Nicotinamide Prolongs Survival of Primary Cultured Hepatocytes without Involving Loss of Hepatocyte-specific Functions*

(Received for publication, November 21, 1988)

Chiyoko Inoue§, Hiroshi Yamamoto§, Toshikazu Nakamura§, Akira Ichihara§, and Hiroshi Okamoto**

From the Department of Biochemistry, Tohoku University School of Medicine, Sendai 980, Miyagi, Japan, the Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka 812, Japan, and the Institute for Enzyme Research, the University of Tokushima, Tokushima 770, Japan

When hepatocytes isolated from adult rats were cultured in the presence of 10 mM nicotinamide, insulin- and epidermal growth factor-induced DNA synthesis and cell proliferation were found to be greatly stimulated, and the cells were able to be kept alive for more than one month. In the nicotinamide-treated hepatocytes, albumin and tryptophan 2,3-dioxygenase mRNAs were present at much higher levels than in the untreated control, and the inducibility of tryptophan oxygenase gene expression by dexamethasone and glucagon was also preserved. Without nicotinamide, primary cultured hepatocytes were viable for only 5–7 days and the hepatocyte-specific phenotypes were rapidly lost. The intracellular NAD level was maintained in the nicotinamide-treated hepatocytes at or above the level in intact liver but depleted in hepatocytes without nicotinamide. These results suggest that the maintenance of the intracellular NAD level is essential for the growth and functioning of hepatocytes and that nicotinamide can preserve the NAD level by blocking NAD degradation as well as by acting as a precursor for NAD synthesis.

Mature hepatocytes have been observed to proliferate in vitro when they are cultivated at a low cell density in the presence of insulin and EGF and are used for studying the mechanisms by which the growth and functions of differentiated cells are regulated (1–5). However, under the culture conditions employed so far, hepatocytes have rapidly lost their specific functions and have usually deteriorated within 5–7 days (1–5).

We and other authors have previously demonstrated that nicotinamide can provide effective protection against chemically induced deterioration of pancreatic β-cells (6–11) and induce the regeneration of β-cells in partially pancreatectomized rats (11–13).

In the present study, we show that hepatocytes cultured with nicotinamide exhibited increased cell replication and survived for more than 1 month without loss of parenchymal hepatocyte-specific phenotypes such as the albumin and tryptophan oxygenase gene expressions. The nicotinamide effect was attributable to protection against intracellular NAD depletion during hepatocyte culture in vitro.

EXPERIMENTAL PROCEDURES

Materials—Collagenses (type I) and nicotinamide were obtained from Wako Pure Chemical Industries, Osaka; dexamethasone and glucagon from Sigma; human insulin from Lilly; human EGF from Wako Pharmaceutical Co., Osaka; [methyl-3H]thymidine (91 Ci/mmol) and [3H]CTP (3,000 Ci/mmol) from Amersham Corp.; Morris hepatoma cells (MH1C1 strain) from Dainippon Pharmaceutical Co., Osaka. Male Wistar rats weighing 250–270 g were used for all experiments.

Primary Culture of Hepatocytes—Parenchymal hepatocytes were isolated from rat liver as described (14). The hepatocytes were cultivated on type I collagen (Nitta Gelatin Co., Osaka) coated dishes at 37 °C under a 5% CO2 atmosphere at an initial density of 4 × 104 cells/cm2 in Williams' medium E which had been supplemented with 5% calf serum, 20 ng/ml EGF, 10−7 M insulin, 10−9 M dexamethasone, and with or without 10 mM nicotinamide. This concentration of nicotinamide almost completely inhibits poly(ADP-ribose) synthesis (9). The medium was renewed after the first 2 h and then every 48 h.

Assay of DNA Synthesis—Hepatocytes were incubated in the presence of 1.25 μCi/ml [3H]thymidine, and the 3H incorporation into DNA was measured as described (4).

Morphological Examination and Cell Count—Hepatocytes were morphologically examined using a Nikon inverted phase-contrast microscope. For cell count determination, at least four different fields were chosen at random and photographed at a magnification of ×100. The cell nuclei in each field were counted and the results were averaged.

RNA Blot Hybridization Analysis—RNA was isolated, electrophoresed on a 1.5% agarose gel, transferred onto a nitrocellulose filter (15), and hybridized to cDNA probes labeled with [α-32P]CTP by the random-primer technique (16). The cloned rat albumin cDNA (17) and the cloned rat tryptophan oxygenase cDNA (18) were used as probes.

Determination of NAD Content—Hepatocytes grown on a 60-mm culture dish and 0.1–0.2 g of excised liver were disrupted by sonication in 2 ml of cold 0.5% perchloric acid. NAD content in the acid-soluble extracts was determined as described (19).

RESULTS AND DISCUSSION

Fig. 1A shows the time course of DNA synthesis of hepatocytes cultured in the presence or absence of 10 mM nicotinamide. Without nicotinamide, [3H]thymidine incorporation increased from 40 h after plating, reached a peak at 55 h, and then decreased. In the presence of nicotinamide, [3H]thymidine incorporation rose sharply from 55 h and peaked at a level 2 times that in the absence of nicotinamide at 62 h. The rate of DNA synthesis was sustained at a significantly high level until 96 h but had declined by 120 h.

As shown in Fig. 1B, hepatocytes grown without nicotinamide showed an increase in cell number between day 2 and day 3, but were then detached, which resulted in a significant cell loss from day 4 on. In the presence of 10 mM nicotinamide, the cell number continued to increase, reaching a plateau on day 5, by which time it was 2.7-fold as high as on day 1; cells
were not lost into the medium, and the cell count remained unchanged from day 5 to day 16. Even 33 days after plating, approximately 70% of the cells were still alive in nicotinamide-treated cultures. Ten mM of nicotinamide was the most effective concentration among those tested (1, 5, 10, 20, and 25 mM). The nicotinamide-treated hepatocytes formed the cord-like structure of individualized and packed cells, round or polygonal in shape, characteristic of differentiated parenchymal hepatocytes (Fig. 2A). When nicotinamide-treated hepatocytes were subjected to autoradiography 16 days after plating, nuclei from about 1% of the cells were still labeled (Fig. 2B), indicating that long-surviving hepatocytes retained the ability to resume proliferation.

Previous reports showed that when hepatocytes were grown in culture, they underwent a rapid loss of liver-specific functions (3, 4). We examined the expression of albumin and tryptophan oxygenase genes as a marker of hepatocyte functions. In hepatocytes grown without nicotinamide, albumin mRNA level declined to 80 and 20% of the initial value of intact liver 1 and 2 days after seeding (Fig. 3A). In nicotinamide-treated cultures, albumin mRNA was at the same level as in intact liver for 2 days after plating, and the abundance was partially retained for 33 days in culture, ranging from 20 to 60% of that in intact liver; nicotinamide-treated hepatocytes on day 33 still maintained the albumin mRNA at a level equal to that in the albumin-producing Morris hepatoma cells (Fig. 3B). Tryptophan oxygenase mRNA level in control culture became undetectable from day 3 on (Fig. 3C). In nicotinamide-treated cultures, the tryptophan oxygenase mRNA was detected at least 14 days after plating, and the inducibility of tryptophan oxygenase gene expression by dexamethasone and glucagon (20–21) was observed for as long as 12 days (Fig. 3D).

We next examined the effect of nicotinamide on the intracellular NAD level in the cultured hepatocytes. As shown in Fig. 4, hepatocytes cultured without nicotinamide had a drastically reduced NAD level such that at 20 h after plating the intracellular NAD content was 42% of that of intact liver; the extent of the NAD reduction was compatible with that of the earlier observation (22). Although the NAD level tended to increase around 76 h after plating, the amount of intracellular NAD did not reach the level in intact liver throughout the entire culture period. In contrast, NAD levels in nicotinamide-treated cultures were significantly higher than those in untreated cultures; the NAD level was highest at 20 h after plating and then gradually decreased with slight fluctuations till 120 h and stayed at level equal to or higher than that in intact liver by day 14 of culture.

The present study showed that nicotinamide significantly enhanced insulin- and EGF-induced DNA replication and cell proliferation in primary cultured hepatocytes. We also demonstrated that a long term exposure of hepatocytes to nicotinamide markedly improved cell viability; the hepatocyte cultures with nicotinamide could be kept alive for more than 1 month, while in the absence of nicotinamide primary cultured hepatocytes were irreversibly on their way to cell death within 5–7 days of seeding. The surviving nicotinamide-treated hepatocytes retained parenchymal hepatocyte-specific phenotypes, as evidenced by their abundance of albumin and tryptophan oxygenase mRNAs and by the inducibility of tryptophan oxygenase gene expression. Furthermore, it was found that intracellular NAD level in hepatocytes cultured without nicotinamide was unphysiologically low throughout the culture period, and that in the nicotinamide-treated hepatocytes the NAD level was maintained at or above the level of intact liver. Since NAD is the most abundant cellular coenzyme and participates in many biological reactions in mammalian cells, it may be reasonable to assume that the reduction in intracellular NAD to such a nonphysiological level during culture may severely affect hepatocyte functions, resulting in cell death, and that nicotinamide may preserve the hepatocyte growth and functions by protection against NAD depletion, which has been shown to be the primary molecular mechanism behind the destruction of pancreatic β-cells (11).

Nicotinamide serves as a precursor for NAD synthesis (23–25). As can be seen in Fig. 4, the NAD content in nicotinamide-treated hepatocytes was maintained at a level equal to or higher than that in intact liver. The maintenance of the NAD level can be ascribed to nicotinamide’s acting as a precursor of NAD synthesis. In addition, nicotinamide is also known to be an inhibitor of poly(ADP-ribose) synthetase, an enzyme which catalyzes polymerization of the ADP-ribosyl moiety of NAD and plays a main role in NAD degradation (26). Althaus et al. (27) reported that in primary cultured hepatocytes poly(ADP-ribose) synthetase activity sharply increased after plating, probably as an adaptive change to the in vitro environment. We observed that 3-aminobenzamide and 5-bromodeoxyuridine, other potent inhibitors of poly(ADP-ribose) synthetase (28), also prolonged the lifespan of, and prevented NAD depletion in, cultured hepatocytes, though less efficiently than did nicotinamide (data not
shown). Therefore, the effects of nicotinamide on primary cultured hepatocytes can be due to its acting as a poly(ADP-ribose) synthetase inhibitor as well as an NAD precursor.

A possible role of poly(ADP-ribosylation) in the process of DNA replication has been suggested (29). Burzio and Koide (30) showed that the formation of poly(ADP-ribose) suppresses the template activation for DNA synthesis in rat liver nuclei. Tanigawa et al. (31) reported that ADP-ribosylation of the nuclear proteins of adult chick liver resulted in the inhibition of DNA synthesis. In the present study, using primary cultured hepatocytes, nicotinamide remarkably increased DNA synthesis and cell proliferation (Fig. 1). 3-Aminobenzamide was also observed to stimulate DNA synthesis in the hepatocytes (data not shown). These observations suggest that poly(ADP-ribose) synthetase may play a role in restricting hepatocyte replication and that inhibitors of the enzyme may relieve restriction of DNA synthesis and so cause hepatocyte proliferation. Thus, our previous observations regarding the ability of poly(ADP-ribose) synthetase inhibitors to prevent the deterioration of β-cells by contributing to the maintenance of the intracellular NAD level (8-11) and to induce the proliferation of these cells (11-13) have been extended and confirmed by this study.

REFERENCES
1. Richman, R. A., Claus, T. H., Piliks, S. J., and Friedman, D. L. (1976). Proc. Natl. Acad. Sci. U. S. A. 73, 3589-3593
2. Koch, K. S., and Leffert, H. L. (1980) Ann. N. Y. Acad. Sci. 349, 111-127
3. Enat, R., Jefferson, D. M., Ruiz-Opazo, N., Gatmaitan, Z. Leinwand, L. A., and Reid, L. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1411-1415
4. Nakamura, T., and Ichihara, A. (1985) Cell Struct. Functions. 10, 1-16
5. Inoue, C., Igarashi, K., Kitagawa, M., Terazono, K., Takasawa, S., Obata, K., Iwata, K., Yamamoto, H., and Okamoto, H. (1988) Biochem. Biophys. Res. Commun. 150, 1302-1308
6. Lazrow, A., Lammies, J., and Tausch, A. J. (1950) J. Lab. Clin. Med. 25, 249-258
7. Rakieten, N., Rakieten, M. L., and Nadkarni, M. V. (1963) Cancer Chemother. Rep. 29, 91-98
8. Yamamoto, H., and Okamoto, H. (1980) Biochem. Biophys. Res. Commun. 95, 474-481
9. Uchigata, Y., Yamamoto, H., Kawamura, A., and Okamoto, H. (1982) J. Biol. Chem. 257, 6084-6088
10. Uchigata, Y., Yamamoto, H., Nagai, H., and Okamoto, H. (1983) Diabetes 32, 316-318
11. Okamoto, H. (1985) BioEssays 2, 15-21
12. Yonemura, Y., Takashima, T., Miwa, K., Miyazaki, I., Yamamoto, H., and Okamoto, H. (1984) Diabetes 33, 401-404
13. Terazono, K., Yamamoto, H., Takasawa, S., Shiga, K., Yonemura, Y., Tochino, Y., and Okamoto, H. (1988) J. Biol. Chem. 263, 2111-2114
14. Seglen, P. O. (1976) Methods Cell Biol. 13, 29-83
15. Inoue, C., Shiga, K., Takasawa, S., Kitagawa, M., Yamamoto, H., and Okamoto, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6659-6662
Nicotinamide Effect on Primary Cultured Hepatocytes

16. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
17. Sargent, T. D., Yang, M., and Bonner, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 243–246
18. Nakamura, T., Niimi, S., Nawa, K., Noda, C., Ichihara, A., Takagi, Y., Anai, M., and Sakaki, Y. (1987) J. Biol. Chem. 262, 727–733
19. Yamamoto, H., Uchigata, Y., and Okamoto, H. (1981) Nature 294, 284–286
20. Greengard, O. (1977) Essays Biochem. 7, 159–205
21. Killewich, L. A., and Feigelson, P. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5392–5396
22. Paine, A. J., Hockin, L. J., and Legg, R. F. (1979) Biochem J. 184, 461–463
23. Dietrich, L. S., Fuller, L., Yero, I. L., and Martinez, L. (1966) J. Biol. Chem. 241, 188–191
24. Blake, R. L., Blake, S. L., Loh, H. H., and Kun, E. (1967) Mol. Pharmacol. 3, 412–422
25. Johnson, G. S. (1980) Eur. J. Biochem. 112, 635–641
26. Suhadolnik, R. J., Baur, R., Lichtenwalner, D. M., and Uematsu, T. (1977) J. Biol. Chem. 252, 4134–4144
27. Althaus, F. R., Lawrence, S. D., He, Y.-Z., Sattler, G. L., Tsukada, Y., and Pitot, H. C. (1982) Nature 300, 366–368
28. Preiss, J., Schlaeger, R., and Hilz, H. (1971) FEBS Lett. 19, 244–246
29. Althaus, F. R. (1987) in ADP-Ribosylation of Proteins (Althaus, F. R., and Richter, C., eds) pp. 93–100, Springer-Verlag, Berlin, Heidelberg
30. Burao, L., and Koide, S. S. (1970) Biochem. Biophys. Res. Commun. 40, 1015–1020
31. Tanigawa, Y., Kawamura, M., Kitamura, A., and Shimoyama, M. (1978) Biochem. Biophys. Res. Commun. 81, 1278–1285
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
33. Nakamura, T., Tohuti, Y., and Ichihara, A. (1983) J. Biochem. (Tokyo) 94, 1029–1035