Increased assembly of cytoskeletal proteins associated with the transformation of human liver cells into fibroblast-like cells

A. Tsukamoto, Y. Kaneko and K. Kurokawa

First Department of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)
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Abstract. A topoisomerase II inhibitor, novobiocin, and a deacetylase inhibitor, butyrate, synergistically transformed human liver cells into fibroblast-like cells. This morphological change was associated with an increased production of procollagen type III peptide and a simultaneous assembly of actin, tubulin, vimentin and cytokeratin. Novobiocin and butyrate had no marked effect on the phosphorylation state of cytokeratin proteins, but synergistically enhanced [^{3}H]acetate uptake. From these results, it can be speculated that protein acetylation plays an important role in inducing the assembly of cytoskeletal proteins and the morphological transformation of human liver cells.
Key words. Cytoskeletal proteins; differentiation; hepatocyte; butyrate; novobiocin.

Nuclear proteins play important roles in regulating gene expression and cellular differentiation, and the chemical modulation of nuclear proteins may change chromatin structure and thereby alter gene expression and the state of differentiation of cells. Butyrate, which is an inhibitor of nuclear deacetylase, causes the hyperacetylation of histones, reduces nucleosome core particle linking number, and alters the cellular differentiation state [1, 2]. Novobiocin, an inhibitor of nuclear topoisomerase II, prevents the structural alteration of chromatin organization of the heat shock gene, and has profound effects on gene expression [3–5]. These data suggest that the effects of novobiocin and butyrate may be brought about by altering chromatin organization [2, 3]. During a study to analyze the effects of novobiocin and butyrate on various cell lines we found that these reagents, used in combination, changed Chang liver cells of epithelial cell origin into fibroblast-like cells [6]. It was reported recently that in cultured bladder carcinoma cells epithelial-mesenchymal transition-like morphological alteration is associated with the assembly and disassembly of cytoskeletal proteins [7]. In the present study, therefore, we analyzed the cytoskeletal proteins of Chang liver cells of epithelial and fibroblast-like appearance. In addition, the production of procollagen type III peptide (P-III-P), a characteristic of fibroblast-like cells of liver, was measured to clarify whether the morphological change into fibroblast-like cells was associated with functional alteration [8, 9].

Chang liver cells (5×10^5 cells) were cultured in RPMI1640 medium containing 5% fetal bovine serum [10]. Novobiocin, butyrate, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7), obtained from the Sigma Chemical Company, were added into the culture medium at the start of culture. The immunofluorescent microscopic study was carried out as reported previously [11]. Briefly, the cells were fixed with ethanol and acetone, and the cytoskeletal proteins were stained by the indirect immunofluorescent antibody technique using rabbit antibodies against tubulin, vimentin and cytokeratin and FITC-conjugated anti-rabbit IgG antibody. Actin was stained with rhodamine-phalloidin (Molecular Probe Inc., USA). Immunoblot analysis was carried out as described previously [11]. Monoclonal anti-phosphotyrosine antibody was obtained from Amersham. Cellular uptake of [^{3}H]leucine and [^{3}H]acetate was measured by the TCA precipitation method [12]. P-III-P produced and secreted into the culture medium was measured as described previously [13].

Figure 1 A demonstrates the morphological appearance of Chang liver cells cultured with or without novobiocin

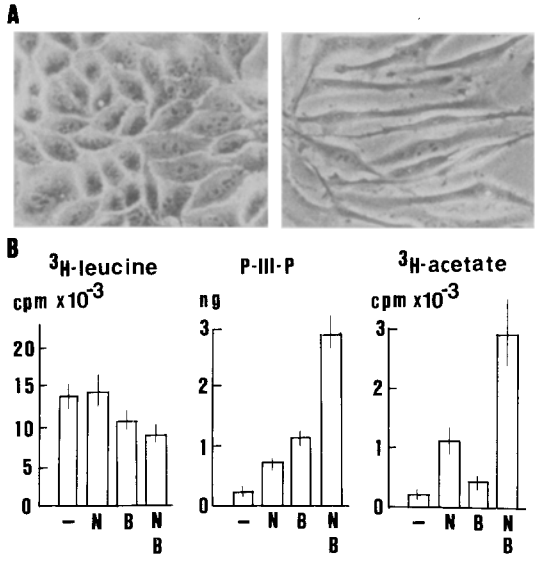

Figure 1. Effects of novobiocin and butyrate on the morphology and function of Chang liver cells.
A Cell morphology. Cells (5×10^5) were cultured for 3 days without (left) or with (right) novobiocin (100 μM) plus butyrate (1 mM). ×400.
B Protein synthesis and acetate uptake. Cells (5×10^5) were cultured for 3 days with no additions (–), 100 μM novobiocin (N), 1 mM butyrate (B), or 100 μM novobiocin plus 1 mM butyrate (NB). Then, the cells were incubated for a further 3 h with 1 μCi/ml of [^{3}H]leucine (left) or 5 μCi/ml of [^{3}H]acetate (right) and the radioactivity incorporated into the TCA precipitable materials was measured with a spectrophotometer. To measure P-III-P produced and secreted into the culture medium (center) the cells cultured for 2 days with or without novibiocin (100 μM) and/or butyrate (1 mM) were washed in serum-free medium 5 times and were cultured for a further 2 days in serum-free RPMI1640 medium with or without novobiocin and/or butyrate. The P-III-P in the medium was measured by radioimmunoassay.

plus butyrate. Chang liver cells in the control culture showed the morphological features of epithelial cells (fig. 1 A, left). Culturing the cells for 3 days with novobiocin (100 μM) or butyrate (1 mM) resulted in a reduction of the cell number by approximately 20%, but their morphological appearance was not markedly altered (photographs not shown). However, the cells cultured for 3 days in the simultaneous presence of novobiocin (100 μM) and butyrate (1 mM) transformed into fibroblast-like cells (fig. 1 A, right).

Protein kinase inhibitor H7 (100 μM) and calmodulin-dependent protein kinase antagonist W7 (10 μM) had no significant effect on the cell morphology and did not antagonize the effects of novobiocin and butyrate (not shown). Figure 1 B demonstrates that novobiocin and butyrate synergistically enhanced [^{3}H]acetate uptake. P-III-P is known to be produced by hepatocytes in chronic liver disease, and is one of the markers for hepatic fibrosis[8,9]. As shown in figure 1 B, P-III-P production was remarkably increased in the fibroblast-like cells in the culture with novobiocin plus butyrate. In contrast, total protein synthesis as measured by [^{3}H]leucine uptake was not altered (fig. 1 B). These results indicate that the morphological change induced by novobiocin and butyrate is associated with a selective increase in P-III-P production.

The effects of novobiocin and butyrate on the state of assembly of cytoskeletal proteins were analyzed by immunofluorescent microscopy using specific antibodies and rhodamine-phalloidin. The assembly of actin, tubulin, vimentin, and cytokeratin was remarkably enhanced when cells were cultured with novobiocin (100 μM) plus butyrate (1 mM) (fig. 2). The fibrous structures of these cytoskeletal proteins appeared in the perinuclear region after 2 days of culture, and some of the fibrous structures extended to the cytoplasm in the tips of elongated cells after the third day of culture.

Figure 3 demonstrates the immunoblot analysis of actin, tubulin, vimentin and cytokeratin. The amounts of these cytoskeletal proteins appear not to be significantly increased in those cells cultured with novobiocin plus butyrate. Cytokeratin proteins of the control cells, and those cultured with novobiocin plus butyrate, show similar banding patterns, indicating that the expression of cytokeratin subtypes was not altered. Tyrosine kinases of the non-receptor type such as v-src, vfps, and v-yes proteins, are also associated with cytoskeletal proteins, and tyrosine phosphorylation alters the structure and function of actin and tubulin[14–16]. Therefore, the phosphorylation state of the tyrosine residues of cytoskeletal proteins was examined. The immunoblot analysis using monoclonal anti-phosphotyrosine antibody revealed that the phosphorylation state of the tyrosine residues of cytoskeletal proteins was not altered significantly.

As shown in the present study, novobiocin and butyrate synergistically transformed Chang liver cells into fibroblasts-like cells, probably by enhancing the assembly of cytoskeletal proteins. It seemed of interest to clarify by what mechanism the assembly of these four cytoskeletal proteins was enhanced simultaneously. A post-synthetic modification seemed to be responsible for the increased assembly, since the quantity of cytoskeletal proteins present was not altered. It has been reported that the assembly of cytoskeletal proteins is regulated by phosphorylation and dephosphorylation events[17–19]. How-

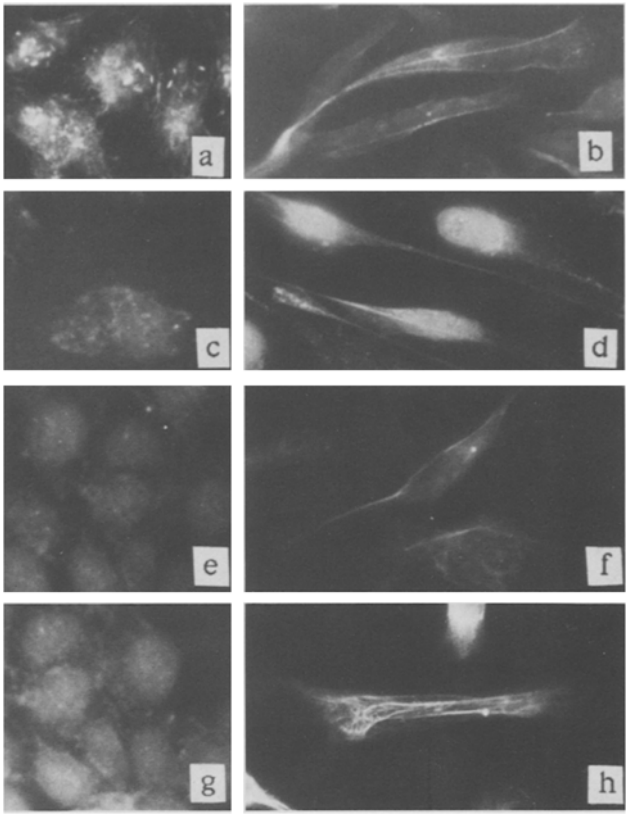

Figure 2. Effect of novobiocin and butyrate on the assembly of cytoskeletal proteins of Chang liver cells.
Chang liver cells (5×10^5) were cultured for 3 days with (b, d, f, h) or without (a, c, e, g) novobiocin (1 μM) plus butyrate (1 mM). The cells were stained with rhodamine-phalloidin (a, b) or immunostained with antibodies against tubulin (c, d), vimentin (e, f) and cytokeratin (g, h). ×1600.

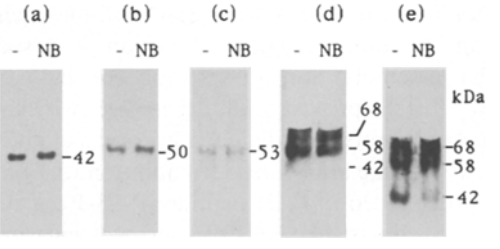

Figure 3. Immunoblot analysis of cytoskeletal proteins of Chang liver cells cultured with or without novobiocin plus butyrate.
Chang liver cells were cultured for 3 days with (NB) or without (−) novobiocin (100 μM) plus butyrate (1 mM). Cytosol fractions of 1×10^5 cells were used for immunoblotting using antibodies against actin (a) and tubulin (b). Triton X100-insoluble fractions obtained from 1×10^6 cells were used for immunoblot analysis using antibodies against vimentin (c), cytokeratin (d) and phosphotyrosine (e).

ever, neither novobiocin nor butyrate appeared to have any significant effect on the phosphorylation state of tyrosine residues of cytoskeletal proteins (fig. 3). In addition, protein kinase inhibitors such as H7 and W7 did not antagonize the effects of novobiocin and butyrate. This suggests that phosphorylation and dephosphorylation of serine and threonine residues may not play a major role in inducing the assembly of cytoskeletal proteins.

Alpha-tubulin has acetylation sites, and their post-translational acetylation correlates with increased stability of assembled α-tubulin [20]. The present study disclosed that novobiocin and butyrate synergistically stimulated [^{3}H]acetate uptake (fig. 1). These data indicate that hyperacetylation may be at least partly responsible for the increased assembly of tubulin. As far as we know, however, there have been no data showing that the assembly of actin or of intermediate filaments is enhanced by protein acetylation. Therefore, further studies are necessary to clarify whether the assembly of these cytoskeletal proteins is regulated by protein acetylation.

Novobiocin plus butyrate increased the production of P-III-P, a marker of hepatic fibrosis. The fact that the increased production of P-III-P was associated with a marked increase in the uptake of [^{3}H]acetate (fig. 1), suggested that the chemical modulation of nuclear proteins alters the chromatin structure and thereby enhances P-III-P production [1–3]. On the other hand, novobiocin appears to have various actions which are not mediated by topoisomerase II [21]. Butyrate is a well-known inducer of cell differentiation, but many of the biochemical pathways of its actions are not clear [22]. It is possible that biochemical pathways which are still unknown may be involved in the synergistic actions of novobiocin and butyrate.

Recently, it has been reported that cancer cells, such as those from bladder and mammary cancers, can be transformed into fibroblast-like cells in vitro [7, 23]. In these cell systems, as with the liver cells used here, increased cytoskeletal assembly was associated with the morphological transition. Cytoskeletal proteins appear to be connected with both cell surface proteins and nuclear skeletal proteins [24]. Therefore, the possibility exists that a structural alteration in cell surface and cytoskeletal proteins may cause changes in skeletal proteins and thereby alter the gene activity and the cellular differentiation state [25]. Alterations of gene structure and gene expression appear to be related to carcinogenesis as well [26]. Further studies of the assembly of cytoskeletal and nucleoskeletal proteins associated with epithelial-mesenchymal transition-like morphological changes will provide important knowledge about the mechanism of the morphological transformation and of alterations in the cellular differentiation state.

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