

Comparative study of DNA synthesis and nucleolar organizer regions of sinusoid littoral cells in mouse regenerating liver

CARLOS A. MARTÍN, JOSÉ MIGUEL SURUR, MARCELA N. GARCÍA, FÉLIX CORRONS, AND AMADO F. BADRÁN

Instituto de Embriología, Biología e Histología. Facultad de Ciencias Médicas. UNLP. Calle 60 y 120. (1900) La Plata. Argentina.

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ABSTRACT: Variations in DNA synthesis (DNAs) and Nucleolar Organizer Regions (NORs) were studied in the littoral cell population from regenerating liver of C3HS inbred mice standardized for periodicity analysis. Immunohistochemical detection of Bromodeoxyuridine (BrdU) with a monoclonal antibody and silver staining of NORs (AgNORs) were assessed by means of a digital image analysis system in histological sections. Tissue samples were obtained every four hours from the 30th to the 54th hours after a partial hepatectomy. The results showed, in both parameters, a gradual increment of the values during the period studied, with highest values (DNAs 107.1 ± 16.1 SE; AgNORs 77.3 ± 3.4 SE) located at 16:00/54 Time of Day / Hours Post-Hepatectomy (TD/HPH), which were significantly different ($p < 0.001$) from the values of the first sample (DNAs 38.1 ± 9.5 SE; AgNORs 27.3 ± 1.0 SE) taken at 16:00/30 TD/HPH. The results of our experiment demonstrate the existence of a strong correlation of DNA synthesis measured by BrdU immunohistochemistry and AgNORs numbers in sinusoid littoral cells from mouse regenerating liver.

Introduction

The proliferative activity of cellular populations of the mouse has been studied in experimental models through diverse methods measuring different aspects of the replicative process.

The introduction of temporary parameters in our experiments, allowed us to demonstrate the presence of circadian variations in the mitotic index and DNA

synthesis (DNAs) in hepatocytes and sinusoid littoral cell populations of adult intact and hepatectomized male mouse (Badrán *et al.*, 1984; Surur, *et al.*, 1985). Littoral cells represent about 40% of the liver cells, playing an important physiologic role, exerting both stimulating and/or inhibitory influences during hepatic regeneration (Michalopoulos and Defrances, 1997). The existence of at least four different cell populations of the so-called sinusoid littoral cells (Vanderkerken *et al.*, 1995) and also the migration into and out of the liver of some macrophages also considered as littoral (Kupffer) cells, makes the analysis and interpretation of the data somewhat complicated.

In the last years, the quantification of the nucleolar organizer regions (NORs) has been widely used in human tumor pathology to distinguish among benign and malignant lesions (Crocker, 1990; Wolanski *et al.*, 1998) either reflecting the proliferative capacity of cells or

Address correspondence to: Dr. Carlos A. Martín. Instituto de Embriología, Biología e Histología. Facultad de Ciencias Médicas, Universidad Nacional de La Plata. Calle 60 y 120. (1900) La Plata, ARGENTINA. FAX: (+54-221) 425-8989.
E-mail: drmartin@speedy.com.ar

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indicating the degree of malignancy (Derenzini *et al.*, 1990; Egan and Crocker, 1992; Rüschoff *et al.*, 1989).

NORs are segments of DNA that encode ribosomal RNA directly related to protein synthesis and cellular proliferation. Active NORs are associated with a subset of specific proteins (C23 and B23), that reacts with silver nitrate (AgNORs), appearing as secondary constrictions in acrocentric chromosomes during mitosis or as black dots in interphase nuclei. Being more numerous in actively proliferating cells, AgNORs quantity increases during G1 phase to reach a maximum during S phase. This quantity is inversely proportional to the cell cycle duration, short cycles showing higher amount of AgNORs (Hofstädter *et al.*, 1995). The amount of B23 protein increases to maximal values when the percentage of cell in G2 phase increases (Derenzini *et al.*, 1995), as was also demonstrated in cell cultures after 24 h of cell cycle stimulation (Sirri *et al.*, 2000).

Taking into account previous data from our own laboratory concerning the close relationship between

DNAs and AgNORs numbers in hepatocytes (Martín *et al.*, 2000), we plan this study to detect possible relationships between DNAs and AgNORs numbers in the sinusoidal littoral cell population from mouse regenerating liver, during the second day post-hepatectomy.

Material and Methods

Animals

C3H/S adult male mice (90 days old), kept under standard conditions for circadian periodicity analysis during 3 weeks (Halberg *et al.*, 1958), caged singly with food and water *ad libitum* and kept in a specially designed room at a temperature of $22 \pm 1^\circ\text{C}$, illuminated (40 W fluorescent light) from 06:00 to 18:00 h alternating with 12 h of darkness, were used in this experiment. All mice were subjected to a partial hepatectomy (70%) at

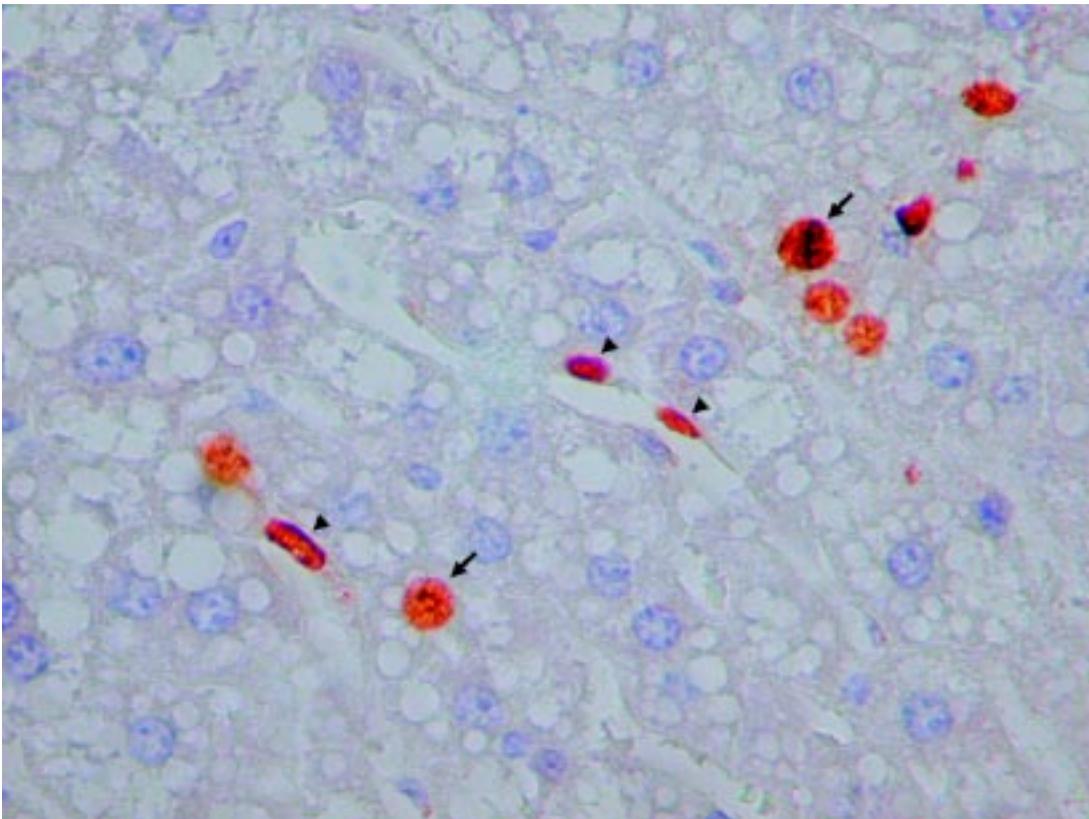


FIGURE 1. Several hepatocyte (arrows) and littoral cell nuclei (arrowheads) are seen immunostained for bromodeoxyuridine (Bu20a X 400).

10:00 h (Souto and Echave Llanos, 1985). One hour before killing, the animals were injected IP with a bromodeoxyuridine solution (BrdU, Sigma, St. Louis, USA) at a dose of 50 mg/kg of body weight. The animals were killed by decapitation and exsanguination at 16:00/30, 20:00/34, 00:00/38, 04:00/42, 08:00/46, 12:00/50 and 16:00/54 time of day/hours post-hepatectomy (TD/HPH). Samples of regenerating liver were removed promptly, fixed in 10% buffered formalin for 24 h, processed routinely and embedded in paraffin.

Immunohistochemistry

Five- μm sections from liver samples were placed onto silanized slides and dried in an oven (58°C) overnight. The sections were deparaffinized in xylene,

rehydrated through graded alcohols and washed in Tris buffer saline (TBS) pH 7.4. Antigen unmasking was done boiling the slides in a plastic container with buffer citrate pH6 in a microwave oven (750 W, 2 cycles of 5 min each), followed by 20 min cooling. After blocking endogenous peroxidase activity with 3% H_2O_2 (15 min), sections were incubated with a mouse monoclonal antibody to BrdU (Bu20a, Dako, Carpinteria, CA, USA), at a 1:50 dilution for 1 h in a moist chamber at room temperature. As a detection system, Envision Peroxidase (Dako) was used for 30 min. After rinsing in TBS, the peroxidase reaction was developed with liquid DAB (Dako), followed by a light hematoxylin counterstain. Slides were then dehydrated, cleared and mounted in synthetic medium.

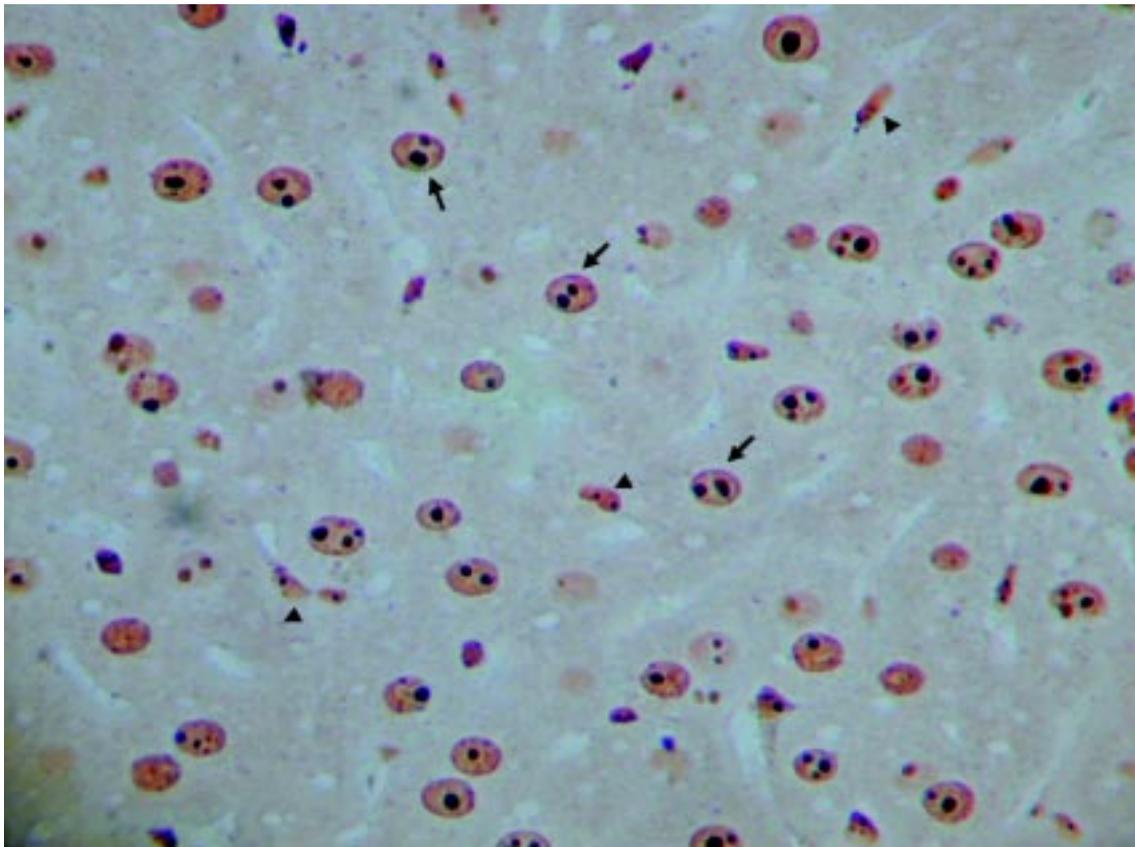


FIGURE 2. AgNORs appearing as black dots into hepatocyte (arrows) and littoral cell nuclei (arrowheads) (Ploton's method X 400).

Silver staining of nucleolar organizer regions

Serial sections taken from the same blocks were stained by means the silver impregnation method of Ploton (1986) as described elsewhere (Martín *et al.*, 2000). In brief, sections were rehydrated to deionized water and placed in a silver nitrate solution consisting of 2 g/dl gelatine in 1 g/dl aqueous formic acid, mixed (1:2 volumes) with a 50 g/dl aqueous silver nitrate solution for 1 h at room temperature. The sections were thoroughly washed in deionized water and fixed in 5% sodium thiosulfate solution (Wolanski *et al.*, 1998) for 5 min. After three washes in deionized water, sections were dehydrated, cleared and mounted in synthetic medium.

Quantification method

BrdU positive nuclei and AgNOR black intranuclear dots, at 40x and 100x magnification, respectively, were counted semi-automatically by means of a digital image analyzer system (Rüschhoff *et al.*, 1990), in littoral cells, i.e. small cells with elongated nuclei lining the hepatic sinusoids. Images of selected fields were captured using a video camera for microscopy and digitized with a PC.

Labeled nuclei for BrdU and AgNORs dots were analyzed quantitatively by means of a software image analyzer.

Positive nuclei for Bu20a antibody or black dots of AgNORs, in 3000 and 250 sinusoid littoral cells per animal, respectively, were marked by using a digital cursor, and the mean values and standard errors (SE) were automatically calculated at the end of the measurement. Values thus obtained were expressed as BrdU positive nuclei x 1000 and AgNOR numbers x 100 sinusoid littoral cells. Statistical significance of the differences was established with the Student's t test.

Results

Figure 1 and 2 are examples of immunostaining for BrdU and silver impregnation of NORs, respectively. The results show a gradual and progressive increment in both values during the 24-hour period studied (Fig. 3). The maximal values of BrdU labelled nuclei and AgNOR numbers (Table I) were registered at 16:00/54 TD/HPH, with $107.1 \pm 16.1SE$ and $77.3 \pm 3.4SE$, respectively, being the differences statistically significant from the value of the first sample taken at 16:00/30 (38.1

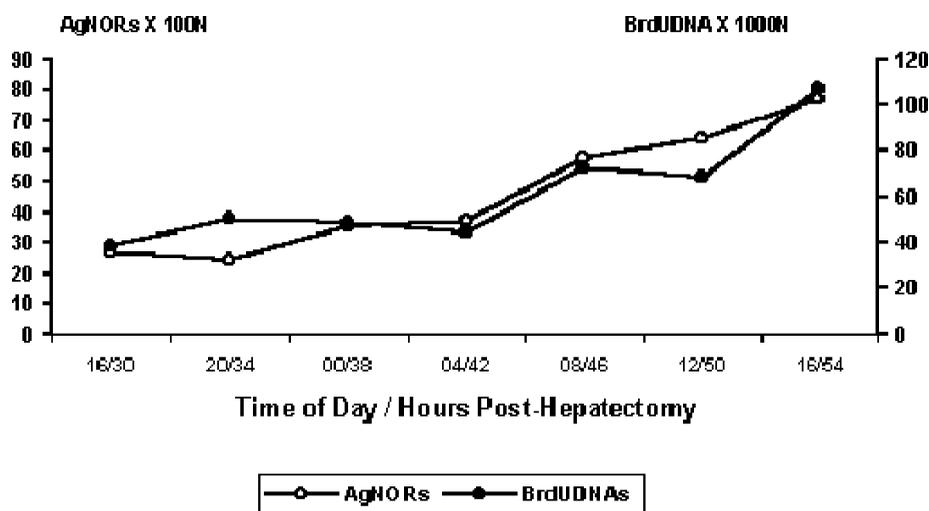


FIGURE 3. Comparative curves of DNA synthesis (BrdU uptake) and nucleolar organizer regions in sinusoid littoral cells related to time of day and hours post-hepatectomy.

TABLE I

DNA synthesis and nucleolar organizer regions of sinusoid littoral cells in regenerating liver

Lot	TD/HPH	AgNORs x 100 N		BrdU x 1000N	
		X ± SE	n	X ± SE	n
1	16:00/30	27.3 ± 1.0	6	38.1 ± 9.5	6
2	20:00/34	24.3 ± 0.4	6	49.8 ± 16.9	6
3	00:00/38	35.5 ± 1.0	6	49.2 ± 21.9	6
4	04:00/42	37.3 ± 1.1	6	44.3 ± 13.1	6
5	08:00/46	58.6 ± 1.2	6	73.3 ± 17.7	6
6	12:00/50	64.2 ± 2.8	6	68.4 ± 4.4	5
7	16:00/54	77.3 ± 3.4	5	107.1 ± 16.1	5
X 24 Hours		46.3 ± 7.7	41	61.4 ± 9.0	40
P		1-7 < 0.001		1-7 < 0.004	
		2-7 < 0.001		2-7 < 0.05	
		4-7 < 0.05		4-7 < 0.02	

References: X ± SE, Mean ± Standard Error; n, Number of animals; TD, Time of Day; HPH: Hours Post-Hepatectomy; p, probability (t test).

± 9.5SE and 27.3 ± 1SE, respectively). Both parameters showed a strong correlation of values throughout the period studied, with a defined circadian rhythm.

Discussion

Liver regeneration is a physiological mechanism which leads to restoration of the hepatic parenchyma following hepatectomy, viral or toxic injury. This process is mediated by a wide variety of cytokines and growth factors. In contrast to other regenerating tissues (bone marrow, skin), liver regeneration is not dependent on a small group of progenitor or stem cells. After partial hepatectomy, regeneration is carried out by proliferation of all the existing mature cellular populations composing the intact organ. The four major types of liver cells include hepatocytes, biliary epithelial cells, fenestrated endothelial cells, Kupffer cells (macrophages in hepatic sinusoids), and stellate cells (Ito's cells). All of these populations proliferate to rebuild the lost hepatic tissue, but the speed of their answer is different. Hepatocytes are the first to proliferate, while other liver cell populations enter into DNA synthesis about 12 h after or later (Rabes *et al.*, 1976). Our observations

demonstrate that DNA synthesis determined by means of BrdU incorporation and the numbers of AgNORs in littoral cells of mouse regenerating liver show a strong correlation of values, as can be deduced from the curves of both parameters (Fig.1), which show circadian variations along the span here considered.

When comparing both cellular populations, the highest values of DNAs and AgNORs in hepatocytes precede in about 12 h those corresponding the littoral cells. (Martín *et al.*, 2000). On the other hand, the increase in both variables precedes in approximately 10 h the first peak of the mitotic activity for this cellular population, as we demonstrated in previous studies carried out in our laboratory (Badrán *et al.*, 1984, Surur *et al.*, 1985). Worthy of note are the striking differences in the magnitude of values for both parameters between hepatocytes (Martín *et al.*, 2000) and littoral cells (Table I), which could be explained by intrinsic metabolic properties of the cells.

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