Lithocholic acid and sulphated lithocholic acid differ in the ability to promote matrix metalloproteinase secretion in the human colon cancer cell line CaCo-2

Bente HALVORSEN*, Anne Cathrine STAFF*, Solveig LIGAARDEN*, Kristian PRYDZ† and Svein Olav KOLSET*¹

*Institute for Nutrition Research, P.O. Box 1046, Blindern, University of Oslo, 0316 Oslo, Norway, and †Department of Biochemistry, P.O. Box 1041, Blindern, University of Oslo, 0316 Oslo, Norway

The human colon carcinoma cell line CaCo-2 has the ability to sulphate the secondary bile acid lithocholic acid (LA), whereas other primary or secondary bile acids were not sulphated [Halvorsen, Kase, Prydz, Gharagozian, Andresen and Kolset (1999) *Biochem. J.* **343**, 533–539]. To study the biological implications of this modification, CaCo-2 cells were incubated with either LA or sulphated lithocholic acid (3-sulpholithocholic acid, SLA), and in some experiments with taurine-conjugated lithocholic acid. Increased secretion of matrix metalloproteinases (MMPs) correlates with transformation of colon epithelial cells. When CaCo-2 cells were incubated with LA, the secretion of MMP-2 was found to increase approx. 60 % when analysed by gelatin zymography, and 80 % when analysed by Western blotting. SLA, in contrast, did not affect the level of MMP-2 secretion, and after zymography the level of enzyme activity was

78 % of control values after 18 h incubation. The secretion of MMPs is linked to increased cellular invasion and, in tumours, to increased capacity for metastasis. The ability of CaCo-2 cells to invade in a chamber assay was stimulated after exposure to LA, whereas SLA-treated cells did not differ from control cells. LA therefore seems to induce a more invasive CaCo-2 cell phenotype, as judged by the two parameters tested, whereas the sulphated counterpart, SLA, did not have these effects. Sulphation of LA in the colon may be an important mechanism to decrease the potential LA has to promote a malignant epithelial phenotype.

Key words: conjugated bile acids, intestine, invasion, metastasis, sulphation.

INTRODUCTION

Absorption of dietary fat is dependent upon the secretion of bile acids from the liver. Primary bile acids are generally conjugated with taurine or glycine. Secondary bile acids, however, are formed in the large intestine as a result of bacterial dehydroxylation. The major portion of intestinal bile acids, primary and secondary, is reabsorbed through the entero-hepatic circulation. Only minor amounts of secondary bile acids escape the body through faeces elimination. Elevated levels of faecal secondary bile acids have, however, been correlated to a higher incidence of colorectal cancer [1]. The biological effects of secondary bile acids in relation to the development of colon cancer have not been defined in any great detail.

Lithocholic acid (LA) has been shown to be the most cytotoxic bile acid towards colon cells [2]. We have recently demonstrated that the secondary bile acid, LA, could be sulphated by cultured human intestinal CaCo-2 cells, whereas the secondary bile acid, deoxycholic acid, and the primary bile acids, cholic acid and chenodeoxycholic acid, were not [3]. This finding suggests that sulphation of LA by colon epithelial cells could be a protective mechanism, probably to promote increased water solubility and decreased cytotoxicity.

Several members of the matrix metalloproteinase (MMP) family are expressed at higher levels in gastrointestinal cancers than in normal tissues and benign adenomas. The most common

MMPs found in colorectal or gastrointestinal cancers are gelatinase A (MMP-2), gelatinase B (MMP-9) and matrilysin (MMP-7) [4]. MMPs are important enzymes in normal processes such as development, tissue repair, wound healing and extravasation of immune cells. In malignancy, such enzymes become important for tumour cells to remodel their local environment, to ensure nutritional support through access to blood supply, and also to provide escape routes to the circulatory system for metastasis to other organs. In the latter process, the ability to migrate through matrix barriers is an important cellular property.

In the present paper we analyse the effects of exposing CaCo-2 cells to LA, 3-sulpholithocholic acid (SLA) and, in some cases, to the taurine-conjugated LA (tauroolithocholic acid, TLA). We reasoned that it would be biologically relevant to test these bile acids on two parameters important to colon cancer cells: the ability to secrete MMPs, and the ability to migrate through barriers. Results presented show that SLA and LA differ in their ability to affect both parameters, suggesting that sulphation may have implications for regulating the effects of LA on human colon cells.

MATERIALS AND METHODS

Chemicals

LA, SLA, TLA and insulin were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM), non-essential

Abbreviations used: TLA, tauroolithocholic acid; LA, lithocholic acid; SLA, 3-sulpholithocholic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MMP, matrix metalloproteinase.

¹ To whom correspondence should be addressed (e-mail s.o.kolset@basalmed.uio.no).

amino acids, penicillin, streptomycin, and fetal calf serum (FCS) were all obtained from BioWhittaker (Walkersville, MD, U.S.A.). Sulphate-free RPMI 1640 culture medium was from Gibco BRL, and trypsin was purchased from Difco (Detroit, MI, U.S.A.). Tissue culture dishes and flasks were supplied by Costar. [35 S]Sulphate and [3 H]leucine were from Amersham International. [3 H]Thymidine and the enhanced chemiluminescence system were purchased from DuPont New England Nuclear (Boston, MA, U.S.A.). Sephadex G-50 Fine was from Amersham Pharmacia Biotech. [14 C]LA was obtained from Amersham International, and [14 C]SLA was obtained by HPLC of conditioned medium from CaCo-2 cells incubated with [14 C]LA (described in [3]). The chromogenic peptide substrate S-2288 (*H*-D-Ile-Pro-Arg-*p*-nitroanilide) was from Chromogenix AB, Mölndal, Sweden.

Cell cultures

The CaCo-2 human intestinal cell line was obtained from A.T.C.C. These cells were chosen as a model system for studying the effects and metabolism of bile acids on human intestinal cells. The cell line was maintained in DMEM medium containing 20 % FCS, 2 mM L-glutamine, insulin (10 μ g/ml), penicillin (50 units/ml), streptomycin (50 μ g/ml) and 1 % non-essential amino acids. For subculture, the cell medium was removed and the cells were detached with 0.25 % trypsin (in PBS containing 0.2 g/l EDTA). FCS was added to terminate trypsin treatment, and the cells were re-seeded at approx. 40 000/cm² in new flasks. Before starting experiments, 500 000 cells were seeded per well (35-mm diameter) and grown to 90 % confluence.

Bile acids

The sodium salts of LA, SLA and TLA were all solubilized in DMEM/ethanol (1:1, v/v). During experiments, control cells were exposed to the same concentration of ethanol (0.5 %) as cells treated with bile acids.

Proliferation and protein synthesis

The viability of CaCo-2 cells exposed to increasing concentrations of LA, SLA, and TLA was examined by measuring the incorporation of [3 H]leucine into cell-associated proteins, as previously described [3]. The proliferative capacity was determined by measuring the incorporation of [3 H]thymidine into cell-associated DNA, as has been outlined by Staff et al. [5].

Labelling of cells

Sulphate-depleted RPMI 1640 medium containing 10 % FCS, L-glutamine and non-essential amino acids was used during [35 S]sulphate-labelling experiments. Before labelling, the cells were washed once with sulphate-depleted RPMI 1640. Thereafter, labelling medium with [35 S]sulphate (200 μ Ci/ml) was added to the cells. Concomitantly, the cells were exposed to LA, SLA, or TLA at concentrations from 2 to 26.6 μ M. After labelling, cell and medium fractions were harvested and [35 S]sulphated macromolecules were recovered, as previously described [3].

In some experiments the cells were labelled with [3 H]glucosamine (200 μ Ci/ml) for 24 h with glucose-free DMEM (Gibco BRL), as described elsewhere [6]. The cells were concomitantly exposed to either LA, SLA, or TLA at concentrations from 1 to 10 μ g/ml (2–26 μ M), and after 24 h incubation [3 H]glucosamine-labelled macromolecules were isolated from the cells and the culture medium as described for isolation of [35 S]sulphated macromolecules [3].

Zymography

Serum-free conditioned medium from CaCo-2 cells was collected to analyse gelatinase activity, as described in [5]. Approx. 5×10^5 cells were seeded per 35-mm well and incubated for one or two days to approx. 90 % confluence. The cells were washed once in DMEM and incubated in 1 ml of DMEM for 9 or 18 h with all additions except serum, but also containing either LA (26.6 μ M), SLA (21.9 μ M), or TLA (2 μ M). The conditioned media were harvested and cell debris removed by centrifugation (167 g, 3 min, 4 °C).

Between 60 and 75 μ g of protein from conditioned media was incubated at room temperature for 10 min with non-reducing loading buffer containing 4 % SDS (w/v). The samples were then subjected to electrophoresis at 4 °C on SDS/polyacrylamide gels containing 0.1 % gelatin (w/v) (Novex, San Diego, CA, U.S.A.). High-molecular-mass markers (14.3–200 kDa; Amersham International) were used as standards. A mixture of purified human MMP-2 and human MMP-9 (Chemicon International, Temecula, CA, U.S.A.) was run in a separate lane. The gels were cleared of SDS, developed for 24 h and stained. Thereafter, the gels were scanned with a laser-scanner densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and quantified using Image Quant software.

Western immunoblotting

CaCo-2 cells were incubated in medium supplemented with LA (26.6 μ M), SLA (21.9 μ M), or TLA (2 μ M) for 9 or 18 h. The cells were washed twice in ice-cold PBS (pH 7.2) prior to the addition of lysis buffer, consisting of 150 mM NaCl, 50 mM Tris/HCl (pH 7.6), 1 mM EDTA, 1 % Triton X-100, and protease inhibitor cocktail (Boehringer Mannheim). The samples were frozen at –70 °C until further analysis. Equal amounts of protein were subjected to SDS/10 % PAGE, and electrophoretically transferred to Immobilon PVDF filters (Millipore, Bedford, MA, U.S.A.). Filters were blocked for 1 h at room temperature with 5 % low-fat milk in Tris-buffered saline containing 0.02 % Tween (v/v), followed by incubation at room temperature for 1 h with the monoclonal anti-human MMP-2 (R & D Systems). Proteins were detected by enhanced chemiluminescence using horseradish peroxidase-labelled anti-mouse IgG (Vector Laboratories, Burlingame, CA, U.S.A.). The enhanced-chemiluminescence-exposed films were quantified by laser-scanning densitometry. To verify the presence of MMP-2 protein, another monoclonal human MMP-2 antibody from an alternative supplier (Oncogene, Cambridge, MA, U.S.A.) was also used. The two different antibodies recognized a protein band with the same molecular mass.

Transwell invasion assay

CaCo-2 cells were labelled for 72 h with [3 H]thymidine in medium, as previously described. The cells were trypsin-treated and washed once in DMEM. Cells (100 000 in 250 μ l) were seeded on 8- μ m Transwell filter (24-wells; Costar) in DMEM with additions as described above but without FCS, either in the presence of ethanol (controls), LA (26.6 μ M), or SLA (21.9 μ M) in the upper (apical) medium. The chambers underneath the filters (basolateral) consisted of DMEM with all additions included (500 μ l). After 48 h incubation, both medium fractions were discarded and adherent non-invaded cells on top of the filters were wiped off with cotton swabs. The filter membranes, containing invaded cells, were cut out and analysed for radioactivity in a scintillation counter. The counted radioactivity was regarded as a measure of invaded CaCo-2.

Enzyme assay

Trypsin-like enzyme activities were measured in serum-free conditioned media from CaCo-2 cells cultured in 16-mm wells (Costar), either untreated or exposed to either LA (26.6 μ M) or SLA (21.9 μ M). Media were harvested after 2, 6 and 20 h incubations. Conditioned medium (100 μ l) was added to wells of 96-well microtitre plates. PBS (100 μ l) was added together with 20 μ l of the chromogenic substrate S-2288 (stock concentration 20 mM). The enzyme activity was recorded by using a Titertek Multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland, U.K.). Media from incubations without cells were used for background subtraction. Measurements were carried out in triplicate.

Statistics

Results are presented as means $\pm$ S.E.M. The Mann-Whitney *U*-test was used to test significant differences between groups. The significance level was set at $P < 0.05$.

RESULTS

In order to compare the effects of LA and SLA on CaCo-2 cells, initial experiments were performed to measure *de novo* protein and DNA synthesis. Consequently, cells were labelled with either [3 H]leucine or [3 H]thymidine in the absence and presence of LA, SLA and TLA. The synthesis of protein and DNA was unaffected by exposure to LA (26.6 μ M) and SLA (21.9 μ M). In contrast the taurine-conjugated LA decreased the level of incorporation of both of [3 H]leucine and [3 H]thymidine at concentrations higher than 2.0 μ M (results not shown). TLA was therefore used in 2.0 μ M concentrations in further studies.

The expression level of [35 S]sulphated molecules was stimulated in the presence of LA, but not the other primary or secondary bile acids tested [3]. We wanted to compare the effects of LA, SLA and another modified form of LA (the taurine-conjugated form), on this particular parameter. Both LA (26.6 μ M) and TLA (2.0 μ M) increased the expression level of [35 S]sulphated molecules in the medium fractions, 3 and 1.6 times above control levels respectively (Table 1). No changes were seen in the cell fractions. In contrast, exposing the cells to SLA did not increase

Table 1 Incorporation of [3 H]glucosamine and [35 S]sulphate into macromolecules in CaCo-2 cells

CaCo-2 cells were incubated in the presence of LA (26.6 μ M), SLA (21.9 μ M) or TLA (2.0 μ M) and labelled with [3 H]glucosamine and [35 S]sulphate for 24 h. The cell and medium fractions were harvested, subjected to gel chromatography, and analysed for radioactivity as described in the Materials and Methods section. Data presented are the means $\pm$ S.E.M from three separate cell dishes. *Statistical significant difference from control ($P < 0.05$).

Fraction	[3 H]Glucosamine (c.p.m./100 μ l)	[35 S]Sulphate (c.p.m./100 μ l)
Medium		
Vehicle, control	2345 $\pm$ 86	1269 $\pm$ 49
LA	2257 $\pm$ 85	4038 $\pm$ 260*
SLA	2323 $\pm$ 105	1288 $\pm$ 76
TLA	2188 $\pm$ 59	2019 $\pm$ 61*
Cells		
Vehicle, control	10951 $\pm$ 135	1162 $\pm$ 104
LA	9689 $\pm$ 701	1343 $\pm$ 47
SLA	10536 $\pm$ 193	1382 $\pm$ 54
TLA	11672 $\pm$ 484	1060 $\pm$ 75

Figure 1 Gelatinolytic activity in CaCo-2 medium

After confluence, CaCo-2 cells were incubated in the presence or absence of LA (26.6 μ M), SLA (21.9 μ M), or TLA (2.0 μ M) for 9 h or 18 h in serum-free medium. Control cells were given ethanol (0.5%). The condition medium was harvested and subjected to zymography, as described in the Materials and methods section. The Figure shows a zymogram from one typical experiment. Each zymography was run on conditioned medium pooled from three different cell cultures. The results are calculated as percentage of controls, and data are presented as means of two experiments with ranges given in parentheses.

the level of [35 S]sulphated molecules, either in the medium or the cell fraction. Furthermore, the incorporation of [3 H]glucosamine into glycoconjugated molecules was not affected by any of the bile acids (Table 1). Consequently, we assume that LA and TLA do not affect the expression level of sulphated glycoconjugates. The lack of effect of SLA suggests that the increased level of [35 S]sulphated molecules after treatment with both LA and TLA is due to a sulphation of these bile acids themselves, in accordance with what has been shown for LA [3].

To compare further the effects of LA and SLA on CaCo-2 cells, conditioned media from cells cultured without serum were analysed for the content of gelatinase activity by zymography. From Figure 1 it is evident that considerable gelatinase activity is present in the medium fractions harvested. Two proteins with the ability to degrade gelatin were seen in all the medium fractions. The level of enzyme activity, however, differed to a large extent between the different conditioned media. Cells exposed to LA released 1.7 times more gelatin-degrading activity than control cells after 9 h incubation, whereas cells exposed to TLA released slightly less than control cells. Medium from cells exposed to SLA, however, contained only 50% of the activity found in control medium after 9 h (66% of control in the experiment shown). After 18 h this level was 78% of the control. At this time point, LA treatment gave a 1.6-fold higher gelatinase level than in the control, whereas the effect of TLA showed a great variation with a mean slightly higher than the control. Accordingly, while LA increased gelatinase activity in the medium fractions, SLA had the opposite effect.

In order to identify the gelatinase activity seen in conditioned medium from CaCo-2 cells, the different fractions were subjected to SDS/PAGE followed by Western blotting. The activity seen after zymography in Figure 1 is clearly MMP-2, as can be seen in Figure 2. We could not detect any MMP-9 activity in these fractions with an anti MMP-9 antibody (results not shown). By scanning the blot it was possible to determine the relative level of MMP-2 protein. Figure 2 (lower panel) shows that the level of MMP-2 was significantly higher in medium from LA-treated cells than in cells exposed to SLA. The level of MMP-2 in the

Figure 2 Effects of LA and SLA on MMP-2 expression in CaCo-2 cells

After confluence, CaCo-2 cells were incubated in the presence or absence of LA (26.6 μ M), or SLA (21.9 μ M) for 9 h or 18 h in serum-free medium. Control cells were given ethanol (0.5%). The cells were harvested and lysed as described in the Materials and methods section. Cell protein (20 μ g) was separated on reducing SDS/PAGE, electrotransferred, and incubated with anti-human MMP-2. The results are calculated as percentage of controls. The bar graph shows the means $\pm$ S.E.M. from four separate experiments, and the immunoblot presented is from one typical experiment.

medium from the latter cells was the same as in medium from control cells. From both zymography and Western-blot experiments we can conclude that LA treatment increases the level of MMP-2 in the medium of CaCo-2 cells, whereas the sulphated counterpart, SLA, does not have such an effect. Both in the zymography and Western-blot experiments a 45 kDa form of MMP-2 could be detected, whereas standard MMP-2 migrated as 65 kDa and 72 kDa proteins. In some experiments we could detect small levels of gelatinase activity with a molecular mass of 65 kDa (results not shown). In parallel experiments with the human trophoblastic cell line JAR MMP-2, gelatinolytic activity could only be detected at a molecular mass of 65 kDa [5]. There is evidently large differences in the processing of MMP-2 between different cell types, and the level of the 65 kDa and 45 kDa form may depend on the level of tissue inhibitor of metalloproteinase-2 in the cell type studied, as has been indicated in [7].

The difference between LA and SLA could be due to differences in the cellular uptake. CaCo-2 cells were incubated with either [14 C]LA or [14 C]SLA and the amount of cell-associated radioactivity determined. The level of [14 C]LA associated with the cells increased up to 2 h (1331 $\pm$ 215 c.p.m.), with no further increase at 4 h. During this incubation time the level of [14 C]SLA was low (115 $\pm$ 33 c.p.m.). However, in several experiments we noted that the level of MMP-2 activity was lower in CaCo-2 cells incubated with SLA for 9 h and 18 h, but significantly higher than in controls after 48 h. These results indicate different rates of uptake for LA and SLA, as has been shown in hepatocytes [8]. Furthermore, in this study the uptake of LA was by simple diffusion and the uptake of SLA by Na⁺-independent carrier, which may possibly lead to different intracellular routing and effects, as is indicated from our data.

Plasminogen activator and other serine proteases are important for the modulation of the cellular microenvironment, as are

Figure 3 Effects of LA and SLA on serine protease activities in CaCo-2 cells

CaCo-2 cells were cultured under serum-free conditions in the presence or absence of LA (26.6 μ M), or SLA (21.9 μ M) for 2 h, 6 h and 20 h. Conditioned media were harvested, incubated with the chromogenic substrate S-2288 and the enzyme activity recorded at 405 nm in a Titertek spectrophotometer. The bar graph shows the means $\pm$ S.E.M. from three experiments.

Table 2 Effect of bile acids on the invasion of [3 H]thymidine-labelled CaCo-2 cells

The invasion of [3 H]thymidine-labelled CaCo-2 cells was performed as described in the Materials and methods section in the absence and presence of LA (26.6 μ M) or SLA (21.9 μ M). Results are given as means $\pm$ S.E.M from 11 to 14 separate cell cultures. The absolute values for controls ($n = 11$) were 8649 $\pm$ 2151 c.p.m./filter. * $P < 0.05$ versus control, § $P < 0.05$ versus SLA.

Bile acid	Invasion (% of control)
Control	100.0 $\pm$ 7.2
LA	163.2 $\pm$ 28.3*
SLA	86.6 $\pm$ 7.5 §

MMPs [9]. To investigate the effect of LA and SLA on the secretion of other proteases, serum-free supernatants from CaCo-2 cells were used to determine the level of serine proteases like plasminogen activator (tissue-type) by using the chromogenic substrate, S-2288. LA stimulated the secretion of S-2288-cleaving activity, whereas SLA did not, as can be seen in supernatants from cells incubated for 6 h (Figure 3). After 20 h incubation, cells exposed to LA released 1.8 times more enzyme activity. Interestingly, at this time point cells treated with SLA had a somewhat higher level (14% increase) of activity in the supernatants than control cells.

Increased expression of MMPs is linked to a more migratory or invasive phenotype in many cell systems. In order to investigate a possible relation between the level of MMPs in the medium and the ability to migrate, [3 H]thymidine-labelled CaCo-2 cells were plated in chambers on filters with 8- μ m pores. The bile acids were exposed to the cells in the upper chambers, and medium in the lower chamber contained serum as chemoattractant. Cell invasion was 1.6 times higher in cells exposed to LA compared with control cells (Table 2). Cells treated with SLA had a migratory capacity slightly lower than control cells.

DISCUSSION

The ability to catalyse sulphation reactions is an important property of all mammalian cells. The most highly sulphated macromolecules are the proteoglycans, found both in the extracellular matrix, on cell surfaces and in intracellular granules [10]. Several other glycoconjugates may also be sulphated, including glycoprotein hormones, glycolipids, secretory proteins, selectins and mucins [11–13]. Most of these sulphation reactions take place in the Golgi apparatus, whereas drugs and xenobiotic compounds are sulphated in the cytosol. The sulphation of LA in CaCo-2 cells is also a cytosolic process [3], but the biological function(s) of this bile acid modification has not been defined. It has been shown that LA has toxic effects on the liver [14], and the cytotoxicity of LA in hepatocytes was greatly reduced by either glucuronidation or sulphation of this bile acid [8]. Sulphation in the intestine could therefore reduce the detrimental effects of LA in the entero-hepatic circulation. It has been shown that SLA is present in substantial quantities in human faeces and that the LA/SLA ratio was 2.1 [15]. It has further been suggested that lowered uptake of sulphated bile acids in the ileum, and lack of uptake in the colon, may contribute to their excretion [16]. Finally, studies with mice have shown that sulphated bile acids may inhibit colon carcinogenesis [17].

In the present paper we show that LA stimulated the secretion of MMP-2 from cultured CaCo-2 cells, whereas SLA did not. Furthermore, the increased invasive capacity seen in these cells after exposure to LA was not observed in cells exposed to SLA. In addition, neither LA nor SLA were found to affect the viability or the ability of CaCo-2 cells to proliferate. From these data it is evident that sulphation of LA leads to the generation of a bile acid derivative with different effects than the parent molecule. The data presented indicate that sulphation of LA can be viewed as a biological inactivation of a compound with possible hazardous effects on colon epithelial cells. MMP-2 secretion and increased invasiveness are clearly parameters of malignant epithelial cells [4]. Our results show that LA treatment led to a stimulation of both these characteristic features in CaCo-2 cells.

The molecular mechanisms of LA effects on CaCo-2 cells or other cells types have not been the subjects of detailed investigations. There are, however, some reports on the effects of bile acids on gene expression. Recently the farnesoid X receptor, defined as an orphan receptor, was shown to bind bile acids and to repress the expression of genes regulating cholesterol metabolism [18,19]. It has furthermore been established that the expression of intestinal bile acid binding protein was promoted more efficiently with lipophilic than hydrophilic bile acids in CaCo-2 cells [20]. Bile acids may also influence cellular signalling processes by increasing the activity of protein kinase C and the transcription factor activator protein 1 in colon cells [21,22]. Although speculative, one possible scenario could be that cellular uptake or intracellular routing of LA and SLA would differ, due to differences in water solubility. SLA and LA may, furthermore, interact with different intracellular proteins or other types of ligands, and LA could reach the nucleus to modulate appropriate target genes, whereas SLA would be secreted and thereby prevented from such gene regulation. Sulphation of bile acids, and LA in particular, may turn out to have implications for the regulation of cell behaviour and development of malignancy in the large intestine.

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