Int J Legal Med (2000) 113 : 244–246

ORIGINAL ARTICLE

M. Rebolledo Godoy · A. P. Rebolledo Godoy
M. Oehmichen

AgNORs during the process of wound healing

Time dependency as evaluated in vital and postmortem biopsy

Received: 29 March 1999 / Final revision received: 30 August 1999

Abstract Silver staining of paraffin sections to detect changes in nucleolar organizer regions (AgNORs) is an established method for detecting cellular proliferation. To determine whether AgNORs are helpful in assessing wound age and vitality, we examined intravital and postmortem skin biopsies from rats surviving incised wounds to both pinnae for defined intervals up to 384 h using, 7 rats per time interval. One biopsy was taken immediately before death, a second 24 h after been sacrificed and storage at 8 °C. Interactive computer-assisted image analysis revealed that in the first 120 h after trauma the total number of AgNORs, the mean AgNOR sum area in the nucleus, and the mean AgNOR area per nucleus were dependent on survival time. Taken as indicators of proliferative activity these morphological phenomena revealed an increase in proliferative activity after survival times ranging from 10 to a maximum of 96 h. The findings were the same in both intravital and postmortem biopsies. These findings are in accordance with those obtained using bromodeoxyuridine. The value of these experimental findings in assessing wound age of human beings is discussed.

Key words AgNOR · Proliferation · Wound · Intravital · Postmortem

Introduction

Nucleolus organizer regions (NORs) are loops of DNA where transcription of rRNA occurs and which are thought to be the rDNA locus in the genome. They are associated with the proliferative activity of cells [3]. Under the control of RNA polymerase a transcriptase, they are responsible for the synthesis of ribosomal ribonucleic acids (rRNA), the principal component of ribosomes and thus constitute the starting point for cellular protein synthesis [4, 16]. NORs can be localized using a silver staining technique on paraffin sections. These silver reaction products are termed argyrophilic NORs (AgNORs) [10] and are a direct index of the proliferative activity of a cell [12, 19]. This activity can be evaluated under standardized conditions [20] by means of computer-assisted image analysis [8, 11]. The AgNOR technique has been applied successfully as a marker of malignancy and to grade overt malignancies [7]. It has proved to be of particular value in assessing prognosis [6, 8, 9, 21]. To our knowledge, this is the first study to apply this method to evaluate the proliferative activity of epidermal basal cells at wound margins [13].

M. Rebolledo Godoy · A. P. Rebolledo Godoy
M. Oehmichen (✉)
Department of Legal Medicine, University of Lübeck,
Kahlhorststrasse 31–35, 23562 Lübeck, Germany
Fax: +49-451-500 2760
e-mail: oehmich@rmed.mu-luebeck.de

Materials and methods

The design of the experiment is described in detail elsewhere [14, 15]. Briefly, the material investigated in this study originated from

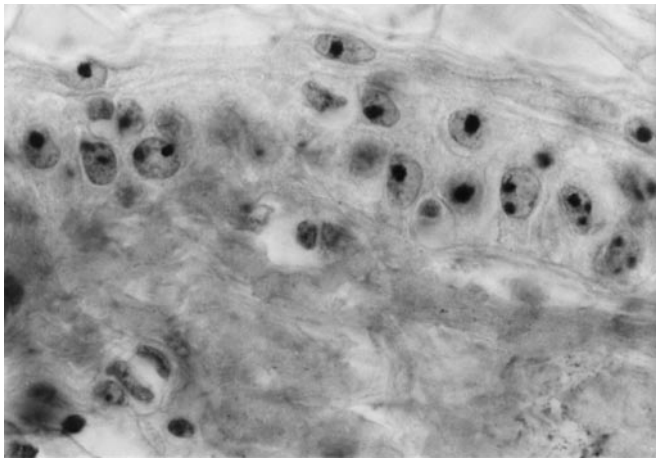

Fig. 1 Demonstration of AgNORs in the nuclei of epidermal basal cells of the rat (silver staining according to Korek et al. [10], magnification × 1000)

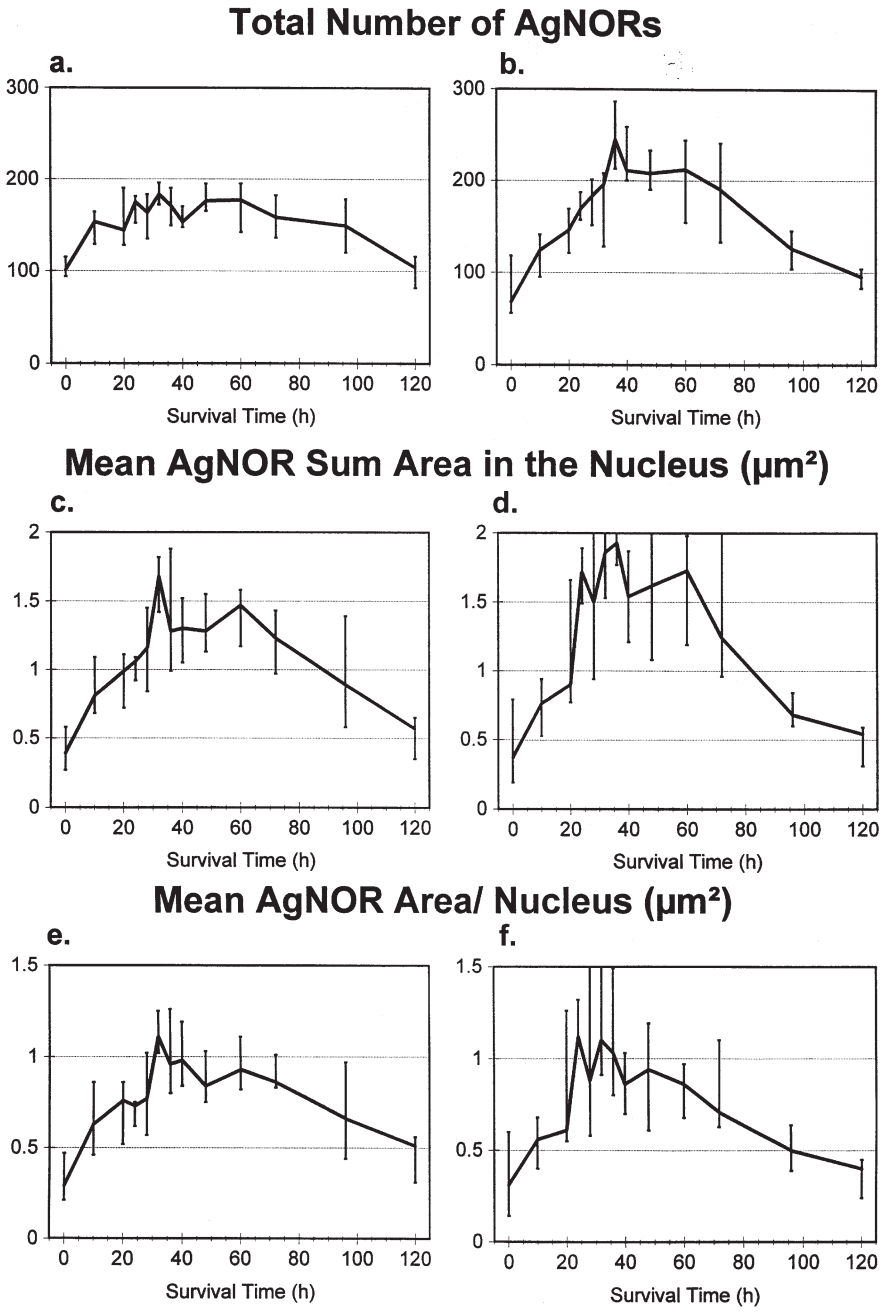

Fig. 2 a–f Quantitative evaluation of AgNORs in epidermal basal cells at the wound border as determined in the first 120 cells – in relation to survival time. **a, c, e** Vital biopsies and **b, d, f** postmortem biopsies after evaluation of the total number of AgNORs, the mean AgNOR sum area in the nucleus, and the mean AgNOR area per nucleus

incised wounds on both pinnae of each rat. One biopsy was taken intravital, the second postmortem after 24 h storage at 8 °C. The wound age varied between 0 and 384 h and each interval 7 rats were sacrificed.

The biopsies were prepared in the following steps: fixation in 4% formaldehyde solution, extraction of 3–5 µm thick paraffin sections, staining for 32 min (our own optimal staining duration) with a combination of Feulgen reaction and silver nitrate according to a modified method for the detection of AgNORs [10, 18], dewaxing and hydration.

A computer-assisted image analysis was used for the quantitative evaluation of the AgNORs. Software (SK-RM1, SK-STAT) was created especially for this purpose (Dipl.-Ing. O. Wegner, Adalbertstr. 25, D-24106 Kiel) in accordance with our specifications.

A total of 120 epidermal basal cells were evaluated per sample. Moving outward from the wound margin, the cells were counted in 3 segments each containing 40 cells. For each cell the size of the nucleus plus the number and size of the AgNORs were determined. The evaluation of the following selected cytological criteria are demonstrated in the present paper: total number of AgNORs, mean sum area of AgNORs in the nucleus (µm^2) and mean AgNOR area/nucleus (µm^2).

Statistical calculations, median and limit values (min, max), were done separately for each group of seven animals, for each survival interval, and for each value measured. Analysis of variance was used to calculate the significance of differences for each value. Finally, multiple comparisons and one-way multiple range tests according to Scheffé were performed with $p < 0.05$ being regarded as significant.

Results

Silver staining proved to be a simple technique for the quantitative evaluation of AgNORs (Fig. 1). The results

revealed a consistent dependence on survival time regardless of the distance from wound border. The differences between the three segments were so slight that all basal cells together (1–120) could be evaluated.

The selected parameters exhibited an especially pronounced dependence on survival time and showed significant intravital and postmortem increases from 10–96 h posttrauma. After 60 h a maximum was passed and after 120 h the starting point was reached again. The values for each parameter peaked after 32 h (intravital) and 36 h (postmortem) survival time. No significant difference was found between intravital versus postmortem biopsies (Fig. 2). A consistent relatively large scattering was found which may be attributable to the small sample of seven animals per survival interval.

Discussion

Activation of the proliferative activity of epidermal basal cells on wound margins with a technique such as bromodeoxyuridine (BrdU) has been demonstrated in animal experiments, and even on postmortem tissue [14]. Antibodies against the Ki-67 epitope, by contrast, do not show a clear increase in proliferation [1]. The question arose whether computer-assisted image analysis of AgNORs can produce a proliferation pattern comparable to that obtained with the BrdU technique, also on postmortem tissue. Theoretically this would provide a marker of proliferation that can be applied – even retrospectively – on paraffin embedded material but would not require the time-consuming incubation in a BrdU solution.

In order to be able to utilize standardized material, the present study was based on previous animal experiments [14, 15]. Use of the same material also enabled direct comparison of the different markers.

Our results show that AgNORs in skin tissue are also an index of proliferation, as was already demonstrated in tumor tissue [2, 5, 17]. Our findings revealed no significant differences between intravital versus postmortem results, except a notable difference in the peaks, intravitally after 32 h, and after 36 h postmortem. This difference may be readily accounted for by the continuation of proliferation after death.

The presented proliferation kinetics applying the AgNOR technique are comparable with those obtained with BrdU [14]. The results confirm our previous BrdU findings regarding the postmortem proliferation behaviour of epidermal basal cells of the skin following wounding. Computer-assisted image analysis of AgNORs and the BrdU technique, can help in the estimation of wound age and provides an only slightly limited aid in the classification of the temporal course of the wound healing process.

References

1. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993) The time-dependent localization of Ki 67 antigen-positive cells in human skin wounds. Int J Legal Med 106: 35–40
2. Crocker J, Macartney JC, Smith PJ (1988) Correlation between DNA flow cytometric and nucleolar organizer region data in non-Hodgkin's lymphomas. J Pathol 154(2): 151–156
3. Crocker J (1990) Nucleolar organizer regions. Curr Top Pathol 82: 91–149
4. Fakan S, Hernandez-Verdun D (1986) The nucleolus and the nucleolar organizer regions. Biol Cell 56: 189–206
5. Field D, Fitzgerald P, Sin F (1984) Nucleolar silver staining pattern related to cell cycle phase and cell generation of PHA stimulated human lymphocytes. Cytobios 41: 23–33
6. Fisseler-Eckhoff A, Becker T, Sudhoff H, Müller KM (1994) AgNOR counts in preneoplastic lesions of the bronchus. Path Res Pract 190: 389–393
7. Hörlin A, Krause J, Radü U, Hübner K, Stutte HJ, Müller H (1992) Tumorproliferation, MDR-Phänotyp und tumorassoziierte T-Lymphozyten in Non Hodgkin-Lymphomen. Verh Dtsch Ges Pathol 76: 248–252
8. Hufnagl P, Beil M, Wenzelides K, Martin H (1991) Die Vermessung der Anzahl, Größe und Lage von AgNORs in histologischen Schnitten von Astrozytomen und Harnblasenkarzinomen. Zentralbl Pathol 137: 493–497
9. Kindermann D, Nikorowitsch R, Pfeifer U (1991) Nucleolus organisierende Regionen (AgNORs) bei benignen und malignen Schilddrüsenläsionen im Vergleich zu DNS-(Mikroskop-) Zytometrie – Hilfen bei der Diagnostik? Verh Dtsch Ges Pathol 75: 439
10. Korek G, Martin H, Wenzelides K (1991) A modified method for the detection of nucleolar organizer regions (AgNORs). Acta Histochem 90: 155–157
11. Martin H, Beil M, Hufnagl P, Wolf G, Korek G (1991) Computer-assisted image analysis of nucleolar organizer regions (NORs): a pilot study of astrocytomas and glioblastomas. Acta Histochem 90: 189–196
12. Nagao T, Ishida Y, Yamazaki K, Kondo Y (1995) Nucleolar organizer regions in hepatocellular carcinoma related to the cell cycle, cell proliferation and histologic grade. Pathol Res Pract 191: 967–972
13. Oehmichen M (1990) Die Wundheilung. Springer, Berlin Heidelberg New York
14. Oehmichen M, Cröpelin A (1995) Temporal course of intravital and postmortem proliferation of epidermal cells after mechanical injury. An immunohistochemical study using bromodeoxyuridine in rats. Int J Legal Med 107: 257–262
15. Oehmichen M, Lagodka T (1991) Time-dependent RNA synthesis in different skin layers after wounding. Experimental investigations in vital and postmortem biopsies. Int J Legal Med 104: 153–159
16. Oehmichen M, Lagodka T, Cröpelin A (1997) RNA and DNA synthesis of epidermal basal cells after wounding. Comparsion of vital and postmortem investigations. Exp Toxic Pathol 49:233–237
17. Orita T, Kajiwara K, Nishizaki T, Ikeda N, Kamiryo T, Aoki H (1990) Nucleolar organizer regions in meningioma. Neurosurgery 26: 43–46
18. Ploton D, Menager P, Jeannesson, Himber G, Pigeon F, Adnet JJ (1986) Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 18: 5–14
19. Roussel P, Hernandez-Verdun D (1994) Identification of AgNOR proteins, markers of proliferation related to ribosomal gene activity. Exp Cell Res 214: 465–472
20. Rüschoff J, Barth P (1992) Theorie und Praxis der Silberfärbung Nukleolus organisierender Regionen (AgNOR). Pathologe 13: 13–19
21. Rüschoff J, Knüchel R, Plate KH (1991) Reaktionsmuster der Silberfärbung Nukleolus organisierender Regionen (AgNOR) – Zellbiologische Grundlagen und diagnostische Bedeutung. Verh Dtsch Ges Path 75: 477