Variability of Argyrophilic Nucleolar Organizer Regions in Osteoblastic Cells

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Various diagnostic parameters are currently available and used for estimating the prognosis of malignant tumors7). One of the methods estimating prognosis is argyrophilic nucleolar organizer region (AgNOR) technique introduced by Goodpasture and Bloom (1975)3). Nucleolar organizer regions (NORs) are loops of DNA in the nucleolus which code for ribosomal RNA (rRNA) and are associated with rRNA activity, protein synthesis and cell proliferation3). NORs can be visualized as black dots by AgNOR technique. The structures thus demonstrated as dots are termed 'AgNORs'. The AgNORs are identified as NOR-associated proteins rather than NORs themselves6). Recently, more sensitive staining method for NORs is available5).

Although numerous histopathological studies of AgNORs have been carried out on benign or malignant tumors6,7), scant attentions have been paid to normal tissues or cells. For applying diagnostic aid in histopathology, variability of AgNORs in normal cells remains to be evaluated.

A clonal murine osteoblastic cell line, MC3T3-E1, is a useful in vitro model system for studying the process of osteoblastic development8). In the present study, we examined the number of AgNORs in cultured MC3T3-E1 cells during cell proliferation and matrix maturation.

Materials and Methods

I. Cell culture

Osteoblastic MC3T3-E1 cells, provided by Dr. Kumegawa (Meikai University Dental School, Japan), were maintained in alpha modification of Eagle's minimum essential medium (Gibco; Grand Island, NY) supplemented with 10% fetal bovine serum under the atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were plated at a density of 8.4×10⁵ cells/35-mm dish. For staining AgNORs, cells were grown on sterile coverslips in the plastic culture dishes. Experiments were conducted three times to obtain a sufficient number of reproducible
histological and biochemical data.

II. AgNOR method

Cells grown on coverslips were fixed with 4% formaldehyde for 1 hr at 4°C. AgNOR staining was performed according to the method described by Hozák et al. (1992). Briefly, solution A (0.5 g/ml AgNO₃ in distilled water) and solution B (1 g gelatin in 100 ml of water containing 1% formic acid) were mixed (2:1, v/v) in a darkroom. The cells were incubated for 30 min in the mixed solution at ambient temperature. To prevent heterogeneous background, coverslips were rinsed thoroughly with deionized distilled water. After rinsing, coverslips were mounted for observation. The specimens were examined under an Olympus BX40 microscope and the microphotographs were taken on Fuji minicopy films using the automatic exposure.

III. AgNOR analysis

The AgNORs were counted according to the recommendation of Crocker (1990). Three hundred nuclei were evaluated per group at a 1000-fold magnification using oil immersion. The significance of the differences in the number of AgNORs in each group was determined by Student's t-test. All statistical manipulations were performed using a computer statistics package (Stat View, version 4.02, Abacus Concepts; Berkeley, CA). Data were expressed as means ± standard deviation.

IV. Assay of alkaline phosphatase activity

Production of alkaline phosphatase (ALP) was measured spectroscopically. Osteoblast cultures of appropriate periods were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline and then frozen. Upon thawing, the cells were scraped from culture dishes with 1 ml of 50 mM Tris-HCl buffer (pH 9.1) and sonicated for 1 min at 100 W (50/60 Hz) on ice. The sonicates were centrifuged for 10 min at 10000 g and the supernatants were used for enzyme assay. Aliquots of 50 µl samples were incubated at 37°C for 30 min with 450 µl of the reaction buffer containing 50 mM Tris-HCl (pH 9.1) and 100 mM p-nitrophenyl phosphate. The reaction was stopped by addition of 500 µl of 1 N NaOH. The production of p-nitrophenol in the presence of ALP was measured at 405 nm. One unit (U) of enzyme was defined as the activity causing the release of 1 nmol of product per min under the assay conditions described above. The protein content of the supernatants was determined colorimetrically (Bio-Rad Protein Assay Kit).

Results

I. Light microscopic observation of AgNORs in osteoblastic cells

AgNORs were clearly visible as black dots in the nuclei (Fig. 1). The size of the dots at the day 3 after plating was large and the shape was jagged (Fig. 1a). In contrast, the dots at the days 6, 9 and 20 were small and round (Figs. 1b, 1c and 1d).

II. Analysis of the number of AgNORs

As shown in Fig. 2, the mean number of AgNORs per nucleus reached to maximum (11.7 ± 2.79) at the day 6, decreased to 7.9 ± 2.25 at the day 9 and then retained the levels to
Fig. 1 AgNORs staining in murine osteoblastic MC3T3-E1 cells. AgNORs in the nucleus were observed as black dots: a, at the day 3; b, at the day 6; c, at the day 9; d, at the day 20. Scale bar, 10 μm.

Fig. 2 Variability of the number of AgNORs per nucleus in MC3T3-E1 cells. Error bars designate means ± standard deviation for 300 nuclei.

Fig. 3 Time course of the ALP activity in MC3T3-E1 cells. Each plot indicates the average of three independent cultures; the bars represent standard deviation.
the day 20 (7.7 ± 2.19). Significant differences were found in the mean number of AgNORs between groups at the days 3 and 6 (p<0.0001). The significance was also found between the groups at the days 6 and 9 (p<0.0001).

### III. ALP activity

Figure 3 shows that specific activity of ALP gradually increased as culture time proceeded and reached to the maximal level at the day 9 after plating. The ALP activity was then decreased till 13 day and leveled to 20 day after plating.

**Discussion**

AgNOR method is reported to assist in the distinction between high- and low-grade non-Hodgkin's lymphomas\(^1\). This technique has been used successfully to differentiate between benign and malignant lesions of cutaneous\(^2\), intestinal\(^3\), and salivary gland tumors\(^4\). The application of AgNOR method to tumor pathology has diagnostic value in the defined situations. The mean number of AgNORs per nucleus was higher in malignant than that found in benign tissues, higher in high grade than in low grade malignancies, and higher in tumors with a poor prognosis compared with a good prognosis. Recently, Xie et al. (1997)\(^5\) presented a cut-off point of AgNORs enumeration, pAgNOR > 1, for distinguishing between normal epithelium, dysplasia, and squamous cell carcinomas of the oral cavity. However, in general, there is no guideline of AgNOR counts for distinction between benign and malignant or high- and low-grade lesions.

Our results demonstrate that the number of AgNORs in osteoblastic cells varied during cell differentiation. According to Crocker et al. (1989)\(^6\), a single, large and round AgNOR is characteristic of normal cells. However, there are several types of AgNORs among the normal tissues\(^7,8\). The number of AgNORs provides a marker of cell proliferation and clinical behavior of many types of neoplasm\(^9,10\).

The high ALP activity is a marker of the osteoblast matrix maturation stage, whereas actively proliferating cells committed to the osteoblast lineage typically express low levels of ALP activity. In the present study, however, the number of AgNORs remarkably increased in the earlier matrix maturation stage, not in the proliferating stage. Many cell types exhibit an inverse relationship between growth and differentiation *in vitro*\(^11\). There is evidence from other cell lines that the number of AgNORs declines as cells become more differentiated. The results presented here are consistent with the results showing that variations in the expression of AgNORs were more related to phenomena of cellular maturation than to proliferative activity\(^12\).

In conclusion, the present study demonstrates that the number of AgNORs varied in osteoblastic cells *in vitro*. This evidence raises the possibility that in the normal cells *in vivo* the number of AgNORs may be variable in accordance with the cell development. These findings suggest that we should take the degrees of cell growth and of cell differentiation into consideration when applying an AgNOR method to diagnosis and prognosis of neoplasm in clinical pathology. By using MC3T3-E1 cells, we are now conducting further work to
elucidate the role of AgNORs in osteoblast differentiation.

**Summary**

The changes in the number of AgNORs per nucleus in normal cells are obscure. In the present study, we examined the variability of AgNORs in osteoblastic MC3T3-E1 cells. We designed to enumerate the number of AgNORs during cell differentiation. The cells were grown for up to 20 days in the presence of fetal bovine serum. At the days 3, 6, 9, 13 and 20 after plating, the cells were stained by AgNOR method and also used for measurement of ALP activity. The number of AgNORs per nucleus increased from 6.9 ± 2.15 (at the day 3) to 11.7 ± 2.79 (at the day 6), decreased to 7.9 ± 2.25 (at the day 9) and then leveled to 7.7 ± 2.19 (at the day 20). The ALP activity was maximal at the day 9 after plating. In osteoblastic cells, the number of AgNORs was maximized in the earlier matrix maturation stage, not in the proliferating stage and was variable during cell differentiation.

**References**

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骨芽細胞様細胞 MC3T3-E1 における AgNORs の変化

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鍍銀染色法の一つである AgNORs 法は細胞増殖能を知ることができることから腫瘍病理学の分野では、病理学的診断と新生物の予後をはかるマーカーとして注目を集めている。本法は免疫組織学的手法に依らないことから、短時間で検索できる点でも迅速な診断を求める臨床の場において広く応用される可能性を秘めている。現在までに、AgNORs 法を用いて悪性腫瘍の診断と予後をはかる報告は数多くみられるが、ある一つの細胞を対象に細胞分化と AgNORs 数との関連を調べたものはほとんどない。今回我々は骨細胞に分化する細胞の骨芽細胞様細胞 MC3T3-E1 を用いて細胞增殖期から細胞外基質成熟期まで核内の AgNORs 数の変化を調べ、以下の結論を得た。

1. 骨芽細胞の核内 AgNORs 数は一定でない。
2. 細胞が基質成熟期の初期のとき AgNORs 数が最大を示す。

AgNORs 法は細胞増殖能をよく反映することから新生物の病理組織診断や予後を応用されつつあるが、本法を応用する際には細胞の成長と分化度を考慮する必要があることが示唆された。