Dose-Dependent Stimulatory and Inhibitory Effects of Luminal and Serosal n-Butyric Acid on Epithelial Cell Proliferation of Pig Distal Colonic Mucosa

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Summary Large bowel bacteria convert various carbohydrates into short-chain fatty acids (SCFA). SCFA stimulate epithelial cell proliferation of the large intestine in vivo and inhibit that of various cells in vitro. Supposing that too high concentration of SCFA on the serosal side is responsible for their inhibitory effect in vitro, we studied effects of luminal and serosal n-butyric acid (0, 0.1, 1, or 10 mmol/L, adjusted to neutral pH) on the epithelial cell proliferation rate of pig colonic mucosa in organ culture taking crypt cell production rate (CCPR) as the measure of proliferative activity. With 0 or 0.1 mmol/L n-butyric acid on the serosal side, luminal n-butyric acid increased CCPR at 1.0 mmol/L, and decreased CCPR at 10 mmol/L when compared to the luminal 0 mmol/L control. With 1.0 or 10 mmol/L serosal n-butyric acid, luminal n-butyric acid depressed CCPR dose-dependently. The above results indicated that n-butyric acid stimulated colonic epithelial cell proliferation at low concentration and inhibit it at high concentration with interaction effect to enhance the inhibitory action. The stimulatory effect of a low dose of serosal n-butyric acid may be responsible for the distant trophic effect of SCFA.

Key Words n-butyric acid, colon, cell division, pig, organ culture

Epithelial cell number is one of the most essential determinants of functions of gut epithelium such as absorption, secretion, metabolism and barrier function. Failure in maintaining the normal level of epithelial cell proliferation can lead to the ulceration of the mucosa, bacterial translocation and cancer formation. Thus, the regulation of gut epithelial cell proliferation is important.

Many indigestible and fermentable carbohydrates such as pectin (1), guar gum (2), or fructooligosaccharides (3) stimulate epithelial cell proliferation and increase epithelial cell number. Bacterial fermentation of these carbohydrates produces short-chain fatty acids (SCFA) such as acetic, propionic, n-butyric and n-valeric acids (4). Administration of a mixture of SCFA or of individual SCFA either into the lumen of the stomach (5), cecum (6, 7), colon (8) or rectum (5), or into the peripheral vein (9) stimulates large intestinal as well as small intestinal epithelial cell (10) proliferation in vivo. Therefore, SCFA should be responsible for the trophic effects of various indigestible and fermentable carbohydrates in vivo (11).

On the other hand, SCFA inhibit the proliferation of various cells in culture (12) both by G1- and G2-arrest (13). This is different from the above in vivo findings. This discrepancy, sometimes called the “butyrate paradox” (14), has been the target of debate. One possible explanation to sublate the butyrate paradox is the presence of neural and humoral systemic transmission of the signal of SCFA recepted locally at the cecum or colon, which is stronger than the local inhibitory effect of SCFA. This is really the case for ruminal epithelium, a classic model for the trophic effect of luminal dietary factors (15). In these animals ruminal administration of propionic or n-butyric acid stimulates insulin release. Insulin, at the blood level after SCFA administration, stimulates ruminal epithelial cell proliferation even in the presence of n-butyric acid (16). However, it is not likely that the physiological level of SCFA stimulates insulin secretion in humans or non-ruminant animals such as rats or pigs (17, 18). The humoral mediation does not explain the trophic effect of intra-venous SCFA administration (8) either.

Interestingly, most in vitro studies used the same concentration of SCFA (1 to 10 mmol/L) for luminal and serosal sides of cells, which cannot be the case in vivo. Consumption of SCFA, especially of n-butyric acid, by epithelial cells (19) and blood flow should remove SCFA to make their concentration in the lamina propria of the mucosa far lower than that in the lumen bulk phase. Thus, there should be a concentration gradient between the luminal and baso-lateral sides of epithelial cells.

The dose of SCFA used for in vitro studies may be arguable. The intact lumen contents of the large intestine are more than 500 times as viscous as their liquid phase (20). Physically, high viscosity retards the diffusion of solutes in the fluid. On the other hand, absorption of SCFA is rapid (21). Therefore, the SCFA concen-
tion at the mucosal surface should not be as high as that in the lumen bulk phase. Further, we should recall that proliferative cells are sitting at the bottom one third of the crypt in the large intestine. Thus, SCFA should travel to this zone against the absorption and metabolism by epithelial cells at the flat surface as well as in the upper part of the crypt and against the bottom-to-surface flow of mucus. Therefore, SCFA concentration at the luminal side of proliferating epithelial cells in the crypt bottom should be far lower than that in the bulk phase.

Accordingly, the purpose of the present study was to learn the influence of luminal and serosal n-butyric acid on colonic epithelial cell proliferation using an organ culture system that enabled us to set different concentrations for luminal and serosal side of the mucosa. Since we do not know the actual n-butyric acid concentration around crypt bottom cells, we took a dose-response approach as an alternative.

**MATERIALS AND METHODS**

**Experimental design.** We adopted a two-way factorial randomized bloc design with a replication of 4. One factor was the concentration of n-butyric acid on the luminal side and another factor was that on the serosal side.

**Organ culture.** We took a segment approximately 30 cm long from the distal colon of a castrated male pig of approximately 70 kg body mass at the Miyagi Prefectural Meat Inspection Center, Yoneyama, Miyagi, Japan. We opened the segment longitudinally at the antimesenteric side, rinsed it in phosphate buffered saline (PBS; KCl 2.7 mmol/L, KH₂PO₄ 1.5 mmol/L, Na₂HPO₄ 8.1 mmol/L, NaCl 136.6 mmol/L) at 4°C and transferred it into PBS supplemented with 50 IU/mL penicillin and 50 μg/mL streptomycin (Cosmo Bio, Japan) and 50 μg/mL gentamicin sulfate (Wako Pure Chemical Industries, Ltd., Japan) at 4°C. Then, we transported the tissue to our laboratory within 1 h.

We rinsed the tissue in PBS and blotted it on filter paper to remove the lumen contents. Thereafter, we removed the *muscularis externa* and most of the submucosa, and cut the mucosa into approximately 10×10 mm sheets in PBS at 4°C. We mounted these mucosal sheets on a 1.5 mL plastic sample tube with its tip removed (Fig. 1). The luminal side of the mucosa faced the lumen of the sample tube. We poured 1 mL PBS containing 0, 0.1, 1, or 10 mmol/L n-butyric acid into the tube (luminal solution). The pH of the luminal solution was adjusted to 7.4. Then, we immersed every 2 tubes in a petri dish that contained 10 mL of F-12 medium (Invitrogen Corp., USA) with antibiotics (50 U/mL penicillin, 50 μg/mL streptomycin and 50 μg/mL gentamicin sulfate) containing 0, 0.1, 1, or 10 mmol/L n-butyric acid at pH 7.4 (serosal solution) and cultured the tissue at 37°C. The gas phase of the culture consisted of 5% CO₂ and 95% O₂ (v/v). Petri dishes were shaken at 10 r.p.m. using a shaker (Mild Mixer PR-24, Taitec, Japan). Such incubations were replicated four times using four different donor pigs.

**Measurement of CCPR.** We employed crypt cell production rate (CCPR) (6) as the measure of epithelial cell proliferation. After 7 h of incubation we harvested 1 mucosal sheet from each dish and added vincristine sulfate (Wako, final 1 μg/mL) to the serosal solution in order to arrest proliferating epithelial cells at the metaphase of mitosis. The other mucosal sheet was cultured for a further 2 h to be harvested at 9 h of culture to measure the CCPR between 7 and 9 h of culture. This time-window was based on our preliminary in vivo study in rats, where intracolonic continuous administration of a mixture of physiologic concentration of short-chain fatty acids stimulated colonic epithelial cell proliferation within 9 h (Inagaki and Sakata, unpublished observation).

The harvested mucosal sheets of the colon were fixed in a mixture of acetic acid and ethyl alcohol (1 : 3, v/v) for at least 3 h. We stained harvested tissues with Feulgen reaction *en bloc*, then dissected colonic crypts from the colonic mucosa, squashed the crypts onto a glass slide, and counted the number of metaphase cells per crypt in randomly selected 20 crypts per specimen. Thus, we obtained 40 measurements of metaphase frequency per crypt for each experimental group in a replication, from which we calculated 40 linear regression equations using 40 datasets for each experimental group in a replication after jackknife re-sampling. The slopes of these equations represented CCPR (n=40 for each experimental group per replication).

**Statistics.** We expressed our results as the mean and pooled standard error of means, i.e. the square root of error mean square. Effects of luminal and serosal n-butyric acid concentration were analyzed by two-way analysis of variance (ANOVA) taking the effect of donor pig as the bloc effect using Stat View 4.0 program (Abacus Concept Inc., Berkeley, CA). Since there was a significant interaction effect, we conducted Tukey’s post hoc comparison among each experimental group using the error mean square of the preliminary ANOVA. Difference between means was considered significant at *p*<0.05.
Table 1. Crypt cell production rate of pig distal colonic mucosa exposed to various luminal and serosal n-butyric acid concentrations in organ culture: mean (SD), n=40 (after Jackknife re-sampling), pooled SE=1.45.

| Serosal concentration (mm) | Luminal concentration (mm) | Crypt cell production rate (cells/crypt/h) | Error probability of 2-way analysis of variance |
|----------------------------|---------------------------|------------------------------------------|-----------------------------------------------|
|                            | 0.0                       | 6.7a (0.6)                               | <0.001                                        |
|                            | 0.1                       | 10.4a (0.7)                              | <0.001                                        |
|                            | 1.0                       | 7.0a (1.1)                               | <0.001                                        |
|                            | 10                        | 3.6a (1.3)                               | <0.001                                        |

Factors: Luminal butyrate concentration (X) = 0.0 0.1 1 10
Serosal butyrate concentration (Y) = 0.0 0.1 1 10
Interaction (X×Y) = 0.0 0.1 1 10

Means not sharing a common superscript differ significantly by Turkey’s post-hoc two-way comparison (p<0.05).

RESULTS

The effect of two-way interaction was significant (Table 1) suggesting that the effect of mucosal n-butyric acid concentration vary among different serosal n-butyric acid concentrations and vice versa.

In the presence of no or physiologic concentration of n-butyric acid (0.0 or 0.1 mmol/L) at the serosal side, luminal n-butyric acid had no effect on CCPR at 0.1 mmol/L, increased CCPR at 1.0 mmol/L, and decreased CCPR at 10 mmol/L when compared to luminal 0.0 mmol/L control. In the presence of 1.0 or 10 mmol/L n-butyric acid at the serosal side, luminal n-butyric acid depressed CCPR in a dose-dependent manner.

If we look at the same set of data differently, serosal n-butyric acid increased CCPR at 0.1 mmol/L and decreased CCPR at higher concentrations when the luminal n-butyric acid concentration was 0.0 to 1.0 mmol/L, and serosal n-butyric acid decreased CCPR in a dose-dependent manner when the luminal concentration was 10 mmol/L.

DISCUSSION

The above results indicated the effect of n-butyric acid on epithelial cell proliferation of the colon was complex. Effects of luminal and serosal n-butyric acid depended on each other.

It is astonishing that the effect of n-butyric acid on colonic epithelial cell proliferation was opposite at different concentrations, stimulatory at lower concentration and inhibitory at higher concentration. Our present results agree with a previous in vitro study on the effect of n-butyric acid on intestinal smooth muscle cell proliferation (22), which also demonstrated a stimulatory effect at low concentration and inhibitory effect at higher concentration. Further, the trophic effect of low dose n-butyric acid (up to approximately 200%) in the present study agrees with that observed in in vivo studies (6, 7). The trophic effect of short-chain fatty acids in these in vivo studies accompanied increased tissue mass. Therefore, the amplitude of the trophic effect in the present study (Table 1) should have physiologic significance. The dose-dependent difference in the effect and the above interaction effect should be the key to solve the discrepancy between in vivo and in vitro studies.

Methodologically, there is a theoretical possibility that n-butyric acid on one side was transferred to another side. However, our preliminary experiments (data not shown) demonstrated such a transfer did not occur, at least in concentration gradient used in the present study. That preliminary experiment also indicated that the conversion of n-butyric acid either in luminal or serosal solution did not occur at a detectable level. On the other hand, the concentration of n-butyric acid decreased from 10 to 7 mmol/L, though without detectable appearance in serosal solution, during the incubation for 9 h in that preliminary study. This might have been due to the consumption of this acid by the mucosal tissue as observed in previous studies (19). However, such a fluctuation in the concentration was smaller than the difference in the dose among experimental groups in the present study and should not skew the essential conclusion of the present study.

Most in vitro studies used serosal n-butyric acid concentration above 1 mmol/L level (for reviews (12, 23)). In such a condition, luminal n-butyric acid should show a dose-dependent inhibitory effect on cell proliferation as we observed in the present study. However, it is not likely that such a high concentration of n-butyric acid exists in the lamina propria surrounding crypt bottom cells. A considerable part of n-butyric acid is consumed by epithelial cells (19), very likely at the flat surface and in the upper part of the crypt. The blood flow in the mucosa should be good, because SCFA stimulate mucosal blood flow (24). Thus, there should be little n-butyric acid left in the lamina propria during the passage of “surviving” n-butyric acid from the epithelium to the capillary. Therefore, it may be sound to consider that the n-butyric acid concentration in the lamina propria is not different from that in the portal blood. Accordingly, the results of previous in vitro studies employing too high a serosal n-butyric acid concentration should be
difficult to extrapolate to in vivo conditions.

On the other hand, the inhibitory effect of luminal 10 mmol/L n-butyric acid in the present study shows apparent discrepancy with previous in vivo studies (5–10). However, we should remember that most in vivo studies used animals, which had luminal contents in their large bowel. Our recent studies demonstrated that the luminal contents of the small and large bowel are highly viscous in pigs (20, 25) rats (26) and chickens (20). High viscosity retards the rate of diffusion in the fluid. Considering the rapid absorption of SCFA from the colonic mucosa together with the possible slow diffusion of these acids across the luminal contents, it is likely that the concentration of these acids at the very surface of the mucosa is far lower than the average concentration in the bulk phase. Actually, a direct measurement of SCFA in the core and peripheral contents of rat cecum and colon showed that the peripheral concentration was significantly lower than core concentration (27). Further, our previous study demonstrated that the lumen concentration after the continuous infusion of a physiologic mixture of SCFA (total 150 mmol/L, 33 mL/d) into rat cecum was less than 5 mmol/L (7). Thus, it is reasonable to consider that the actual n-butyric acid concentration at the very surface of the mucosa in most in vivo studies was by far lower than that in the administered experimental solution.

The stimulatory effect of serosal n-butyric acid on cell proliferation observed in the present study (Table 1) may explain the mechanism via which the effect of luminal n-butyric acid is transmitted to the jejunal epithelium to stimulate its cell proliferation in vivo. One of the authors (T.S.) considered that such a distant trophic effect of n-butyric acid or other SCFA administered into the large intestine is mediated by a systemic mechanism such as neural or humoral mediation (10, 28). However, the present results indicated that just a low concentration of SCFA due to the absorption of these acids from the large bowel is able to stimulate the cell proliferation of diverse tissue such as the jejunal epithelium (10) or colonic smooth muscle cells (22). The trophic effect of the low concentration of serosal n-butyric acid demonstrated in the present study explains the stimulatory effect of colonically infused SCFA on epithelial cells of isolated and denervated loop of the jejunum (10). In this regard, it is very important to test if low concentration of SCFA as found in the arterial blood after the consumption of indigestible and fermentable carbohydrates stimulates or inhibits cell proliferation of various tissues, or not. This should help to assess the risk of these food components for cancer promotion or prevention.

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