Resveratrol Enhances the Differentiation Induced by Butyrate in Caco-2 Colon Cancer Cells

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ABSTRACT Butyrate, a short-chain fatty acid produced in the colon by microbial fermentation of fiber, inhibits growth of colonic carcinoma cells while inducing differentiation. Resveratrol, a plant polyphenol found in red wine and peanuts, has been shown to exert chemopreventive properties on colon cancer cells. The aim of this study was to determine whether resveratrol modulates the effects of butyrate on Caco-2, a colon adenocarcinoma cell line. The growth inhibitory effect of resveratrol (50 μmol/L) was more powerful than that of butyrate (2 mmol/L). Butyrate did not intensify the inhibition of proliferation exerted by resveratrol. Although the polyphenol enhanced the differentiation-inducing effect of butyrate, it did not elevate alkaline phosphatase activity or E-cadherin protein expression, markers of epithelial differentiation, when applied alone. Butyrate-induced transforming growth factor-β1 secretion was inhibited by resveratrol. Treatment with the combination of resveratrol and butyrate attenuated levels of p27Kip1, whereas resveratrol enhanced butyrate’s effect on the induction of p21Waf1/Cip1 expression. These data demonstrate a possible combined chemopreventive effect of two substances naturally occurring in the colonic lumen after ingestion of fibers and resveratrol-containing food. J. Nutr. 132: 2082–2086, 2002.

KEY WORDS: • resveratrol • Caco-2 cells • differentiation • butyrate • colon cancer

Colorectal tumors are remarkably common in Western populations. Chemopreventive agents occurring in the diet offer great potential to reduce the incidence of cancer. An association between reduced risk of colorectal cancer and diets high in fruit, fiber or vegetables has been well-established in epidemiologic studies (1).

Butyrate is synthesized by colonic bacteria from the unabsorbed complex carbohydrates present in dietary fiber (2). The short-chain fatty acid (SCFA)3 stimulates growth of normal epithelial cells, whereas it inhibits proliferation and induces rapid cell differentiation in colon cancer cell lines (3–5). At higher concentrations, induction of apoptosis occurs in carcinoma cells treated with butyrate (6). Histone deacetylase inhibition resulting in modified oncogene expression has been considered to be a molecular mechanism by which butyrate modulates growth and differentiation (7). Induction of peroxisome proliferator-activated receptor γ (8) and upregulation of the vitamin D receptor have also been implicated in butyrate-induced differentiation (9).

The polyphenol resveratrol (trans-3,5,4′-trihydroxystilbene) is produced in plants in response to environmental or fungal stress. Dietary sources of this phytoalexin are red wine, grapes and peanuts. The molecule has been shown to inhibit proliferation of Caco-2 cells (10). In addition, treatment with resveratrol leads to accumulation of colon cancer cells in the S phase of the cell cycle and, at high concentrations, to induction of apoptosis (11). Proposed mechanisms of action include inhibition of cyclooxygenases (12–14), ribonucleotide reductase (15) and DNA polymerase (16).

The objective of the present study was to examine whether the combination of these two substances with different modes of action has a beneficial chemopreventive effect over the monotherapeutic application. The approach of combined chemoprevention has already proved to be efficient in the prevention of polyps in an APCMin/+ mouse model, in which an inhibitor of cyclooxygenases was combined with an epidermal growth factor receptor kinase inhibitor (17). We chose Caco-2 cells as an in vitro model because this colorectal cancer cell line displays an undifferentiated phenotype under normal cell culture conditions, but can express an “enterocyte-like” phenotype either in response to various inducers of differentiation, including sodium butyrate or spontaneously after reaching confluence (4).

MATERIALS AND METHODS

Cell culture. The human colon cancer cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells of passages 52–58 were cultured in Dulbecco’s modified Eagle medium, supplemented with 10% fetal calf serum, penicillin (1000 U/L) and streptomycin (1 mg/L) and incubated at 37°C under an atmosphere of 5% CO2 in air. A stock solution of resveratrol (Sigma, Deisenhofen, Germany) was prepared in dimethyl sulfoxide (DMSO) and of sodium butyrate (Merck, Darmstadt, Germany) in PBS. Resveratrol was used at a concentration of 50 μmol/L, which has been shown not to be cytotoxic (11).

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1 Supported by the Else Kröner-Fresenius Foundation, Bad Homburg, Germany.
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3 Abbreviations used: AOM, azoxymethane; AP, alkaline phosphatase; cki, cyclin-dependent kinase inhibitor; DMSO, dimethyl sulfoxide; SCFA, short-chain fatty acid; TGF, transforming growth factor.

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and butyrate at a concentration of 2 mmol/L; untreated cells received the solvent alone (≤0.1% DMSO).

**Cell number.** Determination of cell numbers was carried out using a modification of the method of Matsubara et al. (18). Briefly, cells were plated at a density of 7 × 10⁴ cells/well in 96-well microtiter plates and were allowed to attach overnight. After treatment for 24, 48 and 72 h (resveratrol- and butyrate-containing media were changed after 48 h), the medium was removed and any adherent cells were fixed to the plate with 5% formaldehyde in PBS. The cells were then stained with a 0.5% aqueous solution of crystal violet followed by elution of the dye with 33% aqueous acetic acid. Absorbance at 570 nm was determined with a Tecan SpectraFluor Plus microplate reader (Tecan, Crailsheim, Germany) and the number of cells was determined from a standard curve of absorbance against cell numbers calculated from a mean of six experiments.

**Incorporation of [³H]thymidine and [¹⁴C]leucine.** Caco-2 cells were seeded in 24-well plates (3 × 10⁴/well) and were allowed to attach for 24 h. Together with treatment 18.5 MBq/well [³H]thymidine and 0.925 MBq/well [¹⁴C]leucine (Amersham Pharmacia Biotech, Freiburg, Germany) were applied to the cells for 24 h. Medium was discarded, monolayers were washed three times with PBS and the cellular macromolecules were precipitated using 5% trichloroacetic acid. The acid was aspirated, cells were washed with absolute methanol, and formic acid (2.5 mol/L) was used to solubilize the precipitated macromolecules. Probes were transferred to scintillation vials, 3.0 mL scintillation fluid (Packard Biosciences, Groningen, Netherlands) was added and measurements were carried out with a liquid scintillation counter (Packard Instruments, Meriden, CT). Cellular protein concentrations were determined as described in the Western blot analysis section.

**Determination of alkaline phosphatase (AP).** Alkaline phosphatase activity was measured using p-nitrophenylphosphate as substrate according to the manufacturer’s instructions (Merck, Darmstadt, Germany). Cells were seeded in 6-well plates at a density of 25 × 10⁴/well 24 h before treatment. Cell lysates of Caco-2 cells treated for 24, 96 or 192 h were analyzed in the assay. AP activity was measured in units/mg protein (U/mg protein).

**Transforming growth factor (TGF)-B1 secretion.** TGF-ß1 secretion was determined by use of an immunoassay kit (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s instructions. Caco-2 cells were seeded in 6-well plates at a density of 25 × 10⁴/well and allowed to attach overnight. Substances were added with serum-free medium and media were collected 24 or 48 h later.

**Western blot analysis.** Caco-2 cells were seeded in 80 cm² flasks; 24 h after plating, cells were incubated for 24 or 48 h with substances. Western blot analysis using total protein extracts from cultured cells was performed as previously described (11). Protein content was quantified with the Bio-Rad (Bio-Rad Laboratories, Munich, Germany) colorimetric assay. Reprobing of blots for expression of actin was done routinely. The antibody against p²⁷⁵/WAF1/CIP1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p²¹ WAF1/CIP1 was purchased from Oncogene (Cambridge, MA), and anti-E-cadherin was obtained from Becton Dickinson (Heidelberg, Germany).

**Statistical analysis.** Data are expressed as means ± SD. One-way ANOVA was used to compare means; P-values were corrected by the Bonferroni method for multiple comparisons (Microsoft Excel, Microsoft, Roselle, IL). A P-value < 0.01 was considered to indicate a significant difference.

**RESULTS**

Resveratrol was more potent than butyrate in reducing Caco-2 cell number as assessed by crystal violet assay. After 72 h of treatment, cell counts were reduced to 53% of control values with butyrate and to 31% with resveratrol (Fig. 1). When applied in combination, butyrate did not add to the growth inhibitory effect of resveratrol (27% of control). DNA synthesis rate, measured by [³H]thymidine incorporation, was not affected by butyrate treatment alone for 24 h, whereas resveratrol alone and the combination of both substances exerted an inhibitory effect with comparable potency (Fig. 2A). Protein synthesis rate (as measured by [¹⁴C]leucine incorporation) was reduced to 79% of control values after 24 h of incubation with butyrate, whereas resveratrol diminished the rate to 32% of control (Fig. 2B). There was no difference between growth inhibition exerted by resveratrol or the combination (35% of control).

Figure 3A shows AP activity, a marker of differentiation. We demonstrated earlier that treatment with resveratrol did not increase AP activity in Caco-2 cells (11), whereas butyrate enhanced it after 192 h approximately twofold (P < 0.01). Compared with the spontaneous differentiation occurring in...
untreated cells, the combination of the SCFA with resveratrol induced AP activity 3.3-fold ($P < 0.01$). E-cadherin protein expression is also a specific marker for differentiation of intestinal epithelial cells (19–21). Western blot analysis was used to detect E-cadherin. Treatment with resveratrol alone for 48 h attenuated E-cadherin protein expression, whereas butyrate alone had the opposite effect. In comparison with butyrate-treated cells, the combined treatment enhanced E-cadherin protein levels (Fig. 3B). Transforming growth factor (TGF)-β1 has also been implicated in epithelial differentiation (22). Therefore, TGF-β1 secretion into the medium was measured. Although butyrate induced secretion of the cytokine, TGF-β1 secretion was inhibited in Caco-2 cells treated with resveratrol as well as in cells treated with the combination of resveratrol and butyrate for 48 h (Fig. 4).

To examine the effect on cyclin-dependent kinase inhibitors (ckis), Caco-2 cells treated with resveratrol and butyrate alone and in combination were harvested after 24 and 48 h and subjected to Western blot analysis. As shown in Figure 5, butyrate increased p27$^{kip1}$ levels, whereas resveratrol, alone and in combination with butyrate, attenuated expression of the cki after 24 h as well as after 48 h. Protein expression of p21$^{Waf1/Cip1}$ was enhanced by the addition of butyrate, whereas resveratrol alone did not change expression of the protein (Fig. 6). Although p21$^{Waf1/Cip1}$ levels were not modified 24 h after combined treatment, an increase greater than that due to butyrate alone was observed after 48 h.

**DISCUSSION**

The results of the current study clearly indicate that treatment of colonic cancer cells with the SCFA butyrate and the plant polyphenol resveratrol inhibits proliferation. The growth-inhibiting effect of resveratrol is not potentiated by butyrate, whereas resveratrol enhances the increase of differentiation markers AP activity and E-cadherin protein expression by butyrate in colorectal cancer cells.

Other studies have demonstrated that resveratrol treatment alone leads to differentiation of erythroleukemic (23), osteoblastic (24) and promyelocytic cells (25). In contrast to these findings we did not observe effects on differentiation by resveratrol or its analog, piceatannol, in Caco-2 cells, as previously demonstrated (11,26). TGF-β1 has been implicated in butyrate-induced differentiation (22,27). Butyrate-induced TGF-β1 secretion was inhibited by resveratrol. Therefore we assumed that TGF-β1 was not involved in the combined effect of resveratrol and butyrate on differentiation. Induction of the cell cycle inhibitors p21$^{Waf1/Cip1}$ and p27$^{kip1}$ has been suggested to be involved in butyrate-induced differentiation of Caco-2 cells (28,29). Wächtershäuser and Stein (30) demonstrated that differentiation also occurs independently of p27$^{kip1}$ induction. Because we observed an attenuation of p27$^{kip1}$ expression along with the increase of AP activity after combined treatment with butyrate and resveratrol, p27$^{kip1}$ is
RESVERATROL AND DIFFERENTIATION OF CACO-2 CELLS

unlikely to play a role in this process. We also showed that the combination of butyrate and resveratrol increased butyrate's induction of p21^{Waf1/Cip1} expression, implying that p21^{Waf1/Cip1} rather than p27^{kip1} was involved in the induction of differentiation in this context.

The tumor suppressor p53 is one of the most important regulators of p21^{Waf1/Cip1}-induction, but is frequently mutated in colorectal carcinomas during the development from adenomas to carcinomas (31). Mutation of p53 has been implicated in the defective response to several growth inhibiting agents and this occurs also in Caco-2 cells (32). Activation of the waf1/cip1 promoter by butyrate is independent of p53 induction (33). A likely cause for induction of p21^{Waf1/Cip1} mediated by butyrate could be the histone deacetylase-inhibiting effect (7). It has been shown that resveratrol treatment alone enhances p21^{Waf1/Cip1} expression in erythroleukemic cells lacking p53 (23), an epithelial carcinoma cell line with only one functional p53 allele (34), and cultured bovine pulmonary artery endothelial cells possessing wild-type p53 (35), ruling out an obligatory role for p53 in the induction of p21^{Waf1/Cip1} by resveratrol. In contrast to these findings, p21^{Waf1/Cip1} levels in aoxymethane (AOM)-induced colorectal aberrant crypt foci of rats are attenuated by resveratrol treatment (36). This leads us to speculate that the effect of resveratrol on p21^{Waf1/Cip1} might be dependent on a specific genetic pattern that is altered in the process of carcinogenesis. It has been suggested that chemopreventive substances can compensate for genetic and biochemical alterations in neoplastic lesions. In our work, butyrate modified gene and protein expression in ways that allowed resveratrol to enhance differentiation. This might mean that butyrate creates the intracellular conditions for the differentiation-inducing action of resveratrol, in spite of genetic lesions.

Epidemiologic studies investigating a possible correlation between fiber intake and reduced risk of colorectal carcinoma were rather disconcerting (37). The conflicting results may relate to the heterogeneity of the fibers and the basal diet because almost complete colonic fermentation is achieved solely with soluble fibers. Several studies have demonstrated that the production ratio of SCFA is decreased in patients with colorectal adenomas and cancers [for a review see (38)].

Perrin et al. (39) demonstrated that only fibers that lead to a constant butyrate production decreased the rate of aberrant crypt foci in rats injected with AOM. Direct application of butyrate with enemas reduced the incidence and size of colorectal tumors in the same animal model, even when colitis was induced with trimetironbenzensulfonic acid before AOM injection (40). Further in vivo studies with defined fibers are required to clarify the role of butyrate in chemoprevention of colorectal carcinoma.

Taken together, our findings provide evidence that resveratrol intensifies the differentiation-inducing effect of butyrate. Aberrancy of differentiation occurs during carcinogenesis and the altered states of cell and tissue differentiation are characteristic of premalignant lesions long before they become invasive and metastatic. The principal of using combined strategies may provide dramatic improvements over monotherapeutic regimens in chemoprevention of colorectal carcinoma.

LITERATURE CITED

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