Modification of Pyridine Nucleotide Pools in Hela Cells Grown under Growth Promoting Factors

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Introduction

Numerous studies have shown that pyridine nucleotide levels change in proliferating tissues in comparison to that of non-proliferating tissues (3,4,6,7,10,16,21). The ratio of NAD: NADH also increases as 3T3 cells grow from logarithmic to stationary phase (22). Furthermore, pyridine nucleotide pools have been studied as a function of growth in normal 3T3 cells and SV40 (simian virus #40) transformed 3T3 cells (14). Recently, we have also demonstrated that the reduction of pyridine nucleotide levels in rat liver follows carcinogen (2-acetylaminofluorene) administration (26). Based on these results, it is reasonable to postulate that pyridine nucleotide pools are important in the control of cell division.

In order to see a close correlation between cell replication and the modification of pyridine nucleotide pools, exogenous stimuli for cell proliferation are applied. Fetal calf serum has been the most common supplement used to stimulate growth. Transferrin, a component of serum, has been shown to replace serum in the growth of many cells (13). Similarly, insulin, another component of serum, has been known as an essential growth factor for some cells (1,2). Recently, stimulation of cell growth by impermeable oxidizing agents such as ferricyanide has also been reported (9,17,24,25). Furthermore, evidence for an additive effect on the growth of HeLa cells by insulin and the impermeable oxidant has been demonstrated (27). The application of these exogenous stimuli, which greatly promote cell proliferation in a serum-depleted media, has therefore been performed in order to study the changes in pyridine nucleotide levels.

The regulation and endogenous biosynthesis of pyridine nucleotides is potentially complex since several alternative pathways have been described in mammalian cells (15). Although the significance of the relationship between pyridine nucleotide levels and cell replication is not clear, the possible involvement of pyridine nucleotides in growth control is of great interest.

Materials and Methods

HeLa cells were grown in serum-free Eagle's medium supplemented with growth factors as described previously (25,27). After 2 days of incubation at 37 °C, cells were harvested and a cell survival count was taken immediately. Survival was determined by the eosin Y exclusion method as described by Mishell and Shrigi (19); cell countes were obtained in duplicate after trypsinization. The colorless, viable cells were counted.

The pyridine nucleotide pool was determined by extraction of NAD and NADH from cells with perchloric acid and alkali respectively (14). The extracted pyridine nucleotides were then quantitated by using a cycling assay which involved alcohol dehydrogenase and glucose-6-phosphate dehydrogenase (18).

Results

Pyridine nucleotide pools have been determined in HeLa cells after 48 hour growth in a serum-free medium supplemented with ferricyanide. The optimal concentration of ferricyanide for growth stimulation is 0.01 mM (Figure 1). Total nucleotide pools follow a similar response, with an optimal concentration of ferricyanide at 0.033 mM. As growth

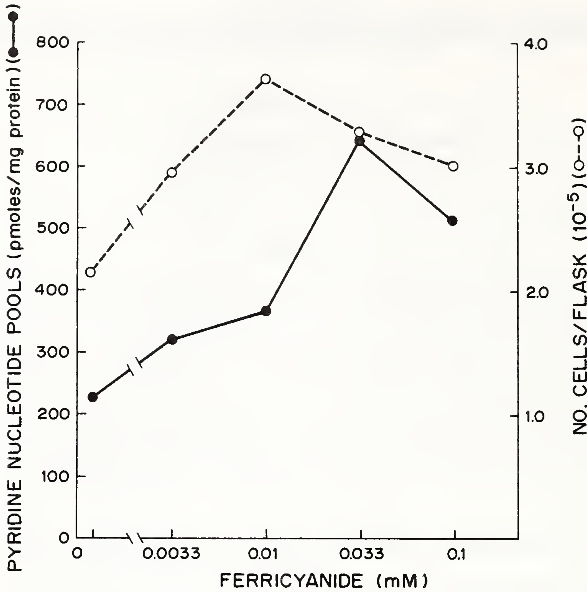

FIGURE 1. Dose-response curve for ferricyanide on cell growth and the induction of pyridine nucleotide pools. The culture of HeLa cells was grown in a serum-free medium for 48 h.

ceases at confluency, pyridine nucleotide levels decline. This correlation between growth and pyridine nucleotides exists for both the reduced form (NADH) and oxidized form (NAD) (Figure 2). When cells are grown with ferricyanide (0.033 mM), NAD concentra-

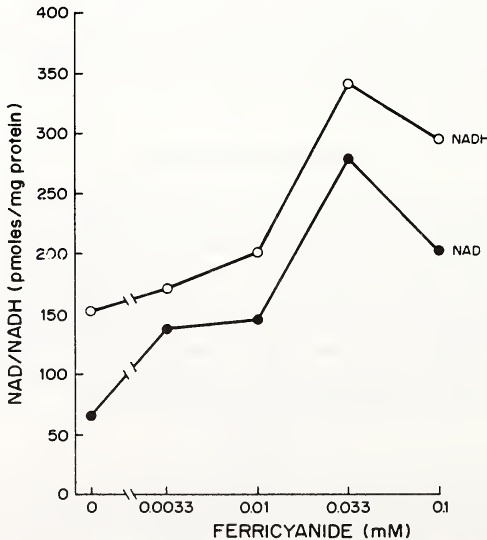

FIGURE 2. Effect of ferricyanide on the pools of NAD and NADH.

tion increases nearly 5-fold over the control, whereas NADH increases 2-fold over the control. Under this growth condition, the levels of the reduced pyridine nucleotide NADH are higher than the oxidized form, NAD.

If the determinatin of pyridine nucleotides pools is performed with HeLa cells grown in serum depleted but insulin-supplemented media, there is a sharp induction of pyridine nucleotide pools as shown in Figure 3, until insulin reaches 3 μg/ml. A downward trend

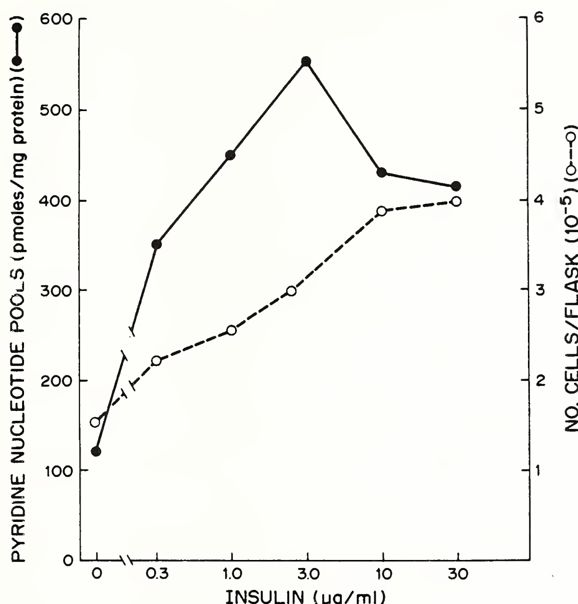

FIGURE 3. Dose-response curve for insulin on the induction of pyridine nucleotide pools and cell growth. Culture was grown in the same way as indicated in Figure 1 except insulin was used as a supplement instead of ferricyanide.

of the pools has been observed when the growth stages shift from logarithmic phase to stationary phase. Each type of pyridine nucleotide is induced in a similar pattern (Figure 4). Again the level of NADH is higher than NAD all the way through the experiment. At the maximum growth, both NAD and NADH are induced to a similar extent.

An additive effect of ferricyanide and insulin on the increase of pyridine nucleotide pools is observed when both agents are applied together (Figure 5). Insulin and ferricyanide also show an additive effect on growth of HeLa cells (27).

Evidence for a relationship between pyridine nucleotide levels and growth in HeLa cells is also seen with the application of diferric transferrin (DFTF). DFTF is a well known growth stimulating agent. As shown in Figure 6, DFTF increases cell growth more than 3 fold above the control at a concentration of 3.4 μM, whereas apotransferrin gives insignificant effects on cell growth. Similar effects have been found on total pyridine nucleotide pools (Figure 7). DFTF induces a 2-fold increase but apotransferrin shows no effect. The effect of DFTF and apotransferrin on the concentration of NAD and NADH is shown in Table 1.

To stimulate cell replication, serum has been used as a supplement in the media. The increase in cell numbers is proportional to the concentration of serum applied (Figure

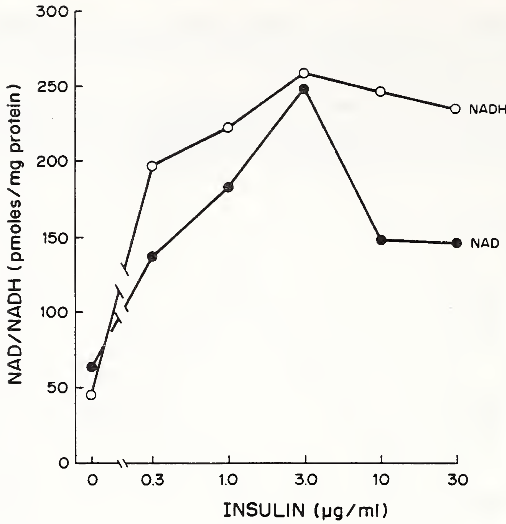

FIGURE 4. Effect of insulin on NAD and NADH pools.

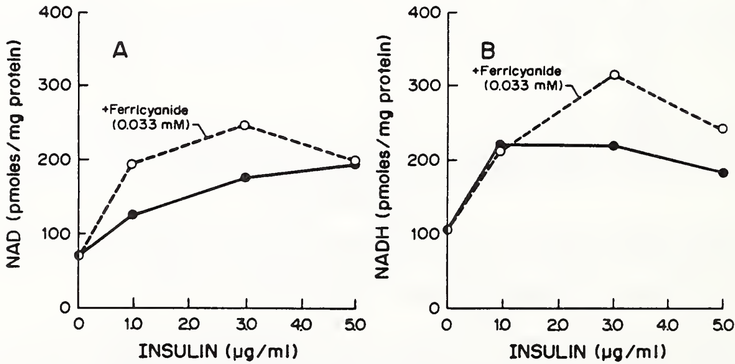

FIGURE 5. An additive effect on the induction of NAD and NADH pools by insulin and ferricyanide.

8). A progressive increase in pyridine nucleotide levels with increasing serum is also demonstrated (Figure 8). Both NAD and NADH increase with added serum (Figure 9).

Discussion

Insulin and transferrin are the major serum factors known to stimulate cell growth (13). However, the stimulation of cell growth by ferricyanide has also been reported by Ellem and Key (14) and from this laboratory (16,18). This electron acceptor can support sufficient electron transport to stimulate cell growth even in the absence of serum factors or serum. It also induces proton release across the membrane (8) which would therefore increase the pH of the cytoplasm. An increase of cytoplasmic pH (alkalinization) is associated with increased cell division (11,12,20).

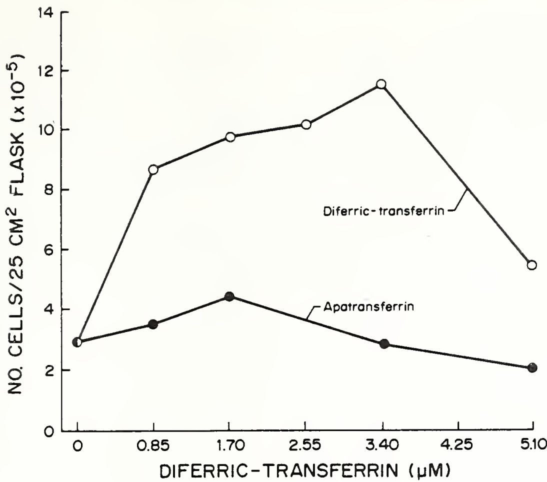

Figure 6. Dose-response cuve for diferric-transferrin and apotransferrin on the growth of HeLa cells.

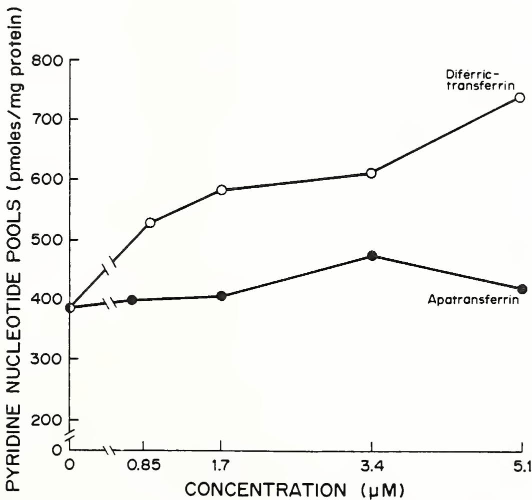

Figure 7. Effect of diferric-transferrin and apotransferrin on pyridine nucleotide pools.

Table 1. Effect of diferrictransferrin and apotransferrin on the distribution of pyridine nucleotide levels.

Addition	NAD	NADH
	(pmoles/mg protein)	
Control	132.5	65.4
Diferric transferrin (0.85 μM)	170.0	174.8
Diferric transferrin (1.7 μM)	190.0	243.4
Apotransferrin (0.85 μM)	138.6	87.9
Apotransferrin (1.7 μM)	129.8	89.1

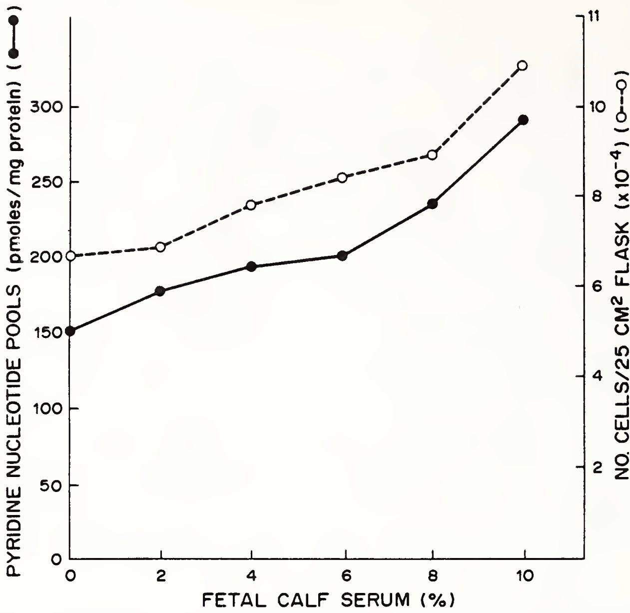

FIGURE 8. Dose-response curve for fetal calf serum on the growth of HeLa cells and the inductin of pyridine nucleotide pools.

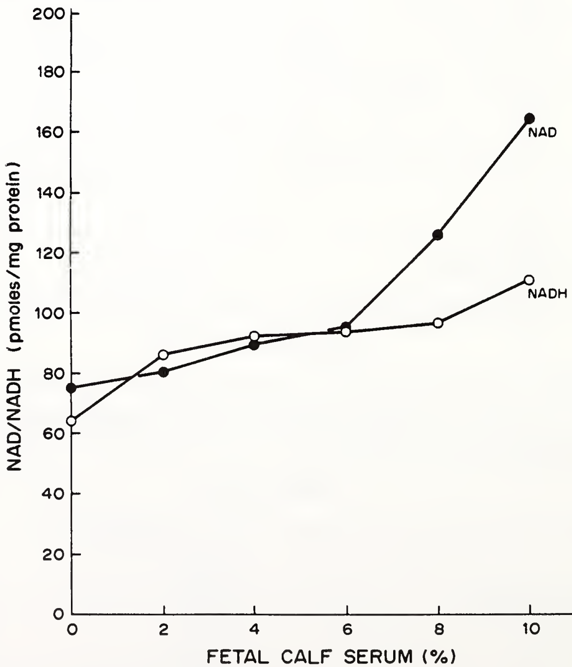

FIGURE 9. Induction of NAD and NADH pools by fetal calf serum.

The effect of growth conditions on NADH pool size and the ratio of oxidized to reduced pyridine nucleotide (NAD:NADH ratio) have been studied in normal and transformed 3T3 cells (22). A decrease in the NAD:NADH ratio was found in transformed cells and the decrease was suggested to relate to the defective growth control in these cells.

Results presented here show that under various growth promoting agents, levels of both NAD and NADH are increased in HeLa cells.

NAD can serve as a substrate in the formation of poly ADP-ribose (poly ADP-Rib), a compound synthesized in mammalian cell nuclei (23). Recent evidence suggests that the accumulation of poly (ADP-Rib) may be involved in turning off DNA synthesis (5.28). The rise in the NADH: NAD ratio which we have observed in this study may therefore regulate DNA synthesis by providing a decrease supply of NAD for poly (ADP-Rib) synthesis. Therefore, cells maintain a continuous synthesis of DNA and growth.

The pyridine nucleotides are important components of the energy-transforming and oxidation-reduction reactions of the cells. The pyridine nucleotide pools can therefore be an indicator of the metabolic capabilities of the cell which varies corresponding to growth conditions. In this paper, an attempt has been made to study the correlation between this indicator and cell replication under the influence of growth promoting agents.

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