline serous tumours of ovary	Rejection of renal allografts

Table I   Techniques for demonstration of NORs

Reagent	Target
Radiolabelled rRNA	rDNA
Silver colloid (AgNOR)	NORAPs
Mercuridibromfluorescein	NORAPs
Bismuth ions	100k NORAP
Antibodies	Various NORAP epitopes

Table IIB   Conditions in which histological evaluation of AgNORs appear to be of prognostic value

Colonic neoplasia	Neuroblastoma
Gastric cancer	Mast cell tumours
? Breast cancer	
Prostatic cancer	

tion currently requires a battery of special procedures including light and electron microscopy, immunohistochemistry, histochemistry and DNA technology. It would not appear that NOR enumeration is of value in the study of NHL in both sections (Crocker & Nar, 1987), and cell imprints (Boldy et al., 1989) and also in the related field of bone marrow aspirate evaluation (Nikicicz & Norback, 1990).

Initial studies have indicated that enumeration (Boldy et al., 1989) or even morphometric analysis of the size of fibrillar centres (Crocker & Egan, 1989; Goodlad et al., 1991) can help in the identification of high from low grade types. Two further studies of follicular lymphoma have shown that when used alone AgNORs cannot distinguish follicular hyperplasia from this distinct subtype of low grade lymphoma (Cronin et al., 1989; Cibull et al., 1989).

Subsequent studies have shown that the number of AgNORS observed are closely related to the percentage of cells recognised by the antibody Ki 67 (Hall et al., 1987). This antibody binds to a nuclear antigen expressed in all cell cycle phases except G. Furthermore, in lymphomas, AgNOR numbers show no relationship to ploidy as assessed by DNA flow cytometry (Crocker et al., 1988), or numbers of AgNORs or chromosomes themselves observed in metaphase spreads (Janmohamed et al., 1989), but have a close correlation with the percentage S phase cells (Crocker et al., 1988) as determined by DNA flow cytometry. It is likely therefore that sizes and numbers of AgNORs are dependent on the proliferative status of the cell or tissue studied.

Gastrointestinal tract

Several studies of rectal and colonic neoplasia have failed to demonstrate the value of evaluation of AgNORs with regard to clinical outcome (Griffiths et al., 1989; Arends & Kate, 1988; Tildsley et al., 1990). Other studies have discriminated accurately between benign and malignant conditions and demonstrated prognostic significance (Ruschoff & Plate, 1990; Young et al., 1990; Ofner et al., 1990; Ruschoff et al., 1990; Derenzini et al., 1991). This apparent contrast may be explicable by incubation time alone. In the former studies, long incubation times may have caused overstaining and led to false fusion of AgNORs lending to decreased scores. It would therefore appear that athough AgNORs have no real advantage over established techniques in the diagnosis of colonic cancer, when used in the assessment of prognosis they may have considerable utility.

Using AgNOR scores as markers of proliferative activity in gastric epithelium Rosa et al. (Mehta & Filipe, 1990a) have demonstrated significant differences between dysplastic, malignant and normal epithelium. As in lymphoma this group subsequently showed no relationship between AgNOR counts and DNA index (Rosa et al., 1990b; Ruschoff & Plate, 1990). Ruschoff et al. demonstrated a prognostic significance in gastric carcinoma. Early studies using longer but variable incubation times showed overlap between benign, regenerative and malignant epithelium (Suarez et al., 1989).

Respiratory tract

Studies of pulmonary tumours have also yielded conflicting results, once again explicable on the basis of the technique employed. We have found AgNOR enumeration to be useful in the evaluation of malignancy in pleural aspirates, pleural biopsies and follow-up post mortem tissue (unpublished observations). The method distinguishes reliably between malignancy and hyperplasia but cannot differentiate mesothelioma from adenocarcinoma (Ayres et al., 1988). Other works have found it to be of limited use in this context (Soosay et al., 1989; Leopardi et al., 1990), but in the opinion of the authors NOR staining enhances the diagnostic yield, and provides information additional to that derived from traditional methods. The method can distinguish small cell carcinoma of bronchus from lymphocytes which is particularly useful in small biopsies (Crocker et al., 1987), but cannot distinguish carcinoids, atypical carcinoids (Benbow & Cromie, 1989) or low grade NHL (Crocker et al., 1987).

In other situations the use of AgNOR counts is yet to be proven. Counts in squamous carcinomas of larynx and bronchus are related inversely to differentiation (Ashworth & Helliwell, 1988; Crocker, 1990); however, squamous tumours of pharynx and larynx cannot be separated on the basis of AgNOR counts (Bryan et al., 1990). It is not yet certain whether AgNORs are related to prognosis for the different histological varieties of lung cancer and investigation are currently underway. Studies of AgNORs and therapeutic response are needed, not only in lung tumours but in many other contexts.

Breast

In the case of breast disease diagnostic AgNOR studies have been published which show enumeration to be useful (Smith & Crocker, 1988; Ohri et al., 1988; Raymond & Leong, 1989) or of limited value (Giri et al., 1989). Prognostic studies have indicated that counting and AgNOR morphology may provide information with regard to nodal metastases, supplementary to that obtained by established methodology (Sividis & Sims, 1990). Currently there is no indication that AgNOR enumeration will prove to be of value in the histological diagnosis of breast lesions, but potentially useful applications are to cytologically suspect, and equivocal fine needle aspirate specimens and to prognosis.

Skin

The method is applicable to skin pathology although it does not appear superior to established methodology. Adnexal tumours and epidermal tumours can be identified on the basis of AgNORs and the spectrum of differentiation of squamous carcinoma is reflected by a range of AgNOR numbers (Egan & Crocker, 1988). Studies of melanocytic tumours are hampered by the argyrophilia of melanin (Crocker & Skilbeck, 1987); nevertheless it has been shown that AgNOR staining is useful in separating melanocytic naevi from malignant melanomas (Crocker & Skilbeck, 1987; Leong & Gilham, 1989; Derenzini et al., 1986) but of limited value as a prognostic indicator (Howat et al., 1988). The value of NOR staining in borderline lesions is currently uncertain (Fallowfield et al., 1988; Fallowfield & Cook, 1989; Howat et al., 1989). Indeed the very existance of dysplastic naevi as a clinical and pathological entity is a matter of considerable debate (Clark et al., 1984; Ackerman, 1988). The only studies of AgNORs in dysplastic naevi suffer from the same overstaining as other earlier AgNOR studies and consequently may have a limited contribution (Fallowfield et al., 1988; Fallowfield & Cook, 1989). Differentiation of melanoma cell lines under the influence of retinoic acid is associated with a reversible decrease in the number of AgNORs (Youngshan & Stanley, 1988). No clinical chemotherapeutic response studies are available.

Genitourinary system

The diagnosis and grading of renal carcinoma using evaluation of AgNORs by light microscopy or image analysis has been shown to be of great value and readily applicable to cytological specimens (Ruschoff et al., 1989). The diagnosis of in situ carcinoma of the testis can be made using AgNOR counts (Loftus et al., 1988) and spermatic seminoma and typical seminoma showed different distributions of AgNORs (Delahunt et al., 1990). AgNOR analysis in prostatic adenocarcinomas has considerable prognostic utility (Contractor et al., 1989). Significant differences of AgNOR counts are seen for pathological endometrium and neoplastic endometrium but since absolute differences are small the use in any single

case is limited (Hansen & Ostergard, 1990; Coumbe et al., 1988). In non neoplastic trophoblastic tissue AgNOR counts reflect ploidy and not proliferation (Suresh et al., 1990), thus apparently being unique in tissues so far studied; the reason for this is obscure. AgNOR counts may be useful in the diagnosis of borderline serous tumours of ovary but not in mucinous tumours (Griffiths et al., 1989; Kinsey et al., 1988; Mauri et al., 1990). Studies of ectocervical and endocervical tissue have to date used long incubation times and have utilised NOR aggregates as single counts. Repeat studies are underway but nevertheless useful data have been obtained. Cervical intraepithelial neoplasia (CIN) shows a progressive increase in numbers of AgNORs with diminishing size from CIN1 to CIN3 (Egan et al., 1988; Egan et al., 1990). Altered nucleolar organisation and AgNOR counts are present in cervical intraepithelial glandular neoplasia (adenocarcinoma in situ, CIGN) (Wood & Egan, 1989) with a spectrum from in situ to invasive disease (Darne et al., 1990). This suggests that CIGN is a premalignant precursor. Additionally, alteration of AgNOR numbers and distribution adjacent to morphological CIGN ('field changes'), not visible in conventional section have been reported as being both absent (Cullimore et al., 1989) and present (Darne et al., 1990). Yet again, differing methods of staining and enumeration may account for this discrepancy.

Endocrine tumours

AgNOR numbers are of limited value in the diagnostic assessment of thyroid neoplasms (Nairn et al., 1988) and certain neuroendocrine neoplasms (Benbow & Cromie, 1989) but appear useful in the assessment of pituitary adenomas (McNichol et al., 1988) and islet cell neoplasms (Ruschof et al., 1991).

Paediatric tumours

Certain childhood tumours have been studied. Fibrosarcoma can be differentiated from fibrous proliferations (Egan et al., 1988a) and neuroblastoma can be differentiated from other small round cell tumours on the basis of AgNOR counts (Egan et al., 1987). AgNOR numbers are strongly correlated with histopathological prognostic indices of neuroblastoma and are an independent prognostic indicator in that condition (Egan et al., 1988b). The series of rhabdomyosarcoma and Ewings sarcoma reported was too small to fully assess prognostic utility (Egan et al., 1988c; Egan et al., 1988d). Experimental studies of neuroblastoma cell lines have shown a strict relationship between doubling time and AgNOR numbers and no clear relationship with synthetic activity or karyotype (Derenzini et al., 1989); indeed, this investigation was seminal in our understanding of AgNOR counts to proliferation. The strict relationship could explain the higher quantity of interphasic NOR which has been repeatedly shown in malignant tumour cells in comparison with the corresponding benign lesions. Cancers are in fact characterised by a tissue growth rate almost always faster than benign or hyperplastic lesions.

Ear, nose and throat tumours

AgNORs have radically altered the diagnosis of cholesteatoma which cannot reliably be made by any other light microscopic method (Cooper & Micheals, 1989). Malignant transitional cell tumours of the nose can be diagnosed using the AgNOR method (Egan et al., 1988) and early results from prospective studies have shown that recurrent benign transitional papillomas have a significantly increased number of AgNORs. Benign and malignant salivary neoplasms can be identified by semiquantitative assessment of silver stained NORs (Morgan et al., 1988).

CNS tumours

Enumeration is useful in the diagnosis of meningiomas (Satoh & Busch, 1981) but appears unable to predict their biological behaviour (Boon & Sharif, 1988). Gliomas and gliosis can be differentiated using AgNORs (Crocker, 1990) but the diagnosis and prediction of behaviour of occular melanomas is not enhanced by their evaluation (Williams et al., 1988).

Other

NORs are of predictive value in canine mast cell tumours and superior to established prognostic indices (Bostock et al., 1989).

Borderline lesions

A continuum of AgNOR numbers is seen in dysplastic laryngeal epithelium (Ashworth & Helliwell, 1988; Crocker, 1990), dysplastic bronchial epithelium and CIN (Egan et al., 1988; Egan et al., 1990; Rowlands, 1988). In situ transitional carcinoma of the nose can be distinguished from benign and invasive disease (Egan et al., 1988). In situ disease of the testis (Loftus et al., 1988; Delahunt et al., 1990) and endocervix (Wood & Egan, 1989) can be identified and the technique may be of value in the assessment of borderline serous tumours of ovary (Griffiths et al., 1989) and atypical endometrial hyperplasia (Coumbe et al., 1988).

Prospective studies of cirrhosis, normal liver and hepatocellular carcinoma showed that AgNOR numbers were predictive in those cases which light microscopy could not distinguish (Crocker & McGovern, 1988).

Non-neoplastic lesions

AgNORs are a sensitive indicator of tubular damage and degree of recovery in renal allografts (Dodd et al., 1988), a phenomenon which may be hard to assess with conventional methods. A physiological increase in AgNORs is seen in rat pituitary corticotrophs following adrenalectomy (Peebles & McNichol, 1988), and the diagnosis of several benign conditions can be enhanced and malignancy excluded for example renal xanthogranuloma (Bryan & Crocker, 1989), cirrhosis of the liver (Crocker & McGovern, 1988).

Conclusions

Currently on the basis of published data it would appear that AgNORs can enhance the diagnostic yield in a number of defined situations. In cholesteotoma it is vastly superior to established methods, but in all other situations the information provided is complimentary to traditional techniques. A recent drive to uniformity has increased the utility of an already reproducible method and will enable further elucidation of the natue of AgNORs. Staining and enumeration is however exacting and inexperience in both can cause poor, uninterpretable or false results. Consequently the place of AgNORs in busy routine laboratories who would carry out the technique intermittently is limited but in certain situations the information provided is indispensible and the results rewarding.

Prospective studies currently underway should define further the prognostic utility of the technique. Overstaining may in the past have contributed to nucleolar rather than NOR staining and diminished confidence in the method but current indications are that when used properly the method is a valuable tool and that the initial promise documented above will continue to find applications in numerous fields of research and diagnosis.

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