Effects of sodium n-butyrate on alpha-fetoprotein and albumin secretion in the human hepatoma cell line PLC/PRF/5

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Summary The in vitro effects of sodium n-butyrate (butyrate) on the growth, morphology and secretion of alpha-fetoprotein (AFP) and albumin by the human hepatoma cell line PLC/PRF/5 were studied. Butyrate caused a marked reduction in the growth rate, colony forming efficiency in soft agar and de novo synthesis of DNA as well as remarkable morphological changes including cell enlargement, flattening and a decreased number of nucleoli. Secretion of AFP was reduced during culture with butyrate, while that of albumin was increased. The requirement of de novo protein synthesis for the increase in albumin and decrease of AFP by butyrate was demonstrated by inhibition studies with cycloheximide. These results suggest that butyrate caused the hepatoma cells to acquire in vitro properties that are considered to be more consistent with normal liver cells.

There is an increased awareness that phasing in gene expression and the concomitant changes that it entails in cell composition, function, structure, and organization are central not only to the problem of developmental and reparative growth but also to that of neoplasia. It has been proposed that malignant transformation of eukaryotic cells results from structural changes in genetic material, loss of growth control, abnormalities in cell differentiation, or misprogramming of normal gene products (Uriel, 1979). Moreover, gene products expressed normally only during embryonal and foetal periods are frequently reexpressed in neoplasia. The production of AFP is often enhanced in tumour-bearing hosts, particularly with hepatocellular carcinoma (hepatoma). While the function of AFP remains undetermined, factors regulating the genomic expression of AFP are even less understood. However, it is clear that the mechanisms controlling the expression of the AFP gene must encompass aspects of both embryonic development and neoplastic transformation (Ruoslahti, 1979). These observations have stimulated our interest in the regulatory mechanisms of AFP gene expression.

Exposure of mouse hepatoma cells to dimethyl sulfoxide (DMSO) stimulates albumin and AFP accumulation in the medium (Higgins et al., 1983). Similarly, changes in hepatocyte gene expression occur during treatment of rat and mouse hepatoma cells with various differentiation-inducing agents (Higgins & Borenfreund, 1980; Schut et al., 1981; Hughes et al., 1982). Among these agents, sodium n-butyrate (butyrate), a 4-carbon fatty acid, is of major interest, since it is a natural fermentation product of colonic bacterial flora, and a potent "differentiation agent" in some cancer cells such as colorectal tumours, erythroleukaemia and a uterine cervical cancer cell line (Tsao et al., 1983; Leder & Leder, 1975; Nozawa et al., 1983).

We have therefore examined the effect of butyrate on AFP and albumin secretion and other phenotypic changes in a human hepatoma PLC/PRF/5-cells.

Materials and methods

Chemicals

Butyrate (Nakarai Chemical Co. Kyoto, Japan) and cycloheximide (Sigma Chemical Co. St Louis, MO.) were dissolved in distilled water. These stock solutions were stored at −20°C and diluted with the culture medium just before use. Non-essential amino acid (NEAA; 100x) for minimal essential medium (MEM) and N-2-hydroxy ethylpiperazine-N'2 ethane-sulfamic acid (HEPES) were purchased from Flow Laboratories (McLean, Va.).

Cell culture

Human hepatoma cell line PLC/PRF/5 (Alexander et al., 1976) was obtained from the National Cancer Institute (NIH, Bethesda, Md.) through the courtesy of Dr S. Watanabe (National Cancer
The radioactivity measured in calf were The mycin Center Research Searle, trichloroacetic 358 laboratories (for 500 ml MEM), 20 mM HEPES, streptomycin (100 µg ml⁻¹) and penicillin (100 IU ml⁻¹). The cells were incubated at 37°C in 5% CO₂ in air, and subcultured once a week using 0.125% trypsin (Flow Laboratories) and 0.02% ethylenediamine tetraacetic acid (EDTA) in PBS.

**Growth study**

PLC/PRF/5 cells were subcultured (in duplicate in 9.6 cm² 6-well multiwell dishes (Becton Dickinson Labware, Oxnard, CA) containing 2 ml Eagles MEM supplemented with 10% FCS (9.6 × 10⁴ cells/well). Twenty-four hours later (Day 0) the medium was changed and butyrate at concentrations of 0.05: 0.1: 0.4: 0.6: 1.0: 2.0 mM was added. The growth medium was changed every fourth day. AFP and albumin contents in the medium and the cell protein content were measured at 24 h intervals over 4 days. In all experiments the cell number determined with a haemocytometer correlated closely with the cell protein content.

**Colony formation in semi-solid agar medium**

Colony formation in semisolid agar medium was performed as previously described (Kim et al., 1980). Cells (2 × 10⁴) were suspended in 0.3% agar (Difco Laboratories, Detroit, MI) in complete growth medium with various butyrate concentrations. Two ml aliquots from these suspensions were layered on a 2 ml based layer of 0.5% agar in the same medium in 60 mm petri dishes. The cells were seeded in agar medium with various concentrations of butyrate. Dishes were incubated at 37°C in 5% CO₂ in air for 2 weeks after which colonies containing ≥ 20 cells were counted.

**Nucleic acid precursor incorporation**

The incorporation of [³H] thymidine (sp. act., 20 Ci mmol⁻¹; New England Nuclear, Boston, MA) was measured during growth in the presence of butyrate; 1 µCi of [³H] thymidine per ml was added for the 3 h pulse labelling. The reaction was terminated by the addition of ice-cold PBS. Cells were harvested by trypsinization, washed with PBS and centrifuged, then precipitated with 5% trichloroacetic acid and washed with methanol as indicated. The radioactivity of NCS (Amersham-Searle, Evanston, IL) solubilized pellets was measured in a liquid scintillation counter (Packard-Instruments) as described previously (Nakao et al., 1983).

**AFP and albumin determinations**

The concentrations of AFP and albumin in the culture medium were determined by enzyme-linked immunoassays (ELISA). Media with treated or untreated cells were centrifuged (3000 g min⁻¹) and the AFP content of the supernatant was assayed in accordance with the instruction of KINOS Laboratories, Ink. (Tokyo, Japan). Albumin content of the supernatant was determined by ELISA using rabbit antihuman albumin and horseradish peroxidase conjugated rabbit antihuman albumin antibodies (Cappel, Cochranville, PA), as described for human AFP (Uotila et al., 1981). The limits of detection in the ELISA were 1.0 ng/tube for both AFP and albumin. Control medium, which was not inoculated with cells, had no detectable levels of AFP or albumin.

**Cell protein determination**

The cells were washed with PBS twice and then 1 ml of 0.08% sodium lauryl sulfate (SDS) was added to the cell layer. Protein concentration was measured by Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as the standard (Bradford, 1976). SDS at this concentration had no interfering effect on protein determination.

**Effect of protein synthesis inhibitors**

A protein synthesis inhibitor cycloheximide was used to examine the de novo synthesis of AFP and albumin by the cells in the presence of butyrate. The inhibitor was added at concentrations of 0.1 and 0.5 µg ml⁻¹ to the culture medium with or without butyrate (Day 0). Cells were cultured on 6 well multiwell dishes for 4 days and then assayed for AFP and albumin levels in the medium and cell protein.

**Morphological studies**

Butyrate-treated or -untreated cells were stained with Giemsa and examined for morphological changes under a light microscope. An aliquot of 2 × 10⁸ cells centrifuged at 900 g was immediately fixed in a mixture of glutaraldehyde and then in 2% buffered osmium. After dehydration in ethanol, they were embedded in an epoxy resin. Ultrathin sections were examined by a JEM-1200 EX electron microscope (Japan Electron Optimal Lab., Tokyo, Japan) after uranyl and lead citrate staining (Nagasaki et al., 1983).

**Statistical analysis**

Data were analysed using Student’s t test on paired differences.
Results

Effect of butyrate on growth properties

Butyrate caused a marked reduction in the growth rate of PLC/PRF/5 cells within 2 days (Figure 1). Normal growth rates were restored upon removal of butyrate. At 2 mM, butyrate had little effect on the viability of the cells as assessed by trypan blue exclusion, and suggested by the continuous increase in cell number from day 0 to day 5 in the treated cells. Furthermore, in both treated and untreated cultures there were no more than 5% floating cells.

![Graph showing growth curves of PLC/PRF/5 cells](image)

Figure 1 Growth curves of PLC/PRF/5 cells in the absence (●) and presence of butyrate (○) 0.4 mM; (△) 1.0 mM; (×) 2.0 mM.

As shown in Figure 2, butyrate induced a significant dose-related inhibition of PLC/PRF/5 cell growth. By day 4, the protein content of cells decreased significantly when treated with 0.4 to 1 mM butyrate. The growth rates were reduced to 90% and 70% of those of untreated cells by 0.4 mM and 1.0 mM butyrate respectively.

The ability of PLC/PRF/5 cells to incorporate DNA as determined by [3H]labeled precursors was reduced in the presence of butyrate (Table I). This DNA synthesis inhibition paralleled the growth inhibition caused by butyrate. Furthermore, colony forming efficiencies in soft agar were also reduced by butyrate treatment.

AFP and albumin levels in the medium

The total amount of accumulated AFP and albumin in the growth medium, normalized per mg cell protein at the time of harvest, were similar for control and the various butyrate concentrations during the initial 24 h of culture. After 2 and 3 days treatment with 1.0 mM butyrate, AFP levels were constantly lower compared with controls, whereas albumin levels were constantly higher than that of control cultures (data not shown). As shown in Figure 3, AFP levels in the medium from 4 days culture were significantly decreased by 0.05 to 1.0 mM butyrate in a dose dependent manner \((P<0.01)\), whereas albumin levels were increased to 201% of controls by 1.0 mM butyrate \((P<0.01)\) and 125% by 0.6 mM butyrate.

Effects of protein synthesis inhibitors

The increase of albumin and decrease of AFP content in the butyrate culture medium was completely inhibited by the addition of cycloheximide \((0.5 \mu g ml^{-1})\) throughout the 4 days of the experiment (Table II). Cycloheximide at 0.5 \(\mu g ml^{-1}\) inhibited 70% of cellular protein synthesis.

![Graph showing dose response of butyrate on the growth of PLC/PRF/5 cells](image)

Figure 2 Dose response of butyrate on the growth of PLC/PRF/5 cells. Cells were subcultured in multiwell dishes and 24 h later the medium was changed and butyrate was added to give the final concentrations indicated. Cells were harvested 4 days later by 0.08% SDS to determine protein content. Each point represents the mean ± s.d. of two replicates from 3 separate experiments. * \(P<0.05\); ** \(P<0.01\).

### Table 1 Effects of butyrate on [3H]thymidine incorporation in PLC/PRF/5 cells and their colony forming efficiency in soft agar.

| Butyrate concentration (mM) | [3H] thymidine incorporation (cpm/well) | Colony forming efficiency (%) |
|----------------------------|------------------------------------------|-------------------------------|
| 0                          | 8998 ± 801                               | 100                           |
| 0.4                        | 6388 ± 205a                              | 57                            |
| 1.0                        | 5304 ± 450a                              | 17                            |
| 2.0                        | 4538 ± 292a                              | 0                             |

*means ± s.d. of 5 wells, * \(P<0.001\).
Figure 3 Effects of butyrate on the extracellular accumulation of AFP and albumin (□, AFP; □, albumin) PLC/PRF/5 cells were cultured as described in Figure 2. After 4 days, culture medium was centrifuged (3000 g min⁻¹), and the supernatant assayed for AFP and albumin by ELISA. Each bar represents mean ± s.d. of two replicates from 3 separate experiments. *, P<0.01; **, P<0.05.

Table II Effects of cycloheximide on AFP and albumin secretion in PLC/PRF/5 cells.

| Butyrate       | AFP      | Albumin   |
|----------------|----------|-----------|
|                | 0 (mM)   | 0.4 (mM)  | 1.0 (mM)  |
|                |          | 0 (mM)    | 0.4 (mM)  | 1.0 (mM)  |
| Cycloheximide  |          | 0         | 0.4        | 1.0        |
| 0              | 1035 ± 64 | 471 ± 4   | 342 ± 15   | 12.9 ± 1.4 | 16.1 ± 0.9 | 25.9 ± 3.9 |
| 0.1 µg ml⁻¹    | 710 ± 37 | 442 ± 1   | 402 ± 1    | 6.8 ± 0.6  | 6.8 ± 0.4  | 7.2 ± 0.1  |
| 0.5 µg ml⁻¹    | 359 ± 15 | 357 ± 16  | 370 ± 33   | 4.7 ± 0.4  | 4.5 ± 0.3  | 4.7 ± 0.3  |

*ng mg⁻¹ cell protein.  
*bµg mg⁻¹ cell protein.  
*cmeans ± s.d. (n = 4)  
*dSignificantly different from butyrate-untreated control (P<0.01)

Morphological changes

Figure 4 shows the morphological changes of PLC/PRF/5 cells after 4 days treatment with 1 mM butyrate. Giemsa-staining of treated cells revealed the disappearance of the formerly prominent nucleolus, altered chromatin network, cellular change in spindle shape and the enlargement and increased number of membranous processes.

These changes were confirmed by electron microscopy. Untreated cells had a dense cytoplasmic matrix with few elements of the endoplasmic reticulum and abundant polysomes and mitochondria. Sparingly distributed glycogen granules were evident. The nuclei were characterised by the absence of condensed chromatin, which was dispersed in clumps around the nucleoplasm. Most cells had more than one nucleolus. Virus particles were not detected (Figure 5a). In cells treated with 1 mM butyrate the number
Figure 4 Giemsa-stained PLC/PRF/5 cells after 4 days culture. Control (untreated) cells had prominent nucleoli and epithelial formations (a,c). One mM butyrate-treated cells (b,d) had an increased number of membranous process. (a,b ×100; c,d ×200).

Discussion

These results show that butyrate caused a significant decrease in the total accumulated extracellular AFP in human hepatoma cell line PLC/PRF/5 (Figure 3). This could have resulted from decreased synthesis, increased protein degradation or interference with the protein secretion process of the hepatoma cells. However, the last two possibilities appear unlikely since butyrate-treated cells continue to secrete at least one other major hepatic protein, albumin (Figure 3). Kaneko et al. (1978) indicated that under culture conditions indentical to those used in the present study, medium AFP levels reflect the amount of AFP synthesized and secreted. Moreover the requirement of de novo protein synthesis for the increase of albumin and decrease of AFP by butyrate is demonstrated by the inhibition study with cycloheximide (Table II). It is therefore possible that the decrease in AFP and increase in albumin in the growth medium was due to altered synthesis rates of proteins in the butyrate-treated cells.

Hirohashi et al. (1979) compared growth, morphology, and production of AFP in human hepatoma cells. The fast- or moderately fast-growing hepatoma cells were moderately differentiated and produced a large amount of AFP. On the other hand, the slowly growing hepatoma cells were well differentiated and produced very little AFP, suggesting that AFP production depends on the degree of cell differentiation. Abelev (1965), who first showed the existence of AFP in the serum of animal foetuses, suggested in his review on production of AFP by hepatomas that each hepatoma corresponds to some stage of liver cell maturation. This assumption could explain the correlation between morphological differentiation and production of
Figure 5 Electron micrographs of PLC/PRF/5 cells after 4 days culture. Untreated cells (a) have a dense cytoplasmic matrix containing mitochondria and glycogen granules and have nuclei with marginal indentification (× 3000). The cells treated with 1 mM butyrate (b; × 4000) show an increased number of surface microvilli and amount of rough endoplasmic reticulum.

AFP in hepatoma. The serum concentration of AFP is reported to be maximal at approximately the 13th week of gestation and to decline rapidly thereafter, while the albumin level increases steadily until about the 26th week of gestation (Gitlin & Boesman, 1966). If the degree of differentiation of human hepatoma parallels the maturation of foetal liver cells, well-differentiated hepatoma cells should produce small amounts of AFP and large amounts of albumin. Such characteristics were in fact observed in the present study, demonstrating the marked phenotypic changes in butyrate-treated PLC/PRF/5 cells, i.e. decrease of AFP and increase of albumin, morphological maturation, reduced growth rate and reduced de novo synthesis of DNA and colony formation in soft agar. Studies with alpha-difluoromethyl ornithine (DFMO) (unpublished data), an irreversible inhibitor of ornithine decarboxylase, the first step in polyamine biosynthesis (Mamont et al., 1978), have demonstrated that the reduction of proliferation in PLC/PRF/5 hepatoma cells by DFMO does not alter the AFP and albumin secretion. These data indicate that the properties of AFP and albumin secretion are not necessarily linked to the rate of proliferation but rather to the degree of differentiation of the hepatoma cells. Such evidence may suggest that butyrate caused PLC/PRF/5 hepatoma cells to acquire in vitro properties which are more consistent with well-differentiated cells.

Butyrate has been noted to affect DNA structure (Terada et al., 1978) and nuclear histone composition through acetylation (Annunziato & Seale, 1983). After the discovery of histone acetylation (Allfrey et al., 1964), it was suggested that this post-synthetic modification of histone structure could provide an enzymatic mechanism for modulating the interactions between histones and
DNA in ways that affect the structure and function of chromatin. Furthermore, numerous correlations have been noted between increased acetylation of the histones and gene activation for RNA synthesis (Ruiz-Carrillo et al., 1975). Thus, further study of butyrate-induced phenotypic changes in the human hepatoma cell line PLC/PRF/5 may provide useful information on the regulatory mechanisms of genes expressing differentiation-associated phenotypes in human hepatocytes and contribute to elucidation of the molecular events involved in their malignant transformation.

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