MITOSIS-TO-G1 PHASE TRANSITION OF HTC CELLS OBTAINED WITH NOCODAZOLE (R 17934; NSC 238159)

Spin-lattice relaxation time and cytofluorometric analysis

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1. Introduction

Analysis of spin-lattice relaxation time (T_1) of water protons in synchronized populations of Hela cells shows that the motional freedom of cellular water is maximum during mitosis (M), then decreases very rapidly during G1 to reach a minimum in the DNA synthetic phase S [1]. The progressive increase in water microviscosity during the M–G1 transition has been attributed to an increase in chromatin diffusiveness [1,2]. This work aims at determining whether variation of T_1 observed during the M–G1 transition of synchronized populations of Hela cells can be detected in other systems.

2. Materials and methods

Measurements were made on cells pre-accumulated in metaphase consecutively treated with Nocodazole, an antimicrotubule drug. Spin-lattice relaxation time was determined during the M–G1 progression monitored by laser cytofluorometry. Under the experimental conditions used here no variation of T_1 has been observed.

2.1. *Cell maintenance and selection*

HTC cells derive from a rat hepatoma [3]. They are grown in monolayer or suspended in medium 213 supplemented with 5% each of decomplemented (30 min at 56°C) calf serum and fetal calf serum as in [4]. To accumulate cells in mitosis, 540 cm^2 Roux flasks inoculated with 4×10^7 HTC cells suspended in 300 ml medium 213 are incubated for 20 h at 37°C. At that time, unattached cells are eliminated following a vigorous shaking of the monolayer with the medium in which they have grown. The cultures are overlaid with 300 ml pre-warmed, fresh growth medium containing 2.41 mM $CaCl_2$ and 0.074 μg/ml Nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate. *Syn:* R 17934 and NSC 238159). After 5 h incubation, the Roux flasks are carefully emptied and mitotic cells detached following 10 consecutive inversions of the Roux flask filled with 40 ml phosphate-buffered saline (10 mM phosphate buffer (pH 7.4), 140 mM NaCl). The mitotic cells are resuspended in fresh growth medium at 7.5×10^5 cells/ml.

At the times indicated below aliquots containing 2×10^6 HTC cells are taken and analyzed by NMR and laser flow cytofluorometry. After a 10 min centrifugation at $700 \times g_{av}$ at room temperature, the pellet of detached cells is resuspended in 10 μl phosphate-buffered saline. Parts of the concentrated cell suspension is aspirated through one end of 25 μl Drummond capillary tube, care being taken not to completely fill the tube. After sealing the empty end in the flame, the cells are packed by a 1 min centrifugation at $12\,000 \times g_{av}$ in a Gelman Hawksley microcentrifuge and used for NMR measurement. The cell pellet is 12–15 mm high in the capillary tube.

2.2. *NMR analysis*

All measurements are performed immediately after collection of each sample at 33°C on a Bruker WP spectrometer working at 60 MHz. The portion of the

sample containing the cells is located in a coaxial NMR tube whose outer part is filled with benzene-d_6 to ensure the field-frequency lock of the spectrometer. Spin-lattice relaxation time of water protons is determined by the standard $180°-\tau-90°$ pulse sequence. For each variable time interval, two free induction decay signals are averaged on 1 k of data memory and submitted to Fourier transform to give an absorption signal in the frequency domain. T_1 values are calculated by a multiparametric exponential regression (Minuit program–Cern library program).

2.3. *Laser flow cytofluorometric analysis*

NP 40-Propidium iodide–ribonuclease [5] solution (1 ml) is added to the concentrated cell suspension which remains in the tube after withdrawal of the sample for NMR spectrometry. The cells remain in that solution overnight at 4°C before analysis by laser flow cytofluorometry in a Biophysics cytofluorograph model FC 200/4800 A equipped with a 100 mW argon ion laser.

2.4. *Chemicals*

Propidium iodide and Nonidet P40 are from Sigma and Shell (UK), respectively. Nocodazole (methyl [5-(2-thienyl-carbonyl)-1H-benzimidazol-2-yl] carbamate. *Syn:* R 17934 and NSC 238159) is a gift from Janssen Pharmaceutica (Beerse-Belgium).

3. Results

Preliminary experiments to determine the minimum length of time and the optimum drug concentration necessary to get a sufficient number of cells accumulated in metaphase showed that moderate shaking of monolayer cultures incubated for 5 h in presence of 0.075 μg/ml Nocodazole gives 98% of cells in mitosis (channel 45). G1 cells amount to only 2% (channel 23) (fig.1A). The coefficient of variation of this peak is 3.23%. It increases to 6.10% 30 min after removal of the inhibitor (fig.1B). Between 30–60 min later, the cells synchronously leave M and enter G1 (channel 23). G1-cells constitute now 67.5% of the population (CV = 6.02%) (fig.1C).

A new population appears (17% of the total) characterized by a broader and lower fluorescence intensity (channels 10–20) than that of G1 cells (low fluorescence cells). It most probably represents micronuclei containing a reduced number of chromosomes [6]. It is not known in fig.1C whether the 15.5% cells with fluorescence intensity at channel 43, represent cells that were in early G2 at the time of

Table 1
Spin-lattice relaxation time T_1 of 'NCD-cells' during the M–G1 transition

Time after resuspension of 'NCD-cells' in fresh growth medium	T_1 in ms[a]	
	Exp. 1	Exp. 2
0	996	1194
30 min	1098	1166
1 h	1037	1031
4 h	1076	1002

[a] T_1 values of random HTC cells: 1078 ms. Values of T_1 obtained at intermediate times (15 min, 2 and 3 h) are <10% of the values indicated in table 1

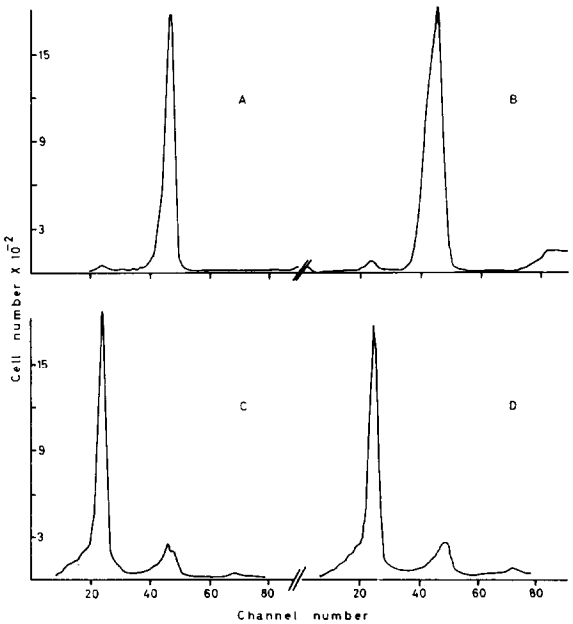

Fig.1. Mitosis–G1 transition of Nocodazole-treated cells. Each histogram represents the fluorescence intensity distribution among cells and gives in ordinate the number of nuclei per channel and in abscissa the relative fluorescence intensity which varies in proportion with the DNA content. To facilitate the comparison between samples in a given figure counting is normalized to pre-determined values. Each graph represents the cell DNA-distribution after resuspension in fresh medium. (A) 0 min; (B) 30 min; (C) 1 h; (D) 4 h.

the selective detachment, cells irreversibly blocked in mitosis or G1 doublets.

Table 1 gives the spin-lattice relaxation times corresponding to the cytofluorometric DNA histograms discussed above. The constancy of T_1 during the M–G1 transition of Nocodazole-treated cells is obvious and the values observed do not significantly differ from that characteristic of asynchronously growing cells.

4. Discussion

Accumulating experimental evidence strongly suggests that regulation of cell reproduction primarily occurs during the G1 period of the cell cycle. However, due to the absence of suitable cell cycle markers very little is known about the succession of metabolic events actually taking place. This situation changed recently with the advent of two new techniques: laser flow cytofluorometry which can monitor the progression of cells during the various stages of the cell cycle [7]; and premature chromosome condensation which characterizes the extent to which a cell has progressed into G1 by the degree of decondensation of its chromatin [8].

In view of [1,2] determination by NMR spectroscopy of the variations of T_1, the spin-lattice relaxation time of water protons seemed to provide a new and very promising approach to dissect the G1 phase.

It therefore surprised us that Nocodazole-treated HTC cells display no significant decrease of T_1 during the M–G1 transition. A possible artefactual cause of our system such as the lack of response of the spectrometer owing to the higher frequency (60 MHz vs 30 MHz in [1]) seems improbable. A more reasonable explanation for this unexpected result may come from the fact that in metaphasic cells obtained following interference with microtubule formation, chromatin decondensation would be precluded. Alternatively, the variations of T_1 observed during the M–G1 phase transition of Hela cells are specific for these cells and generalization cannot be extended to other cell types.

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