Virchows Archiv A Pathol Anat (1992) 420:353–358

Virchows Archiv A
Pathological Anatomy and Histopathology

Nucleolar organizer regions in uterine sarcomas

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Received September 11, 1991 / Received after revision December 23, 1991 / Accepted December 27, 1991

Summary. Nucleolar organizer regions demonstrable by silver staining technique (AgNORs) are loops of nucleolar DNA transcribing to ribosomal RNA. This report quantifies AgNORs in normal endometrium and myometrium, and in leiomyomas and homologous sarcomas of the uterus. The mean AgNOR number in leiomyosarcomas was significantly higher than that in normal myometrium and that in leiomyomas, whereas no significant difference was observed between normal myometrium and leiomyomas. The mean AgNOR count in low-grade endometrial stromal sarcomas was significantly higher than that in normal endometrial stroma, and significantly lower than that in the high-grade variant of the same tumour. The epithelial component of malignant mixed müllerian tumours exhibited a significantly higher mean AgNOR number than normal endometrial epithelium, and the stromal component of these tumours showed a significantly higher mean AgNOR count than normal endometrial stroma and normal myometrium, respectively. The AgNOR count was significantly correlated with the mitotic rate in leiomyosarcomas, in high-grade endometrial stromal sarcomas, and in the epithelial and mesenchymal portions of mixed müllerian tumours, whereas no statistically significant correlation was observed in low-grade endometrial stromal sarcomas. Increased AgNOR counts have been reported for some kinds of malignant tumours in various organs, compared with normal tissues and benign tumours. This study demonstrates a similar increase when homologous uterine sarcomas are compared with histogenetically related normal and neoplastic tissues. AgNOR counting might be a useful adjunct in the classification and grading of uterine tumours.

Key words: Nucleolar organizer regions – Sarcoma – Uterus

Introduction

Uterine sarcomas are rare neoplasms, accounting for less than 5% of all uterine malignancies (Young et al. 1982; Harlow et al. 1986). Ober (1959) suggested a classification of these sarcomas into six major classes, and other classifications have also been proposed. The one most commonly applied nowadays (Hendrickson and Kempson 1980) comprises four major classes of sarcomas (pure sarcomas, mixed sarcomas, mixed müllerian tumours and unclassified sarcomas) which include the main histopathological categories: leiomyosarcomas, endometrial stromal sarcomas and malignant mixed müllerian tumours.

Endometrial stromal sarcomas are subdivided into low-grade and high-grade variants according to the mitotic rate. Among unusual uterine neoplasms can be found a low-grade sarcoma resembling ovarian sex-cord tumour (Hendrickson and Kempson 1980; Malfetano and Hussain 1989), a pure malignant giant cell tumour (Kindblom and Seidal 1981), a malignant giant cell tumour associated with leiomyosarcoma (Siein'ski 1990), and an endometrial diffuse clear cell stromal sarcoma identified with the help of immunohistochemistry (Lifschitz-Mercer et al. 1987). Leiomyosarcomas of the uterus and soft tissues are similar to each other, but differ in some respects from those in the gastrointestinal tract (Evans et al. 1988).

Because of the low incidence, little is known about the biochemical and biological characteristics of uterine sarcomas. Moreover, the microscopic identification and classification of uterine sarcomas may be problematic, especially with regard to the differentiation between cellular, atypical leiomyomas and low-grade leiomyosarcomas. Endometrial curettage is of great importance, whereas cervical smears are only rarely of any help in the diagnosis of uterine sarcomas (Kahanpää et al. 1986). Antibodies to vimentin, not to desmin, have been found in low-grade stromal sarcomas, and in the mesenchymal elements of mixed mesodermal tumours (Lifschitz-Mercer et al. 1987).

This study was carried out to see whether determination of argyrophilic nucleolar organizer regions (AgNORs) might be helpful in the diagnosis, classification and grading of uterine sarcomas. Enumeration of AgNORs has in recent years been used for discrimination between benign and malignant tumours, and for grading of neoplasms in different organs. In a previous study, a significantly increased mean AgNOR number was found in parathyroid carcinoma, compared with normal, hyperplastic and adenomatous parathyroid tissues, whereas no evidence was obtained for a role of AgNOR counting in the differentiation among normal, hyperplastic and adenomatous glands (Boquist 1990).

Attention has been paid to AgNORs in a few studies of epithelial tumours of the uterus. Significantly more AgNOR staining has been reported in adenocarcinoma in situ and adenocarcinoma of the endocervix compared with normal endocervical epithelium (Cullimore et al. 1989; Marbaix et al. 1989; Darne et al. 1990), and in endometrial hyperplasia with atypia and adenocarcinoma compared with benign hyperplasia (Coumbe et al. 1990; Wilkinson et al. 1990). AgNOR enumeration has also been used in breast pathology. Increased AgNOR counts in breast carcinoma compared with benign lesions (Smith and Crocker 1988) bear a relationship to tumour growth fractions (Raymond and Leong 1989) and may provide supplementary and prognostic information (Giri et al. 1989; Derenzini et al. 1990; Sivridis and Sims 1990). As regards mesenchymal tissues, fibrous proliferations in childhood can be differentiated from low-grade infantile fibrosarcoma on the basis of AgNOR counting (Egan et al. 1988).

Materials and methods

The material consisted of tissue from women with a primary histopathological diagnosis of uterine sarcoma. The primary diagnoses had been made using routinely embedded tissue from curettage and/or uterine specimens stained with one or more of the following stains: haematoxylin and eosin, van Gieson's stain, periodic acid-Schiff, Masson trichrome, phosphotungstic acid haematoxylin, alcian blue, and reticulum stain. Immunohistochemistry had additionally been performed in selected cases. The primary diagnoses were verified by re-examination of the original slides. When necessary, new sections were prepared and studied after staining, including immunohistochemistry. Antibodies and their dilutions used for immunohistochemistry were monoclonal muscle actin, 1:800; monoclonal cytokeratin, 1:100; polyclonal desmin, 1:1000; polyclonal myoglobin, 1:1000; monoclonal vimentin, 1:80, and polyclonal S-100 protein, 1:2000. All controls were positively stained.

At re-examination, the tumours were classified according to the method of Hendrickson and Kempson (1980). The diagnosis of leiomyosarcomas was based on the occurrence of at least 5 mitotic figures in the additional presence of nuclear polymorphism per 10 consecutive high-power fields, or on the occurrence of at least 10 mitotic figures in the absence of nuclear polymorphism per 10 consecutive high-power fields.

Tumours classified as endometrial stromal sarcomas were composed of spindle-shaped cells having a varying amount of cytoplasm, and a varying degree fo polymorphism. According to the mitotic rate, these tumours were sub-classified into a low-grade variant exhibiting less than 20 mitotic figures per 10 consecutive high-power fields, and a high-grade variant showing more than 20 mitoses per 10 consecutive high-power fields.

The neoplasms classified as malignant mixed müllerian tumours were composed of both epithelial and non-epithelial cells with polymorphism and a varying degree of mitotic activity.

The following tumour categories were chosen for AgNOR enumeration: leiomyosarcoma, endometrial stromal sarcoma and malignant mixed müllerian tumour. Since comparison was made with normally occurring uterine tissues, no cases of heterologous tumours were studied. Unclassified sarcomas (Hendrickson and Kempson 1980) were also excluded.

In attempts to use histogenetically related tissues as control material, normal endometrium and myometrium were chosen for comparison with neoplastic tissue. In addition, leiomyomas were selected for comparison with leiomyosarcomas. The normal tissues were obtained from patients without any kind of uterine neoplasm. In preliminary AgNOR enumerations, endometrium was also studied in three patients with uterine leiomyosarcoma not infiltrating the mucosa. Moreover, in four leiomyosarcoma patients preliminary AgNOR counts were made of the apparently normal myometrium surrounding the tumours. The AgNOR counts obtained in these preliminary studies were similar to those for endometrium and myometrium of women without any neoplasm. Only tissues from women without any uterine neoplasm were used as controls for the final counts.

Consecutive 3 μm sections were cut from blocks containing uterine tissue which had previously been routinely fixed in 10% formalin and embedded in paraffin. In order to minimize non-specific precipitation and background staining, the equipment was scrupulously cleaned and the sections were thoroughly rinsed. Then the sections were dewaxed in xylene, hydrated through decreasing concentrations of ethanol, and washed for 10 min in deionized water. Thereafter a one-step silver nitrate staining was performed. The staining solution was prepared by dissolving 2% gelatin in distilled and deionized water and adding formic acid to a final concentration of 1%. This solution was mixed in a proportion of 1:2 volumes with 50% aqueous silver nitrate solution, and filtered through a 0.22 μm Millipore filter under safelight conditions. After preparation, the working solution was immediately dropped on to the sections and incubation was carried out for 35 min at room temperature in a darkroom. No counterstaining was used. The sections were washed with distilled and deionized water for 10 min, dehydrated in ascending ethanol concentrations, cleared in xylene, and mounted in DPX medium.

The sections were examined by one and the same person, under oil immersion microscopy at a magnification of ×1000. A total of 100 randomly selected nuclei of tumour cells were counted, using a graticule to prevent duplicate counting. The microscope was equipped with a colour filter. AgNORs were visualized as black intranuclear dots. The numbers of discernible and separable dots were counted, and the mean number of AgNORs per cell was calculated. AgNORs that were aggregated or morphologically inseparable were counted as one dot. In preliminary studies, repeat counts varied by 5–8%. Wilcoxon's test was used for statistical treatment of the AgNOR counts. The relationship between AgNOR counts and mitotic rates in each tumour category was estimated by analysis of linear correlation, testing the Pearson's coefficient by means of a *t*-test.

Results

The AgNOR sites appeared as black dots in the nuclei of the normal and neoplastic cells (Fig. 1). They exhibited somewhat varying size, configuration and staining affinity, and occurred in of all kinds of cells investigated. There appeared to be a somewhat greater variation in size and configuration of the AgNORs in malignant than in normal cells, but this was not subjected to any further study.

Non-specific precipitation and background staining

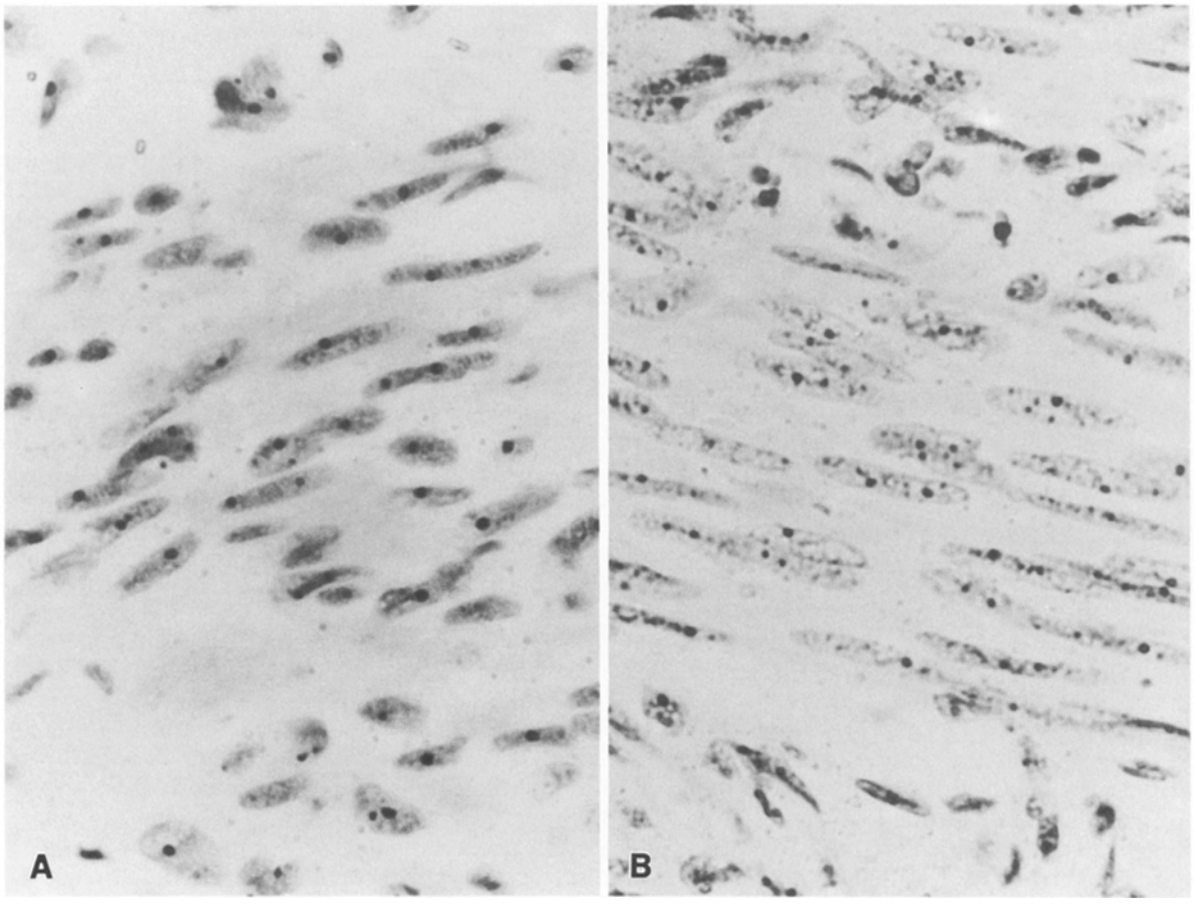

Fig. 1 A, B. Photomicrographs demonstrating AgNORs in a uterine leiomyoma (**A**) and in the mesenchymal component of a malignant mixed müllerian tumour (**B**). Silver colloid staining, ×250

in the sections could not be completely avoided, but only in a few cases did this cause any significant difficulties in the dilineation of the AgNORs. In these cases the influence of non-specific alterations could be considerably reduced by new sectioning and staining.

When treating all uterine sarcomas as one group, the mean number of AgNORs was significantly higher ($P<0.001$) than that in the cells constituting the normal endometrium and myometrium. The mean number of AgNORs in the tumour cells of leiomyomas was not significantly different from that in the muscle cells of the normal uterus (Table 1). In contrast, leiomyosarcomas exhibited a significantly higher mean AgNOR number than the normal uterine muscle cells and the tumour cells of the leiomyomas, respectively.

A significantly higher ($P<0.001$) mean AgNOR number was observed in endometrial stromal sarcomas, when compared with normal endometrial stroma. As regards the two grades of endometrial stromal sarcomas, the mean AgNOR count in the low-grade variant was significantly higher than that in normal endometrial stroma, and the mean AgNOR number in the high-grade variant was significantly higher than that in the normal stroma and in the low-grade variant, respectively.

In comparison with the mean AgNOR numbers for the stromal cells of normal endometrium and the muscle cells of normal myometrium, a significant increase in mean AgNOR count was found for the mesenchymal component of malignant mixed müllerian tumours. Moreover, the epithelial component of malignant mixed müllerian tumours showed a significantly higher mean AgNOR count than that of the epithelium of normal endometrium.

A significant correlation was observed between the AgNOR count and the mitotic rate in leiomyosarcomas ($r=0.759$, $P<0.001$) and high-grade endometrial stromal sarcomas ($r=0.746$, $P<0.005$). A slight, although statistically significant, correlation between these variables was also found in both the epithelial ($r=0.636$, $P<0.05$) and the mesenchymal ($r=0.601$, $P<0.05$) components of mixed müllerian tumours. In contrast, there was no significant correlation between the number of AgNORs and the frequency of mitotic figures in low-grade endometrial stromal sarcomas.

Discussion

Although there are still some uncertainties as to the histogenesis of the sarcomas studied, it seems adequate for the present purpose to compare the data for the sarcomas with those for normal endometrium and myo-

Table 1. Mean AgNORs counts in 100 nuclei of normal and neoplastic uterine cells

Diagnosis (group)	No. of cases	AgNOR count (mean ± SEM)	Statistical significance
Endometrium; epithelium (I)	8	104.4 ± 9.1	
Endometrium; stroma (II)	8	129.9 ± 11.1	
Myometrium (III)	26	144.8 ± 9.2	
Leiomyoma (IV)	15	151.1 ± 7.6	III–IV NS
Leiomyosarcoma (V)	18	218.0 ± 16.7	III–V $p<0.001$ IV–V $P<0.001$
Endometrial stromal sarcoma; low-grade (VI)	6	199.3 ± 21.9	II–VI $P<0.01$
Endometrial stromal sarcoma; high-grade (VII)	8	264.9 ± 20.5	II–VII $P<0.001$ VI–VII $P<0.05$
Malignant mixed müllerian tumour; epithelial component (VIII)	14	309.9 ± 27.6	I–VIII $P<0.001$
Malignant mixed müllerian tumour; mesenchymal component (IX)	14	281.5 ± 33.6	II–IX $P<0.001$ III–IX $P<0.001$

NS, Not significantly altered

metrium. Leiomyosarcomas are usually considered to develop from myometrial muscle cells, or from leiomyomas undergoing malignant transformation. Hence the data for the leiomyosarcomas were compared both with those for normal myometrium and with those for leiomyomas. Since endometrial stromal sarcomas appear to be derived from the stromal cells of the endometrium, AgNOR counts for the latter cells were used for statistical evaluation of the data for these sarcomas. Mixed müllerian tumours are considered to originate from undifferentiated cells of the müllerian system with the potential to differentiate into either epithelial or stromal tumour cells (Sternberg et al. 1954). Since data were not obtained for such undifferentiated cells, it seems preferable to compare the data for malignant mixed müllerian tumours with those for epithelium in normal endometrium and those for stroma both in normal endometrium and myometrium.

"Dedifferentiated leiomyosarcomas" possessing poorly differentiated areas not recognizable as leiomyosarcoma have been reported to bear a similar prognosis as other leiomyosarcomas (Evans et al. 1988). Mitotic activity or nuclear polymorphism have no prognostic significance for mixed müllerian tumours (Williamson and Christopherson 1972; Kahanpää et al. 1986). As regards uterine leiomyosarcomas, there are highly different opinions in the literature both about diagnostic and prognostic factors. For the microscopic diagnosis, estimation of mitotic activity has in most studies been ascribed a prominent role in the discrimination between benign and malignant neoplasms. Some authors have based the discrimination between leiomyomas and leiomyosarcomas entirely on mitotic frequency (Vongtama et al. 1976; Silverberg 1979), whereas others diagnose a leiomyosarcoma when there are 5 or more mitotic figures in 10 consecutive high-power fields in the most active area of the tumor, but also when there is lower mitotic rate but extensive tumour necrosis (Evans et al. 1988). Still others base the diagnosis of leiomyosarcomas on the occurrence of at least 5 mitotic figures and in addition nuclear polymorphism, or on the absence of nuclear polymorphism but the occurrence of 10 or more mitoses per 10 consecutive high-power fields (Hendrickson and Kempson 1980). There are also authors who use nuclear polymorphism as the main criterion for malignancy and do not require any minimum mitotic frequency (Spiro and Koss 1965). Leiomyoma variants which might be difficult to differentiate from leiomyosarcomas include atypical leiomyoma, palisaded leiomyoma and plexiform leiomyoma, as well as intravenous leiomyomatosis and peritoneal leiomyomatosis (Evans et al. 1988).

The observations in this study suggest a value of quantification of AgNORs in the diagnosis of uterine sarcomas. The mean AgNOR numbers for all tumours studied were significantly higher than that for the histogenetically related normal tissues, and leiomyosarcomas exhibited significantly higher mean AgNOR counts than leiomyomas.

Since the mitotic rate plays a role in the discrimination between leiomyomas and leiomyosarcomas, and between low-grade and high-grade endometrial stromal sarcomas, it is also of interest that a correlation was observed between the AgNOR count and the mitotic rate in leiomyosarcomas and in high-grade endometrial stromal sarcomas. A similar correlation was seen in both the epithelial and mesenchymal parts of mixed müllerian tumours, whereas no such correlation could be demonstrated in low-grade endometrial stromal sarcomas.

As referred to above the differentiation between leiomyosarcomas and at least some kinds of leiomyomas in the uterus is occasionally problematic, and there are

sometimes difficulties in the discrimination between endometrial stromal sarcomas, on the one hand, and benign proliferative conditions and benign neoplasms, on the other. As regards mixed müllerian tumours, it might be difficult to assess whether the epithelial component is benign ("adenosarcoma") or malignant. Moreover, there are cases of uterine adenocarcinoma in which one might question whether structural alterations in the stromal tissue are of reactive or neoplastic nature. In such cases AgNOR counts might give sufficient additional information to reach an accurate histopathological diagnosis. Moreover, since significantly different mean AgNOR counts were observed in low-grade compared with high-grade endometrial stromal sarcomas, it appears that quantification of AgNORs also might be of importance in the grading of these sarcomas.

The limitations of using AgNOR counting for classification and grading of uterine sarcomas are apparently similar to those associated with the use of the AgNOR technique for the study of other neoplasms. Non-specific background staining, aggregation of dark-stained nuclear components, silver staining of nucleoli, problems of reproducibility, and inter- and intra-observer variations (Howat et al. 1988; Giri et al. 1989; Griffiths et al. 1989; Raymond and Leong 1989) limit the usefulness of the AgNOR technique for investigation of uterine neoplasms. Previous experience of the use of AgNOR technique for the study of proliferative and neoplastic conditions (Boquist 1990) suggests that such problems are not negligible, and necessitate consideration both as regards methodology and interpretation. In this study the estimated intra-observer variation was rather low, and similar to that previously recorded by the author when studying parathyroid glands (Boquist 1990) and esentially similar to that reported by others (Raymond and Leong 1989). Such problems might be pronounced for those using the AgNOR technique only infrequently.

The mean AgNOR count for normal endometrium in this study is essentially similar to that reported by others for normal endocervix (Cullimore et al. 1989; Darne et al. 1990). The number of AgNORs in a cell may be related to cellular activity (Ploton et al. 1986; Crocker and Nar 1987), ploidy (Giri et al. 1989; Raymond and Leong 1989) and metastatic potential in malignant cells (Gillen et al. 1988). It has been reported that the AgNOR count is higher in invasive adenocarcinoma of the endocervix than in adenocarcinoma in situ, and that the latter in turn exhibits a higher AgNOR count than normal endocervical cells (Cullimore et al. 1989; Darne et al. 1990). The observations that the mean number of AgNORs in this study was higher in sarcomas than in normal tissues and leiomyomas, and higher in high-grade than in low-grade endometrial stromal sarcomas suggest an association between AgNOR number and malignant potential in uterine tumours with a pure sarcomatous or mixed carcinomatous and sarcomatous appearance.

Acknowledgement. Supported by a grant from Lion's Research Foundation, Department of Oncology, University of Umeå, Umeå, Sweden.

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