Upregulation of activin A gene by butyrate in human colon cancer cell lines

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Sonoyama, Kei, Pimara Pholnukulkit, Masahiko Toyoda, Suriya Rutatip, and Takanori Kasai. Upregulation of activin A gene by butyrate in human colon cancer cell lines. Am J Physiol Gastrointest Liver Physiol 284: G989–G995, 2003. First published January 22, 2003; 10.1152/ajpgi.00384.2002.—Activin A has been reported to play a role in the progression of colorectal cancer. Because dietary fiber protects against colorectal cancer, we hypothesized that butyrate, a fermentation product of dietary fiber, may affect the expression of activin A in colon cancer cells. Semiquantitative RT-PCR demonstrated that the activin A gene was upregulated by sodium butyrate in the human colon cancer cell lines HT-29 and Caco-2 in a concentration- and time-dependent manner. However, the activin A gene did not respond to sodium butyrate in the human normal colonic cell line FHC, rat normal intestinal epithelial cell (IEC) line IEC-6, and the explant of rat colon. Flow cytometry and agarose gel electrophoresis of genomic DNA revealed that cell cycle arrest and apoptosis were induced by sodium butyrate but not exogenous activin A in HT-29 cells, indicating that activin A could not act as an autocrine factor in colon cancer cells. By assuming that activin A promotes colorectal cancer spread as a paracrine factor, our findings suggest that butyrate could act as a tumor promoter in some circumstances.

ACTIVINS, which are members of the transforming growth factor-β (TGF-β) superfamily, have been shown to regulate several different cell functions, including cell proliferation and differentiation (13, 17). These molecules are synthesized as either a single homo- or heterodimer of two highly related β-subunits (βA and βB), resulting in three different isoforms of activins: activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (15, 32). In addition, the βA- and βB-subunits also form heterodimers with another dissimilar α-subunit, generating inhibin A (αβA) and inhibin B (αβB) (17). Activins and inhibins act as functional antagonists in some cell systems (15, 32). Activins bind to binary cell surface receptors, which are those receptors composed of two single membrane-spanning serine-threonine kinases, designated as type I and type II (18). Another type of activin-binding protein is called follistatin (20). Follistatin, a family of proteins generated by alternative splicing and proteolytic processing, has a specific and high affinity for activin; it neutralizes the effect of activin in a variety of systems. Thus the biological function of activins is regulated, in concert with its receptors, antagonists (inhibins), and neutralizing binding proteins (follistatins).

We (28) reported the expression and possible function of activin A in the intestinal epithelium. The results demonstrated that rat jejunal, rat intestinal epithelial cell line IEC-6, and the human colon adenocarcinoma cell line Caco-2 expressed mRNA encoding the βA-subunit of activin, cell surface receptors type IB and type IIA, and the activin-binding protein follistatin. In addition, we observed that the βA-subunit mRNA was expressed more abundantly in the villus cells than in crypt cells in the rat jejunum and that activin A protein was expressed in the upper villus cells, suggesting that expression of activin A is associated with a termination of proliferation, initiation, and/or a maintenance of differentiation of intestinal epithelial cells. It was also shown that gene expression of the βA-subunit of activin was coupled with cellular differentiation in Caco-2 intestinal cells. Furthermore, it was shown that supplementation of activin A into the culture medium suppressed the proliferation and increased the expression of apolipoprotein AIV gene, a differentiation marker of enterocytes, in IEC-6 cells. These results suggest that activin system is involved in the maintenance of homeostasis in the intestinal epithelium.

More recently, Wildi et al. (35) demonstrated that human colorectal cancers overexpressed βA-subunit mRNA of activin A compared with normal colon, and that the overexpression was greatest in stage IV tumors. Because TGF-β, the prototype of the superfamily derived from cancer cells has been thought to exert paracrine effects to promote angiogenesis, suppress cancer-directed immune mechanisms, and alter the extracellular matrix in a manner that enhances cancer spread and metastasis (11), Wildi et al. (35) speculated that activin A, like TGF-β, may act via paracrine mechanism to promote the progression of colorectal cancer.
Dietary fiber has been hypothesized to protect against colorectal cancer, and the following potential mechanisms have been proposed: 1) increased stool bulk (3); 2) binding with potential carcinogens (25, 27); 3) binding with bile acids (23, 29); 4) lowered fecal pH (25, 27) altered colonic microflora (7, 6) fermentation by fecal flora to short-chain fatty acids (SCFAs) (21, 26); and 7) prevention of insulin resistance and hyperinsulinemia (6, 19). SCFAs, especially butyrate, have been reported to inhibit growth and induce differentiation and apoptosis in colonic tumor cell lines (1, 8, 12). In addition, it has been shown that butyrate modifies the expression of specific genes (14, 31, 34), raising the possibility that butyrate may prevent colorectal cancer progression via changing the expression of some genes. In this context, assuming that activin A derived from colorectal cancer cells promotes the progression of the cancer in paracrine manner as suggested by Wildi et al. (35), butyrate may contribute to protection against colorectal cancer by suppressing the expression of activin A in cancer cells. Therefore, this study was carried out to investigate whether butyrate affects the expression of activin A in colon cancer cells.

MATERIALS AND METHODS

Cell culture. HT-29, Caco-2, FHC, and IEC-6 cells were obtained from the American Type Culture Collection. Cells were maintained in a 100-mm plastic dish (Nippon Becton Dickinson; Tokyo, Japan) in a standard culture medium at 37°C in a humidified atmosphere of 5% CO2-95% O2. The standard culture medium for HT-29, Caco-2, and IEC-6 cells consisted of Dulbecco’s modified Eagle’s medium supplemented with 4 mM l-glutamine, 25 mM glucose, 1× nontissue amino acids (from 100× liquid, GIBCO-BRL; Tokyo, Japan), 100,000 IU penicillin, 100 mg/l streptomycin, 50 mg/l gentamycin, and varying concentrations of FBS. HT-29 and Caco-2 cells were cultured in the presence of 10% and 20% FBS, respectively, whereas IEC-6 cells were cultured in the presence of 5% FBS and 5 mg/l insulin for optimal growth. For FHC cells, the medium consisted of 1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium supplemented with 10 mg/l cholera toxin, 5 mg/l insulin, 5 mg/l transferrin, 100 mg/l hydrocortisone, and 10% FBS. Media were replaced every 2 days or every day, depending on harvest times and degree of confluence.

To examine the effect of sodium butyrate on the expression of activin A gene in HT-29, FHC, and IEC-6 cells, the preconfluent cells were plated onto six-well plastic plates (Nippon Becton Dickinson). In the case of Caco-2 cells, which have been known to differentiate spontaneously after reaching confluence (9), to compare the effect of sodium butyrate on gene expression in undifferentiated and differentiated cells, preconfluent and 14 days-postconfluent cells cultured in 23.4-mm cell culture inserts (pore size: 0.45 μm, Nippon Becton Dickinson) were used for the experiment. The cells were cultured in the standard medium supplemented with sodium butyrate. In the dose-response experiment, the cells were cultured in the medium supplemented with different concentrations (0, 0.1, 0.5, 1, 2, 5, and 5 mM) of sodium butyrate (Wako Pure Chemical; Osaka, Japan) for 6 h. In the time-course experiment, the cells were cultured in the medium supplemented with 5 mM of sodium butyrate for 0, 0.5, 1, 3, 6, 12, and 24 h. Caco-2 cells were cultured for up to 72 h. In addition, HT-29 cells were cultured in the standard medium supplemented with 0.3 μM trichostatin A (TSA; Wako), a histone deacetylase inhibitor, for 0, 0.5, 1, 3, 6, 12, and 24 h. The cells were then harvested for isolation of RNA.

In separate experiments, preconfluent HT-29 cells were cultured in the standard medium supplemented with different concentrations (0, 0.1, 0.5, 1, 2, and 5 mM) of sodium butyrate or 100 ng/ml of recombinant human activin A (Genzyme) for 48 h. Cells were harvested by trypsinization, washed with PBS, then fixed in 70% ethanol, and stored at −20°C for flow cytometric analysis of cell cycle. Cells were also subjected to analysis for DNA fragmentation.

Explant colonic organ culture. Male Wistar rats (Japan SLC; Hamamatsu, Japan) were housed in individual cages in a temperature-controlled (23 ± 2°C) room with a dark period from 7 PM to 5 AM. They were allowed free access to water and to a purified diet prepared according to AIN-93G (24). After undergoing an overnight fast, the rats weighing ~200 g were anesthetized by an intraperitoneal injection of mixed solution of ketamine hydrochloride (70 mg/kg body wt; Wako) and xylazine hydrochloride (8 mg/kg body wt; ICN Biomedicals). After a laparotomy was performed, the rats were euthanized by an aortic cut. The large intestine was opened longitudinally, and the luminal contents were thoroughly washed with ice-cold saline. Six pieces of explant (2.5 mm in diameter) were excised from one animal and transferred into a six-well culture plate (6 pieces/well) containing RPMI-1640 medium supplemented with 10% FBS, 100,000 UI/l penicillin, 100 mg/l streptomycin, and 50 mg/l gentamycin. Explants were incubated with or without 5 mM sodium butyrate for 0, 1, 2, 3, 4.5, 6, and 18 h at 37°C in a humidified atmosphere of 5% CO2-95% O2. Explants were then subjected to isolation of RNA.

This study was approved by the Hokkaido University Animal Use Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Isolation and analysis of RNA. Total RNA was isolated from harvested cells and colonic explants using Isogen (Nippon Gene; Tokyo, Japan) according to the manufacturer’s protocol. Total RNA samples were treated with DNase RQ1 (Promega) to remove any genomic DNA. The samples were subjected to RT-PCR, as previously described (28). For semiquantitative PCR, the kinetics of amplification was studied for each combination of primers in preliminary experiments, and PCR was performed at an exponential range. Two microliters of each PCR product were spotted onto a nylon membrane (Biodyne Plus; Pall, NY), followed by fixation with UV cross linking, and the blots were then hybridized with each inner oligonucleotide probe labeled with digoxigenin with the use of a DIG oligonucleotide tailing kit (Roche Diagnostics; Tokyo, Japan). Prehybridization, hybridization, and detection were all carried out with a luminescence detection kit (Roche Diagnostics), as previously described (28). The signals developed on X-ray film were quantitated with the use of NIH Image software, and the signal intensity was normalized by comparison with the intensity of GAPDH. The sequences of PCR primers and oligoprobes were previously described (28).

Flow cytometric analysis of cell cycle. After the removal of ethanol by centrifugation, cells were then incubated with PBS containing 0.25 mg/ml of RNase A (Sigma; St. Louis, MO) at 37°C for 30 min. Cells were then stained with propidium iodide (PI) (50 μg/ml at final concentration; Wako) at 4°C for 5 min. Stained nuclei were analyzed for DNA-PI fluorescence using Epics XL flow cytometer (Beckman Coulter; Tokyo, Japan). Resulting DNA distributions were analyzed by MacCycle AV (Phoenix Flow Systems) for the

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proportion of cells in apoptotic, G1, S, and G2-M phases of the cell cycle.

Analysis of DNA fragmentation. Cells were harvested by trypsinization and washed twice with PBS. Cells were then lysed in 100 µl of the lysis buffer containing 10 mM Tris-HCl buffer (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100, and were kept on ice for 10 min. The samples were then centrifuged at 12,000 g for 20 min at 4°C. After centrifugation, the supernatant was incubated with 40 µg RNase A (Wako) at 37°C for 1 h, followed by a further 1-h incubation at 37°C with 40 µg proteinase K (Wako). DNA was then precipitated with 20 µl of 5 M NaCl and 120 µl of 2-propanol, and kept overnight at −20°C. After centrifugation for 15 min, DNA was separated by electrophoresis using 2% agarose gel and visualized by ultraviolet illumination after SYBR gold (Molecular Probes; Eugene, OR) staining.

Statistical analysis. Results were expressed as means ± SE. To compare the mean values, unpaired t-test was applied. The statistical calculations were carried out using StatView version 5.0 computer software (SAS Institute). Differences were considered significant if P < 0.05.

RESULTS

Semi-quantitative RT-PCR showed that the mRNA levels of βA-subunit of activin A in human colon cancer cell line HT-29 were significantly increased by cultivation in the presence of 0.5, 1, 2, and 5 mM sodium butyrate for 6 h in a concentration-dependent manner (Fig. 1A). However, the levels of mRNA encoding TGF-β1, the prototype of the superfamily, were unchanged. A time-course experiment in HT-29 cells showed that activin A mRNA levels began increasing significantly at 3 h after the addition of 5 mM sodium butyrate, and continued increasing to 24 h, whereas the mRNA levels in the cells cultured without sodium butyrate were unchanged throughout the culture period (Fig. 1B). Although activin A mRNA levels were also increased at 6 and 12 h after adding TSA, the levels were decreased to control level at 24 h. The relatively smaller effect of TSA on activin A mRNA levels may be dependent of its shorter half life.

Figure 2A showed the time course of changes in activin A mRNA levels in human colon cancer cell line Caco-2 cultured in the presence of 5 mM sodium butyrate. The mRNA encoding apolipoprotein AIV were also investigated as an enterocyte differentiation marker in Caco-2 cells (Fig. 2B). The mRNA levels of apolipoprotein AIV were significantly higher in postconfluent cells than in preconfluent cells at 0 h (i.e., before sodium butyrate was added), indicating the enterocytic differentiation of Caco-2 cells after reaching confluence (data not shown). Activin A mRNA levels were significantly increased at 24 and 72 h after the addition of 5 mM sodium butyrate in preconfluent (undifferentiated) Caco-2 cells (Fig. 2A). Similarly, postconfluent (differentiated) Caco-2 cells responded to sodium butyrate with increased activin A mRNA. In preconfluent Caco-2 cells, apolipoprotein AIV mRNA levels continued increasing to 72 h after adding 5 mM sodium butyrate, whereas postconfluent cells did not respond to sodium butyrate throughout the culture period (Fig. 2B).

To examine whether normal cells respond to butyrate with increased expression of activin A gene, normal cell lines (FHC and IEC-6) and explant of rat colon were cultured in the presence or absence of 5 mM sodium butyrate for 24 h. Activin A mRNA levels in fetal human colonic cell line FHC and rat intestinal cell line IEC-6 were not significantly changed throughout the culture period, regardless of treatment of sodium butyrate, whereas the mRNA levels in butyrate-treated FHC cells tended to be higher than those in the control cells (Fig. 3, A and B). In explants of rat colon, activin A mRNA levels began increasing significantly at 3 h, achieved a maximum by 6 h, and remained stable up to 18 h after cultivation was started in both control and butyrate-treated explants (Fig. 3C). However, there was no significant difference between control and butyrate-treated explants at all time points.

The effect of different concentrations of sodium butyrate and exogenous activin A on cell cycle of HT-29 cells was evaluated via flow cytometry, and the cell cycle phase distribution is summarized in Fig. 4A. Treatment with 0.1–1 mM sodium butyrate for 48 h induced accumulation of cells in G1 phase with a decrease in the percentage of cells in S phase and G2/M phase relative to controls. In addition, higher concen-
Concentrations (~0.5–5 mM) of butyrate induced accumulation of apoptotic cells in a concentration-dependent manner. The butyrate-induced apoptosis was further confirmed by a demonstration of the DNA fragmentation (Fig. 4B). DNA fragmentation shown by DNA ladder on agarose gel electrophoresis is considered to be a hallmark of cell apoptosis. As shown in Fig. 4B, agarose gel electrophoresis clearly demonstrated the typical DNA ladders in butyrate-treated HT-29 cells in a concentration-dependent manner. However, treatment of HT-29 cells with exogenous activin A had no effect on cell cycle phase distribution and induction of apoptosis.

**DISCUSSION**

Our previous study suggested that activin A plays a role in the regulation of cell renewal process, i.e., cell proliferation and differentiation, in intestinal epithelium (28). Theoretically, it would be possible that activin A pathway is involved in the colorectal carcinogenesis because disregulation of the renewal process in the epithelium is pathological of colorectal cancer. Actually, the mutation of intracellular signaling proteins in TGF-β/activin A pathway is involved in colorectal carcinogenesis. It has been reported that 16% of primary colorectal tumors harbored alterations of smad4 (30), and that missense mutations of smad2 were identified in 6% of cases (5). In addition, smad3 mutant mice uniformly develop metastatic colorectal cancer (36). Recently, Wildi et al. (35) reported that activin A was overexpressed in human colorectal tumors, especially in stage IV cancer. The authors speculated that, like TGF-β, activin A may promote colorectal cancer spread as a paracrine factor (35). Thus activin A may be associated with the development of colorectal cancer through not only impairment of its signal transduction in cancer cells but also paracrine effects on ambient cells.

Dietary fiber is one of several factors whose role in colorectal carcinogenesis has been extensively studied. SCFAs, especially butyrate, produced by fermentation of dietary fiber by colonic bacteria have been thought to
Contrary to our expectancy, the present study clearly demonstrated that butyrate upregulates the expression of activin A gene in colorectal cancer cells. In colon cancer cell line HT-29, physiological concentrations of butyrate stimulated the expression of activin A gene in concentration- and time-dependent manner. The other colon cancer cell line Caco-2 also responded to 5 mM butyrate with significant increase of activin A mRNA. Therefore, butyrate may rather contribute to cancer spread via upregulation of activin A in some circumstances.

One may point out that the observed increase in activin A mRNA in colon cancer cell lines would merely reflect the cellular differentiation by butyrate. Actually, butyrate has been shown to stimulate the differentiation of Caco-2 cells (4), and the present study showed that butyrate stimulated the expression of apolipoprotein AIV gene, an enterocytic differentiation marker, in undifferentiated preconfluent Caco-2 cells. In addition, our previous study showed that the expression of activin A gene was upregulated after reaching confluence in Caco-2 cells grown on filter, suggesting the relationship between activin A and cell differentiation in intestinal epithelium (28). In the present study, activin A mRNA levels in 14 days postconfluent Caco-2 cells were significantly higher than those in the preconfluent cells in the absence of butyrate (data not shown). In the postconfluent cells, however, butyrate no longer stimulated the expression of apolipoprotein AIV gene while activin A gene was still upregulated by butyrate. Thus the findings suggest that upregulation of activin A gene by butyrate does not merely reflect the differentiation.

Butyrate has been shown to act as a histone deacetylase inhibitor, which regulates expression of specific genes (31, 34). To examine whether upregulation of activin A gene by butyrate is due to inhibition of histone deacetylase, in the present study, we used TSA, a specific histone deacetylase inhibitor. The results indicated that TSA also stimulated activin A gene whereas the effect was smaller than butyrate. Thus it is suggested that upregulation of activin A gene by butyrate in colon cancer cells is due to the enhancement of transcription via histone hyperacetylation.

To investigate whether upregulation of activin A gene by butyrate is specific to cancer cells, human normal colonic cell line FHC, rat normal intestinal cell line IEC-6, and rat colon explant were cultured in the presence or absence of butyrate. In these normal cells, activin A mRNA levels were unchanged by butyrate. The results suggest that upregulation of activin A gene by butyrate in colon cancer cells is due to the enhancement of transcription via histone hyperacetylation.

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A mRNA in the explant may reflect the upregulation of activin A gene in the repair process.

A series of studies (1, 8, 12) have shown that butyrate-induced cell cycle arrest, apoptosis, and differentiation in colon cancer cell lines, and these effects of butyrate have been regarded as an important mechanism by which dietary fiber protects against colorectal cancer. In the present study, we observed the induction of G1 arrest and apoptosis in HT-29 cells by supplementation with butyrate but not activin A. In addition, our preliminary experiments showed that DNA synthesis evaluated by incorporation of bromodeoxyuridine was significantly suppressed in nontransformed IEC-6 cells but not HT-29 cells by treatment with 100 ng/ml activin A (K. Sonoyama, S. Rutatip, and T. Kasai, unpublished data). Thus the findings suggest that activin A could not act as an autocrine factor in colon cancer cells, even though its expression is increased by butyrate. In other words, activin A does not seem to mediate the growth suppressing effect of butyrate in colon cancer cells. In general, it has been known that cancer cells are resistant to growth-suppressing effect of TGF-β because of the mutation of type II TGF-β receptor (16), smad2 (30), and smad4 (24). Our preliminary experiment showed that exogenous activin A and TGF-β1 induced phosphorylation of smad2 but not complex formation of phosphorylated smad2 with smad4 in HT-29 cells (K. Sonoyama, P. Pholnukulkit, and T. Kasai, unpublished data). The observations suggest that unresponsiveness of HT-29 cells to activin A is due to the mutation of smad4.

In conclusion, assuming that activin A is a paracrine factor to promote the progression of colorectal cancer as suggested by Wildi et al. (35), the present study suggests that butyrate could act as a tumor promoter. On the other hand, butyrate also suppressed growth and induced apoptosis in colon cancer cells. Thus it may be dependent of such conditions as the phenotype of cancer cells and circumstances around cancer cells whether butyrate promotes or suppresses cancer growth.

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REFERENCES


