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FIBROBLAST-LIKE TRANSFORMATION AND c-myc GENE ALTERATION OF HUMAN HEPATOCYTES INDUCED BY NOVOBIOCIN AND BUTYRATE

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SUMMARY: Topoisomerase II inhibitor novobiocin and deacetylase inhibitor sodium butyrate synnergistically transformed Chang liver cells into fibroblast-like cells. In these fibroblast-like cells, the production of type III procollagen was increased and the DNase I hypersensitivity of c-myc gene was reduced. In addition, these changes were associated with an increased acetylation of nuclear proteins, especially those of DNase I sensitive nucleosomes. Therefore, it is suggested that chemical modulation of nuclear proteins by novobiocin and butyrate may be responsible, at least partly, for the alteration of the chromatin structure of c-myc gene and the fibroblast-like transformation of Chang liver cells. © 1988 Academic Press, Inc.

Nuclear proteins are considered to be playing important roles in regulating gene activity. For example, a nuclear enzyme topoisomerase II which alters the higher dimentional structure of DNA is known to change the transcriptional activity of genes(1, 2). On the other hand, nuclear deacetylase alters nucleosome structure and gene activity through deacetylation of histones and non-histone nuclear proteins(3, 4). Therefore, it is supposed that functional changes of these nuclear enzymes may have a profound influence on the c-onc activity and the differentiation state of human liver and hepatoma cells as well(5). To test this supposition, the effects of topoisomerase II inhibitor novobiocin and deacetylase inhibitor sodium butyrate on the structure and function of c-myc gene of human liver cells were examined. These reagents synnergistically enhanced nuclear protein acetylation and brought remarkable changes of the chromatin structure of c-myc gene and the cell morphology.

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MATERIALS AND METHODS

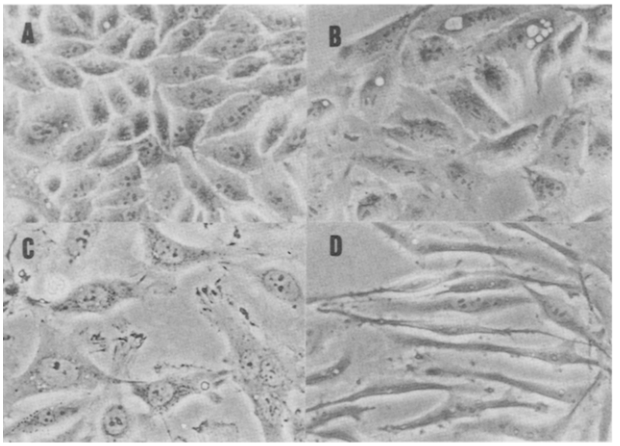
Novobiocin, sodium butyrate, retinoic acid, phorbol ester and adriamycin were obtained from Sigma Co.. 3×10^5 of Chang liver cells. HuH7 and PLC/PRF/5 hepatoma cells were cultured in RPMI1640 medium + 5% fetal bovine serum for 3 days with or without 200 μM novobiocin and/or 2 mM sodium butyrate(6, 7). These cells were incubated for further 3 h with 2 μCi/ ml of [^{3}H]leucine, and the amount of [^{3}H]leucine incorporated into TCA-precipitable materials was determined as described previously(7). To measure the production of type III procollagen(P-III-P), the cells were washed 3 times in the serum-free RPMI1640 medium and were cultured for further 24 h in the serum-free medium. The amount of P-III-P in the culture medium was determined with radioimmunoassay kit from Hoechst Co.(8).

DNase I hypersensitivity of c-myc gene was examined as reported previously(7). Cells cultured for 3 days with or without novobiocin and/or sodium butyrate were treated with a nuclear buffer(10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.5% NP40 solution. Nuclei separated by centrifugation and washed 3 times in the nuclear buffer were treated for 15 min at 37°C with different concentrations of DNase I. DNA was extracted from the nuclei by phenol methods, cleaved with Eco RI and subjected for Southern blot hybridization using ^{32}P-labelled Cla I-Eco RI fragment containing the third exon of c-myc(5, 7). The amount of c-myc mRNA was measured by Northern blot hybridization. The cells were dissolved in 4M guanidine isothiocyanate and was ultracentrifuged through CsCl solution. RNA was electrophoresed in formaldehyde-1% agarose gels, transferred to a nylon filter and was hybridized with the c-myc probe in a solution containing 50% folmamide and 10% dextran-sulfate(7).

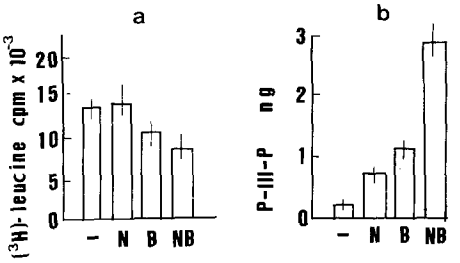
Topoisomerase II activity of nuclear extract was measured as described (2, 10). Nuclei isolated as described above were incubated in 0.3M NaCl solution for 30 min at 4°C. Different amounts of the 0.3M NaCl extracts were added to the reaction mixture containing 1 μg pBR322 and 1 mM ATP. After 15 min at 37°C, the reaction mixtures were electrophoresed in a 0.8% agarose gel containing 2 μg/ml of ethidiumbromide. [^{3}H]acetate incorporation into cells, nuclei, non-histone nuclear proteins and nucleosomes was measured as reported previously(9). Cells cultured for 3 days with or without novobiocin and/or butyrate were incubated for 4 h with 5 μCi/ml of [^{3}H]acetate(New England Nuclear). The cells were washed 3 times in RPMI1640 and the nuclei were isolated as described above. [^{3}H]acetate incorporation into non-histone nuclear protein was measured by extracting the proteins with 0.3M NaCl solution. To measure the amount of [^{3}H]acetate incorporated into nucleosomes which are considered to contain transcriptionally active genes the nuclei were treated with 1 μg/ml of DNase I for 10 min at 37°C and were centrifuged. The supernatant contained nucleosomes cleavable with DNase I.

RESULTS

Chang liver cells of normal human hepatocyte origin showed morphological nature of epithelial cells(6). Either novobiocin alone or sodium butyrate alone had no remarkable effect on their morphological appearance. However, when treated simultaneously with novobiocin and sodium butyrate, Chang liver cells were transformed into fibroblast-like cells with markedly elongated cytoplasm(Fig. 1). These fibroblast-like cells regained the morphological



(1)



(2)

Fig. 1. The effects of novobiocin and sodium butyrate on the morphology of Chang liver cells. 3 x 10^5 of Chang liver cells were cultured for 3 days under the presence or absence of novobiocin or sodium butyrate. A, control; B, novobiocin 200 μM; C, sodium butyrate 2 mM; D, novobiocin 200 μM + sodim butyrate, 2 mM.

Fig. 2. The effects of novobiocin and sodium butyrate on the prctein synthesis and type III procollagen production by Chang liver cells. 3 x 10^5 of Chang liver cells were cultured for 3 days with 200 μM novobiocin and/or 2 mM sodium butyrate. -, control; N, novobiocin; B, sodium butyrate; NB, novobiocin + sodium butyrate. (a) [^{3}H]leucine incorporation into TCA-precipitable materials. cpm x 10^{-3}/3h/10^6 cells. (b)Type III procollagen (P-III-P) synthesis(ng/24h/10^6 cells). The values are the mean ± SD of triplicates(n=3).

nature of epithelial cells after 3 days of culture without these reagents. In contrast, HuH7 and PLC/PRF/5 hepatoma cells were not transformed by the same treatment, indicating that this synnergistic effect of novobiocin and butyrate was specific to Chang liver cells. Reagents such as ethanol, retinoic acid, phorbol esters, adriamycin, etc could not mimic nor antagonize the synnergistic action of novobiocin and sodium butyrate on Chang liver cells.

In addition to the morphological effect, novobiocin and sodium butyrate synnergistically enhanced the production of the type III procollagen. Their stimulatory effect seemed to be specific for type III procollagen since no significant increase was found in non-collagen protein synthesis(Fig. 2).

Fig. 3 demonstrates that novobiocin reduced the DNase I hypersensitivity of c-myc gene of Chang liver cells. The hypersensitive sites on or near the second and the third c-myc exon were affected preferentially(Fig. 3A and B). Sodium butyrate itself had no remarkable effect on the DNase I hyper-

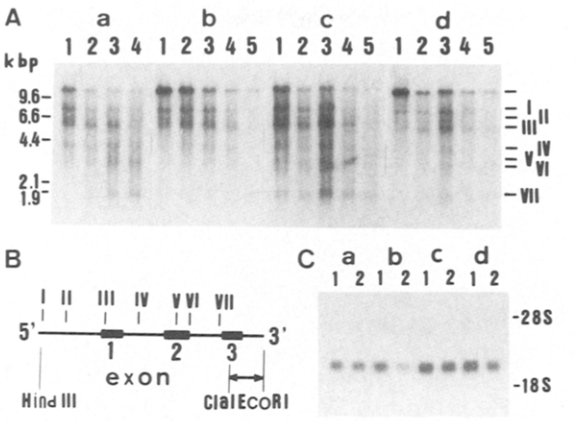


Fig. 3. The effects of novobiocin and sodium butyrate on the DNase I hypersensitivity and the transcriptional activity of c-myc gene. (A)DNase I hypersensitivity of c-myc gene. Chang liver cells were cultured for 3 days with or without 200 μM novobiocin and/or 2 mM sodium butyrate. a, control; b, novobiocin; c, butyrate; d, novobiocin + butyrate. Nuclei were treated with different concentrations of DNase I(lane 1, 0 μg/ml; lane 2, 0.5 μg/ml; lane 3, 1 μg/ml; lane 4, 2 μg/ml; lane 5, 5 μg/ml). (B) Restriction map of c-myc gene. DNase I hypersensitive sites were indicated by Roman numerals. The cleavage at each sensitive site gave c-myc fragment indicated by the same Roman numerals in A. The Cla I-Eco RI fragment used as a hybridization probe is also shown. (C) c-myc mRNA levels of Chang liver cells cultured with novobiocin and sodium butyrate. The cells were cultured as described in A. a, control; b, novobiocin; c, butyrate; d, novobiocin + butyrate. lane 1, RNA 20 μg; lane 2, RNA 10 μg.

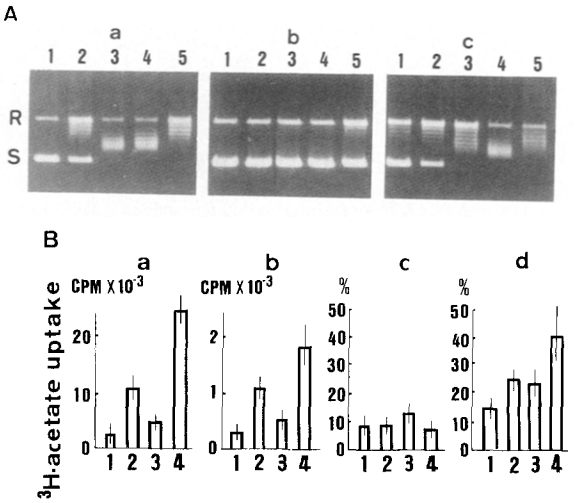


Fig. 4. The effects of novobiocin and sodium butyrate on the topoisomerase II activity and acetylation of nuclear proteins. Chang liver cells were cultured for 3 days with or without 200 μM novobiocin and/or 2 mM butyrate. (A) Topoisomerase II activity of the nuclear extract. Different amounts of nuclear proteins(lane 1, 0 μg; lane 2, 0.5 μg; lane 3, 1 μg; lane 4, 2μg;lane 5, 5 μg) were incubated with pBR322. S, supercoiled pBR322; R, relaxed pBR322. a, control; b, novobiocin; c, butyrate. (B) [^{3}H]acetate incorporation into cells(a), nuclei(b), non-histone nuclear proteins(c) and nucleosomes released from the nuclei by DNase I treatment(d). 1, control; 2, novobiocin; 3, butyrate; 4, novobiocin + butyrate. a, cpm/10^6 cells; b, cpm/10^6 cells; c, cpm of non-histone protein/cpm of nuclei(%); d, cpm of nucleosomes digested with DNase I/cpm of nuclei(%). mean ± SD of triplicates.

sensitivity, but appeared to augment slightly the effect of novobiocin(Fig. 3A). The decrease in DNase I hypersensitivity of c-myc gene was not always accompanied by a decrease in its transcriptional activity(Fig. 3C).

Novobiocin is a specific inhibitor of the nuclear topoisomerase II and the nuclear extract from Chang liver cells cultured with novobiocin showed remarkable decrease in their topoisomerase II activity. Butyrate had no effect on the topoisomerase II activity(Fig. 4A). In addition, novobiocin was disclosed to be a potent stimulator of [^{3}H]acetate incorporation and acted synnergistically with sodium butyrate in enhancing [^{3}H]acetate accumulation into cells, nuclei, and nucleosomes which were released from the nuclei by mild DNase I treatment(Fig. 4B).

DISCUSSION

The present study revealed that novobiocin and butyrate synnergistically transformed Chang liver cells into fibroblastic cells. This morphological transformation could not be ascribed simply to a non-specific toxicity of these reagents, since no loss in cell viability was shown and the production of type III procollagen was increased after 4 days of treatment. An important implication of the present study is that Chang liver cells showing the characters of epithelial cells have a potential to change riversiblly into fibroblastic cells with an increased production of type III procollagen. This in vitro phenomenon may suggest that human hepatocytes like other cell populations of liver such as fat storing cells and myofibroblasts are capable to change into fibroblastic cells under certain in vivo conditions as well(11, 12, 13), and that hepatocytes may play some role in forming extensive fibrosis in cirrhotic liver(14).

The present study also disclosed that novobiocin, an inhibitor of topoisomerase II, was a potent stimulator of protein acetylation. Therefore, the hyperacetylation of nuclear proteins and the reduced topoisomerase II activity induced by novobiocin and butyrate may be responsible, at least partly, for the change of c-myc chromatin structure and the morphological

appearance of Chang liver cells(2, 15, 16). However, novobiocin which had both inhibitory effect on topoisomerase II and stimulatory effect on acetate uptake could not bring the morphological transformation by it self, suggesting that the synnergistic action of novobiocin and sodium butyrate might involve biochemical pathways of still unknown characters.

REFERENCES

1. Hans, S., Udvardy, A. & Schedl, P.(1985) J. Mol. Biol. 183, 13-19.
2. Sahyoun, N., Wolf, M., Besterman, J., Hsieh, T-S., Sander, M., LeVine, H.III, Chang, K-J. & Cuatrecasaa, P.(1986) Proc. Natl. Acad. Sci. USA. 83, 1603-1607.
3. Candido, E.P.M., Reeves, R. & Davie, J.R.(1978) Cell 14, 105-113.
4. Sealy, L. & Chalkley, R.(1978) Cell 14, 115-121.
5. Kaneko, Y., Shibuya, M., Nakayama, T., Hayashida, N., Toda, G., Endo, Y., Oka, H. and Oda, T.(1985) Jpn. J. Cancer Res. 76, 1136-1140.
6. Chang, R.S-M.(1954) Proc. Soc. Exp. Biol. Med. 87, 440-443.
7. Kaneko, Y., Toda, G. & Oka, H(1987) Biochem. Biophys. Res. Commun. 145, 549-555.
8. Rhode, H., Vargas, L., Hahn, E., Kalbfleisch, H., Bruguera, M. & Timple, R. (1987) Eur. J. Clin. Invest. 9, 451-459.
9. Kaneko, Y.(1983) Biochim. Biophys. Acta 762, 111-118.
10. Pommier, Y., Kerrigan, D., Schwartz, R.E., Swack, J.A. & MaCurdy, A. (1986) Cancer Res. 46, 3075-3081.
11. Shiratori, Y., Ichida, T., Kawase, T. & Wisse, E.(1986) Liver 6, 246-251.
12. Savoratori, E-R., Leo, M., Timpl, R. & Lieber, C.S.(1984) Gastroenterology 87, 777-787.
13. Bhathal, P.S.(1972) Pathology 4, 139-144.
14. Sakakibara, K., Ooshima, A., Igarashi, S. & Sakakibara, J.(1986) Virchows Arch. A. 409, 37-46.
15. Piperno, G., LeDizet, M. & Chang, X-J.(1987) J. Cell Biol. 104, 289-302.
16. Chan, T.A. & Allfrey, V.G.(1987) Proc. Natl. Acad. Sci. USA. 84, 5252-5256.