NAD LEVELS IN 3T3 CELLS DURING EXPONENTIAL GROWTH AND DENSITY-DEPENDENT INHIBITION OF GROWTH

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1. Introduction

Recent work with poly(ADP-ribose) (see [1] and [2] for reviews) has implicated this nuclear polymer in the regulation of DNA replication [3,4]. Because poly(ADP-ribose) is synthesized from NAD^+, a relationship may exist between the rate of proliferation of a cell and its content of NAD. To test this possibility, we have measured NAD levels in cultures of 3T3 cells, which exhibit density-dependent inhibition of growth [5]. We have found that the NAD content of 3T3 cells increases during exponential growth to a maximal level just prior to density-dependent inhibition of growth, and then remains at a relatively high level during the density-inhibited state. These findings suggest that intracellular concentrations of NAD are related to the growth of 3T3 cells and are consistent with previous data that link lowered NAD levels with cell proliferation in tumors [6–8] and regenerating tissues [9,10].

2. Methods

2.1. *Culture conditions*

BALB/c 3T3 mouse-embryo fibroblasts, the gift of Dr Charles Hedgcoth, were maintained in 250 cc Falcon plastic bottles and cultured in 60 mm diameter Falcon plastic Petri dishes. The growth medium consisted of 90% Dulbecco's modified Eagle's minimal essential medium and 10% fetal-calf serum, and was supplemented with penicillin (100 units/ml) and streptomycin (100 μgm/ml). The nicotinamide content of this medium is about 3.3×10^{-5} M. All cultures were grown at 37°C in a humidified atmosphere of 90% air, 10% CO_2, and daily changes of medium were made. Growth medium, antibiotics, and trypsin were obtained from Grand Island Biological Co. (Grand Island, New York).

Cells were prepared by treating stock cultures with 2 ml of trypsin (0.5 mg/ml) at room temperature for 5 min, diluting them with growth medium, and plating them in 5 ml at initial densities of 1.4×10^5 cells/dish. Duplicate dishes were prepared for each sampling procedure. Before sampling, the medium was decanted, and the dish was washed twice with 5 ml of phosphate-buffered saline and allowed to drain for 5 min.

2.2. *Cell number*

Cells were detached by treatment with 1 ml of trypsin, the reaction was terminated with 1 ml of growth medium, and the cells were diluted with phosphate-buffered saline and counted with a Coulter counter.

2.3. *Protein*

Dishes for protein determinations were stored at −30°C until all samples were collected. Cells were dissolved with 5 ml of Lowry's reagent 'C', and assayed for protein by the method of Lowry, as modified by Oyama and Eagle [11].

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2.4. *Perchloric Acid Extraction*

Each sample was treated with 1 ml of 0.5 M $HClO_4$, kept on ice for 10 min, transferred to a centrifuge tube together with 0.5 ml of a 0.5 M $NHClO_4$ rinse, and centrifuged in the cold for 20 min at 1500 *g*. The supernatant fractions were used to determine NAD^+, and the sediments were used to determine RNA as described below.

2.5. *RNA*

Measurement of RNA is based on the procedure of Orr, et al. [12]. The perchloric acid sediments from above were dissolved in 1.6 ml of 0.2 M NaOH and stored at −30°C until all samples were collected. The samples were thawed, incubated at 37°C for 1 hr to hydrolyze the RNA, reacidified with 0.4 ml of 3.3 M $HClO_4$, and kept on ice for 15 min. Acid-insoluble material was removed by centrifugation as above, and the supernatant fractions were assayed at 260 nm, using appropriate blanks. RNA values are expressed as A_{260} units (A_{260} × ml of extract).

2.6. *NAD^+*

Supernatant fractions from the perchloric acid extracts were neutralized with 0.5 vol of 1.0 M KOH in 0.33 M K_2HPO_4–KH_2PO_4 (pH 7.5) and centrifuged as above to remove solid $KClO_4$. The resulting supernatant fractions were stored at −30°C until all samples were collected, and then assayed for NAD^+ by a cycling procedure [13].

2.7. *NADH*

Extraction of NADH is based on a previously described procedure [14]. Cells were dissolved with 1.5 ml of 0.25 M NaOH, incubated at 60°C for 5 min, and neutralized with 0.5 vol of 0.5 N H_2SO_4 in 0.33 M K_2HPO_4–KH_2PO_4 (pH 7.5). Extracts were stored at −30°C until all samples were collected, and then assayed by a cycling procedure [13]. In the present studies, NADH was generally 9 to 13% of the total NAD. Only total NAD values (NAD^+ + NADH) are reported.

3. Results

Fig. 1 shows typical results from one of several experiments. During exponential growth, the NAD content of 3T3 cells increases from an initial level of 0.6 nmole/10^6 cells to a maximum level of 1.2 nmoles/10^6 cells, which occurs just prior to the density-dependent inhibition of growth. The NAD level then decreases to a relatively constant value of 0.9 nmole/10^6 cells in the density-inhibited state.

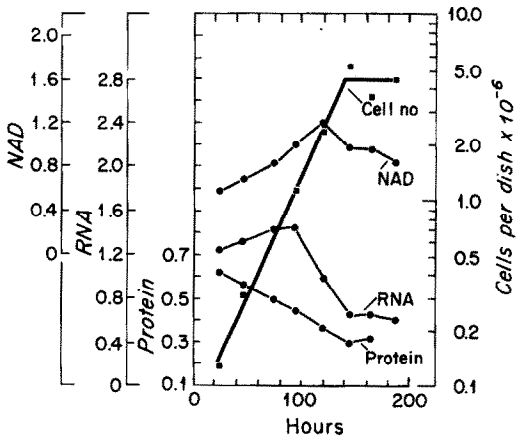

Fig. 1. Levels of protein (mg/10^6 cells), RNA (A_{260} units/10^6 cells), NAD (nmoles/10^6 cells), and cell number as a function of culture time.

During the course of the experiment (fig. 1), there is an overall 2-fold decrease in cellular RNA and protein. The decrease in protein is progressive, whereas the RNA first increases to a maximum value and then decreases. Meisler [15] has noted similar changes in the RNA and protein content of 3T3 cells and has partially associated these with changes in cell volume.

In order to determine the relative changes in the intracellular concentration of NAD in a manner that

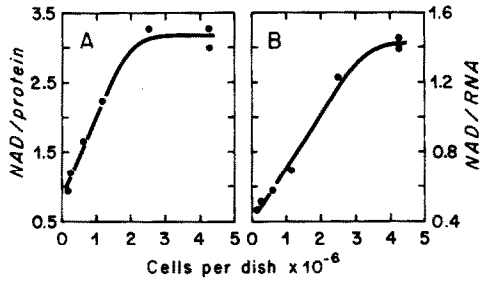

Fig. 2. Changes in NAD relative to protein (A) or RNA (B) as a function of cell density. Units of NAD, RNA, and protein are the same as in fig. 1.

would be less dependent on changes in cell volume, NAD/protein and NAD/RNA ratios were calculated and plotted as a function of cell density (fig. 2). The data in Fig. 2A and 2B show that, relative to protein and RNA, respectively, there is a 3-fold increase in the intracellular concentration of NAD during the growth cycle of 3T3 cells, and that the density-inhibited state is associated with a high level of NAD.

4. Discussion

The increase of NAD associated with the density-dependent inhibition of 3T3 cells is of interest because depressed levels of NAD have been associated with tumors [6–8] and regenerating tissues [9,10]. Cellular NAD would be lowered by an increase in the activity of NAD glycohydrolase [16], or by a decrease in the activity of enzymes that synthesize NAD [17–19]. However, further studies are needed to establish the mechanism of NAD depression and how it may be linked to proliferation.

The density-dependent decrease of cellular RNA and protein observed in 3T3 cells may be rationalized by assuming that the elevation of NAD would facilitate the formation of poly(ADP-ribose), which in turn would interfere with the synthesis of mRNA from a DNA template and lead to a decrease of protein synthesis. This postulated sequence is consistent with the recent finding of Johnson, et al. [20] that growing cultures of 3T3 cells contain 4-times as much mRNA as density-inhibited cultures, and that the rate of protein synthesis is proportional to the amount of mRNA present.

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