HMG-CoA reductase inhibitor mevastatin enhances the growth inhibitory effect of butyrate in the colorectal carcinoma cell line Caco-2

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Mevastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis. Butyrate, a short-chain fatty acid, reduces proliferation and induces differentiation of human colon cancer cells. The aim of our study was to determine the effect of mevastatin, alone or in combination with butyrate, on proliferation, the cell cycle and apoptosis in the human colorectal carcinoma cell line Caco-2. In this report we show that mevastatin combined with butyrate synergistically suppressed growth of Caco-2 cells in a dose- and time-dependent manner. In addition, incubation with mevastatin arrested cells in the G1 phase of the cell cycle after 24 h with a switch to the G2/M phase after 72 h. This was accompanied by a down-regulation of cyclin-dependent kinases (cdk) 4 and cdk 6 as well as cyclin D1, while cdk 2 and cyclin E protein levels remained unchanged during mevastatin treatment. Cell cycle inhibitors p21 and p27 were significantly upregulated by mevastatin. The pro-apoptotic properties of mevastatin were further enhanced by co-incubation with butyrate. Lastly, the effects of mevastatin could be reversed by addition of mevalonate, but not farnesyl- or geranylgeranylpyrophosphate, intermediate products of cholesterol synthesis, to the medium. These results suggest that HMG-CoA reductase inhibitors like mevastatin may enhance the antiproliferative effect of butyrate in colon cancer cells via induction of apoptosis together with a G0/G1 cell cycle arrest.

Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA-) reductase inhibitors (HRI) are widely used and well-tolerated pharmaceuticals for treating clinical hypercholesterolemia (1). They prevent formation of mevalonate, a short-chain fatty acid, from HMG-CoA and thereby inhibit cholesterol synthesis. However, their effects on cultured and malignant cells appear to be mediated by their inhibition of mevalonate and subsequent nonsterol metabolites (2), which have been identified as essential factors for cell cycle progression (3). Interestingly, tumour cells appear to have higher demands for these metabolites than normal cells (4). Farnesyl- (FPP) and geranylgeranylpyrophosphate (GGPP), intermediates in the cholesterol synthetic pathway, are needed for isoprenylation of cellular proteins (like Ras, Rho, Cdc42, Rac), a crucial step for membrane attachment (5,6). Ras proteins are important regulators of cell proliferation (7,8), and post-translational prenylation is essential for their binding to the internal plasma membrane and subsequent acquisition of biological activity (9). Aberrant Ras function has been considered as an important contribution to human colon carcinogenesis, therefore significant progress has been made to antagonize Ras prenylation (7). By inhibiting protein isoprenylation, HRIs exert important cellular effects, including a reduction of cell proliferation and an induction of apoptosis (1). Thus, HRIs, such as mevastatin, pravastatin or simvastatin, may have significant antitumour activity. This was further supported by two clinical studies showing a 43% (10) and 19% (11) reduction in the number of newly diagnosed cases of colorectal cancer during a 5-year follow-up period in patients with coronary artery disease receiving pravastatin and simvastatin, respectively. Furthermore, the chemopreventive effect of pravastatin could be augmented by concomitant ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin (10). In rodents, pravastatin and simvastatin have been shown to reduce carcinogen-induced colon cancers by 50–65% (12,13).

Butyrate, a short-chain fatty acid produced by colonic fermentation of dietary fibre, has been shown to modulate proliferation and differentiation of normal and neoplastic colonocytes (14,15). Butyrate is functionally disparate but structurally similar and metabolically related to mevalonate. Within the colonocyte the oxidative metabolism of butyrate yields metabolic intermediates for the synthesis of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA), the precursor of mevalonate synthesis (16). Colonic butyrate is thought to maintain mucosal differentiation and oppose carcino genesis (17). The underlying molecular mechanisms for the selective antineoplastic effects of butyrate have not been fully elucidated.

Several mechanisms have been proposed, ranging from histone hyperacetylation as a result of histone deacetylase inhibition to influence on oncogene expression and interaction with extracellular matrix proteins (18,19). As recently published by us, there is increasing evidence that a nuclear receptor, termed PPAR-γ, may also have a key role in the differentiation process of colorectal cancer cells (20). In addition, it has been suggested that butyrate’s antiproliferative effects may be linked to the inhibition of mevalonate-mediated cell growth (16).

Recently, it has been shown that lovastatin, another HMG-CoA reductase inhibitor, reduces proliferation of myeloid leukemic and colon cancer (HT-29) cells (21,22) and induces G1 arrest in a wide variety of malignant cells in vitro (23–25). The aim of the present study was to evaluate the effect of HMG-CoA inhibition in combination with butyrate on cell proliferation, cell cycle and apoptosis in the human colorectal adenocarcinoma cell line Caco-2, which undergoes rapid cell differentiation in the presence of butyrate.

Abbreviations: AP, alkaline phosphatase; cdk, cyclin-dependent kinase; FPP, farneslypyrophosphate; GGPP, geranylgeranylpyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HRI, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA-) reductase inhibitor; NSAIDs, non-steroidal anti-inflammatory drugs; PPAR-γ, peroxisome proliferator-activated receptor gamma.
Materials and methods

Cell culture

The human colorectal cancer cell lines Caco-2 and HCT-116 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Caco-2 cells of passages 40–50 were grown in Dulbecco’s modified Eagle medium (DMEM), HCT-116 cells (passages 21–23) were grown in McCoy’s 5A medium, both supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin under 5% CO2 and 95% air. Cells were passaged using Dulbecco’s PBS containing 0.25% trypsin and 1% EDTA. For treatment with sodium butyrate, mevastatin or mevalonate, cells were cultured in DMEM until nearly confluent. Medium was then removed and replaced by a medium containing either the solvent or 1–mevalonate (DL-mevalonic acid lactone; Sigma, St Louis, MO), mevalonate. Butyrate (Merck-Schuchardt, Hohenbrunn, Germany) and mevastatin (Sigma, St Louis, MO) were solubilized in PBS and added to the medium. Mevastatin (Sigma) was solubilized in DMSO. For defining a role of intermediate metabolites of the mevalonate pathway, cells were incubated with 100 µM farnesylpyrophosphate (FPP) or 10 µM geranygeranylpyrophosphate (GGPP) (Sigma). The medium was changed every other day. Cells were then harvested at the times stated in the figure legends.

Cell counts

Cells were suspended and cultured on 96-well dishes at a density of 2 × 10^3/cm² until confluent. Media was then changed every other day and at given time points cell number was assayed by crystal violet staining. Medium was replaced from the plates and cells were fixed in 5% formaldehyde for 5 min. After washing with PBS cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and unstained with 33% acetic acid. Absorption, which correlates with the cell number, was measured at 620 nm.

Cytotoxicity was excluded using a LDH kit (Merck, Darmstadt, Germany) measuring lactate dehydrogenase activity in the supernatant of damaged cells.

Cell cycle analysis

Cells were seeded in 6-well plates at a density of 15 × 10^4/cm² and allowed to attach for 24 h. Twenty-four hours following treatment cells were washed with PBS and harvested by trypsinization (2.5% trypsin/EDTA solution; Gibco, Eggenstein, Germany). DNA contents of cells were measured using a DNA staining kit (CycleTEST PLUS DNA Reagent kit; Becton, Dickinson, Heidelberg, Germany). Propidium iodide stained nuclear fractions were obtained as described in the manufacturer’s instructions. Data were acquired using CellQuest software (Becton Dickinson) with a FACScalibur (Becton Dickinson) flow cytometry system using 10 000 cells per analysis. Cell cycle distributions were calculated by use of ModFit LT 2.0 software (Verity Software House, Topsham, USA).

SDS–PAGE and immunoblot analysis

Cells were washed twice with ice-cold PBS and lysed in protein lysis buffer (Biolabs, Beverly, USA) containing protease inhibitors (Boehringer Mannheim, Germany). Protein extracts were obtained after sonication of cell lysates 3 × 5 s at 50% power and centrifugation at 10 000 r.p.m. at 4 °C. Protein content was quantified with the Biorad Protein Colorimetric assay (Hercules, CA). After addition of sample buffer and boiling of samples at 95 °C for 3 min, 7–50 µg of each total protein lysate were separated on a SDS–polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and the membrane was blocked overnight at 4 °C with 3% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Next, blots were washed and incubated 1 h in TBS-T with a 1:1000 dilution of primary antibody for human cyclin-dependent kinase (cdk) 2, cdk 4, cdk 6, cyclin D1, cyclin E, p21 or p27 (all Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary, horseradish peroxidase-conjugated antibodies (Vector Laboratories, Burlingame, CA) were diluted at 1:2000 and incubated with the membrane for another 30 min. After chemiluminescence reaction (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), bands were detected after exposure to Hyperfilm-MP (Amersham). For quantitative analysis, bands were detected and evaluated densitometrically using the ProVidoc system (Desaga, Wiesloch, Germany).

Apoptosis

Apoptosis was quantified by use of a commercial photometric ELISA (Boehringer), measuring cytoplasmic histone-associated DNA fragments.

Statistical analysis

Data are expressed as mean ± SD. The Mann–Whitney U-test was used to calculate the differences between treatment groups in the experiments. Differences were considered significant at P ≤ 0.05.

Results

Mevastatin enhances the growth inhibitory effect of butyrate

First of all, the effective dose of mevastatin in Caco-2 cells was determined. For this purpose, cells were treated with various concentrations of mevastatin and cell number was assessed after 5 days of incubation. As shown in Figure 1, mevastatin caused a dose-dependent decrease in cell number.

In the first set of experiments, Caco-2 cells were grown under standard conditions or incubated with butyrate-containing medium (1 or 2 mM) and treated with mevalonate (2.5 mM), mevastatin (64 µM) or a combination of both. The dosage of mevalonate and mevastatin, respectively, was changed every other day. Cells were then harvested at the times stated in the figure legends.

The enhancement of the antiproliferative effect of butyrate by mevalonate as well as its reversal by mevalonate was most prominent with 64 µM mevalonate and cell number was measured lactate dehydrogenase activity in the supernatant of damaged cells.

Apoptosis was quantified after 5 days of incubation. Means ± SD, n = 8.

Mevastatin causes an early G0/G1 and a late G2/M cell cycle arrest

Cells were incubated with different concentrations of mevastatin (32, 64 or 128 µM) and the number of cells in each cell cycle phase was determined after 24, 48 and 72 h of treatment. As shown in Figure 4, cell cycle analysis revealed that mevastatin caused a dose-dependent increase of cells in G0/G1 phase of the cell cycle of up to 30% versus control after 24 h of treatment. After 72 h, however, there was an increase of cells in the G2/M phase of the cell cycle, which was most prominent with 64 µM mevastatin (up to 21%
Mevastatin, butyrate and colorectal carcinoma

**Fig. 2.** (A) Cell counts of Caco-2 cells incubated with butyrate (1 or 2 mM) alone or in combination with mevastatin (64 µM) for up to 5 days. Means ± SD, n = 8. (B) Cell counts of Caco-2 cells incubated with butyrate (1 or 2 mM) alone or in combination with mevalonate (2.5 mM), mevastatin (64 µM) or mevalonate (2.5 mM) plus mevastatin (64 µM) for 5 days. Means ± SD, n = 8. *P < 0.05, **P < 0.01.

**Fig. 3.** (A) Cell counts of HCT-116 cells incubated with butyrate (1 or 2 mM) alone or in combination with mevastatin (5 µM) for up to 72 h. Means ± SD, n = 8. (B) Cell counts of HCT-116 cells incubated with butyrate (1 or 2 mM) alone or in combination with mevalonate (2.5 mM), mevastatin (5 µM) or mevalonate (2.5 mM) plus mevastatin (5 µM) for 72 h. Means ± SD, n = 8. *P < 0.05, **P < 0.01.

**Fig. 4.** Cell cycle analysis of Caco-2 cells (A) 24 h and (B) 72 h after incubation with mevastatin (32, 64 or 128 µM) versus control. Means ± SD, n = 2.

Mevastatin induces apoptosis in Caco-2 cells
Caco-2 cells were seeded into 96 wells and incubated with mevastatin. Western blot analysis revealed that mevastatin caused a dose-dependent decrease in cdk 4 (up to 18% with 128 µM mevastatin versus control), cdk 6 (up to 23% with 128 µM mevastatin versus control) and cyclin D1 (up to 45% with 128 µM mevastatin versus control) protein expression, accompanied by an up-regulation of the cell cycle inhibitors p21 (up to 223% with 64 µM mevastatin versus control) and p27 (up to 344% with 64 µM mevastatin versus control), supporting the observed G1 cell cycle arrest (Figure 5A and B). However, at higher concentrations (128 µM mevastatin) cell cycle inhibitors p21 and p27 decreased slightly (p21 up to 118% versus control; p27 up to 320% versus control). The decrease in cdk 4 and cdk 6 protein expression as well as the increase of the cdk inhibitors p21 and p27 by mevastatin could be prevented by co-incubation with mevalonate (2.5 mM) (Figure 5A).

Cdk 2 protein expression decreased significantly with butyrate treatment, as shown in earlier studies (27), but was not influenced by incubation with mevastatin (Figure 5C). The same effect could be observed in terms of cyclin E. The expression of the cell cycle inhibitor p21 increased in both groups by mevastatin treatment, with the more prominent increase in the butyrate group.

In order to assess the specificity of these effects, HCT-116 cells were treated with increasing concentrations of mevastatin. Similar to the effects observed in Caco-2 cells, cyclin D1 protein expression decreased in mevastatin-treated cells in a dose-dependent manner (up to 12% with 20 µM mevastatin versus control) and p27 (up to 344% with 64 µM mevastatin versus control), supporting the observed G1 cell cycle arrest (Figure 5A and B). The expression of the cell cycle inhibitor p21 increased in both groups by mevastatin treatment, with the more prominent increase in the butyrate group.

In order to examine the effect of mevastatin on cell cycle associated proteins, we determined protein expression of cdk 2, cdk 4, cdk 6, cyclin D1, cyclin E, p21 and p27 in Caco-2 cells after 3 days of treatment with various concentrations of mevastatin. Western blot analysis revealed that mevastatin caused a dose-dependent decrease in cdk 4 (up to 18% with 128 µM mevastatin versus control), cdk 6 (up to 23% with 128 µM mevastatin versus control) and cyclin D1 (up to 45% with 128 µM mevastatin versus control) protein expression, accompanied by an up-regulation of the cell cycle inhibitors p21 (up to 223% with 64 µM mevastatin versus control) and p27 (up to 344% with 64 µM mevastatin versus control), supporting the observed G1 cell cycle arrest (Figure 5A and B). However, at higher concentrations (128 µM mevastatin) cell cycle inhibitors p21 and p27 decreased slightly (p21 up to 118% versus control; p27 up to 320% versus control). The decrease in cdk 4 and cdk 6 protein expression as well as the increase of the cdk inhibitors p21 and p27 by mevastatin could be prevented by co-incubation with mevalonate (2.5 mM) (Figure 5A).

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inhibition of the mevalonate pathway by use of mevastatin, the prototype HMG-CoA reductase inhibitor, enhances the antiproliferative effect of butyrate on the colorectal cancer cell line Caco-2 in a time- and dose-dependent manner. This effect could be reversed by co-incubation with mevalonate, bypassing the HMG-CoA reductase inhibition by mevastatin, and was not specific of Caco-2 cells, since another colon cancer cell line, HCT-116, responded in a similar manner, even though earlier than Caco-2 cells and at lower mevastatin concentrations. This is most likely to be due to the higher proliferation rate of HCT-116 cells as compared with Caco-2 cells.

It is generally accepted that butyrate causes a G1 arrest in several cell lines (29–31). The same effect could be observed with HMG-CoA reductase inhibitors in myeloid leukemia cells, human breast cancer cells and mouse fibroblasts (21,23,25). Therefore, we examined the putative influence of the HRI mevastatin on the cell cycle and cell cycle relevant proteins in the colorectal adenocarcinoma cell line Caco-2. Flow
cytometry analysis showed that mevastatin led to an early arrest of cells in the G0/G1 phase (after 24 h of treatment) and a late arrest in the G2/M phase (after 72 h). Again, these effects could be reversed by the addition of mevalonate to the medium.

Cell cycle is governed by a family of cyclin-dependent kinases (cdks), whose activity is regulated by binding of the cyclins, by phosphorylation and by negative regulators, cdk inhibitors, like p21 and p27 (25,32). Cdk 4 and cdk 6 are thought to play a major role in cell cycle progression in early G1 phase, triggering the phosphorylation of cyclin D, which is responsible for the progression of cells through the G1 phase of the cell cycle. Cdk 2 activity is first evident during the middle of G1 and acts together with cyclin E to control progression though G1. With cyclin A cdk 2 may function in controlling the start of DNA synthesis in the S phase (27,31). We could show that in Caco-2 cells mevastatin caused a significant down-regulation of cdk 4, cdk 6 and cyclin D1 protein with a concomitant up-regulation of the cell cycle inhibitors p21 and p27. This is in agreement with our data showing an arrest of cells in the G1 phase after treatment with mevastatin. It has been shown previously that butyrate inhibits cyclin D1 expression at the transcriptional level, an effect which probably could be enhanced by co-incubation with mevastatin (33).

The role of p21 and p27 in the regulation of cell proliferation, differentiation and apoptosis is still controversial (34). Marked up-regulation of endogenous p21 has been demonstrated in various cell types during the apoptotic response to different agents (35). On the other hand, several studies suggested a role of p21 in protection from apoptosis in colon and other cancer cells (36,37). Yamamoto et al. (34) postulated a role of p21 in the early stage of differentiation whereas p27 was thought to play a positive role in both the early and late stages of differentiation (34). It has been stated previously that inhibition of HMG-CoA reductase led to an increase of cell cycle inhibitors in several cell lines (21,23). Furthermore, it has been shown that the increase of p21 and p27 was due to the inhibition of their ubiquitin-proteasome mediated proteolysis by the HRI lovastatin, resulting in an accumulation of p21 and p27 and a subsequent G1 arrest (38,39). It is very likely that the same mechanism is responsible for the increase of the cdk inhibitors in our cell line Caco-2 by mevastatin, although other pathways cannot be ruled out. However, our data suggest a role of p21 and p27 rather in apoptosis than in differentiation, as the latter process is prevented by mevastatin in our cell line, as we have recently shown (40).

Cdk 2 protein expression decreased with butyrate treatment, which has been demonstrated earlier (27), but was not significantly altered by mevastatin. The same was true for cyclin E protein expression. This is in agreement with previous data showing that in human prostate carcinoma cells lovastatin had no effect on cdk 2 and cyclin E protein expression (41). However, the butyrate-mediated down-regulation of cdk 2 and cyclin E, responsible for the progression of cells from G1 to S phase of the cell cycle, could probably contribute to G1 arrest. Taken together, analysis of cell cycle relevant proteins shows that mevastatin acts synergistically with butyrate to keep the cells in G1 phase of the cell cycle by down-regulating the cell cycle promoting kinases cdk 4 and cdk 6 as well as their binding partner cyclin D1 and by increasing the cell cycle inhibitors p21 and p27. However, it cannot be ruled out that other cell cycle related proteins, probably responsible for the late switch to the G2/M phase, are also involved.

In summary, our data show that in the human adenocarcinoma cell line Caco-2 the HRI mevastatin synergistically acts with butyrate to inhibit cell proliferation and to induce cell cycle arrest and apoptosis in this cell line. This seems to be associated with an early arrest of cells in G1/G0 phase of the cell cycle and a late arrest in the G2/M phase, accompanied by a down-regulation of cdk 4, cdk 6 and cyclin D1 and an up-regulation of p21 and p27 protein expression. These results suggest that induction of apoptosis together with the G0/G1 and G2/M cell cycle arrest may be one of the mechanisms of the antiproliferative effect of mevastatin in colon cancer cells. As butyrate has a very short metabolic half-life of ~6 min (56,57), the concomitant provision of HRI, like mevastatin,
may serve to further enhance the butyrate effects. Thus, the combination of butyrate and HRI may provide a novel strategy for the chemoprevention and/or the non-toxic treatment of colon carcinogenesis.

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Mevastatin, butyrate and colorectal carcinoma


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