Blood Biochemical Characteristics, Cecal microbiota and Short-chain Fatty Acid Composition in Fistula Implanted rats

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Abstract: We raised an experimental rat implanted with a cecal fistula and investigated various characteristics of fistula-implanted rats. Male F344/N Slc rats at 14 weeks of age were divided into three groups, the fistula group (n=5) which consisted of fistula-implanted rats, the sham group (n=7) which consisted of sham-operated rats, and the control group (n=7) which were not subjected to any surgical procedure. Four weeks after the fistula implantation surgery, we compared the blood biochemical indices, the microflora composition and the short-chain fatty acids (SCFA) concentration in cecal contents of fistula-implanted rats with those of sham-operated and control rats. The blood albumin concentration of the fistula group was significantly lower than that of the sham group and the control group, and the hematocrit value of the fistula group was significantly lower than that of the control group, but there were no significant differences in the SCFA concentration and the microflora composition among these three groups. In conclusion, it was considered that the fistula-implanted rats are useful for taking cecal contents and determining the microflora composition and the metabolites concentration at any time, without disturbing the physiological functions of the intestinal tract.

Key words: cecal fistula, microflora, SCFA

Introduction

The gastrointestinal tract is the most important part for digestion and immune response. In studies on the effects of several food components on the intestinal metabolism and microflora composition, fecal samples are routinely used for the analyses. However, it is very difficult to estimate the exact changes in microflora

composition and metabolite production in the gastrointestinal tract by using fecal samples. This is because there are some differences in microflora composition and metabolites between cecal contents and feces. Kawadu [5] compared the microflora composition and the metabolite production in cecal contents from rats with those in feces and showed the following differences. Firstly, the counts of Enterobacteriaceae

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(Received 27 August 1998 / Accepted 16 December 1998)

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in cecal contents were higher than those in feces, even though the counts of bifidobacteria and eubacteria in cecal contents were approximately equal to those of feces. Secondly, the pH, the ammonia concentration and the short-chain fatty acid (SCFA) concentration in cecal contents were higher than those in feces and then some amount of propionate and butyrate could be found in cecal contents but not in feces. Therefore, to estimate the exact changes in microflora composition and metabolite production in the gastrointestinal tract, we have to take some samples of the cecal contents and analyze them, but it is impossible to take the samples from the same rat regularly, without disturbing the physiological functions of the intestinal tract. In the present study, we raised an experimental rat implanted with a cecal fistula and investigated the particular characteristics of this fistula-implanted rat.

Materials and Methods

Animals, housing and diets

Male F344/N Slc rats (Japan SLC Inc., Tokyo; 280–300 g body weight) at 14 weeks of age were fed a stock diet, (CE-7; Clea Japan, Tokyo) which contained 5% (w/w) of rich-fiber, for 2 weeks to generate enough cecum volume for surgery. The rats were then divided into three groups, the fistula group (n=5) which consisted of fistula-implanted rats, the sham group (n=7) of sham-operated rats, and the control group (n=7) which were not subjected to any surgical procedure. All the rats were housed in individual plastic cages in a temperature controlled ($24 \pm 2^\circ\text{C}$) room with $50 \pm 10\%$ humidity and a 12-hr light cycle. The rats had *ad libitum* access to the stock diet (CE-7) which was sterilized with gamma irradiation at 30 kGy and unsterilized tap water.

Fistula components

The fistula was composed of 4 parts (Fig. 1): a main body (II) made of acrylic-plastic tube (inside diameter, 5 mm, outside diameter, 8 mm and length, 24 mm) with a screw-shaped tube end, a brim-shaped tube end, and a plate with 3 holes to attach to the abdominal wall, a stainless steel screw cap (IV), an acrylic-plastic stick (III) to store the dead volume of the main body (Natsume Seisakujyo Co. Ltd., Tokyo), and two silicone rings (I).

Fig. 1. Side sectional view of all the fistula parts. I-silicon ring, II-main body, III-acrylic-plastic stick to fill the dead volume of the main body, IV-stainless steel cap. I-1, II-1-vertical view of each part.

Implantation of the fistula

In order to maintain the cecum volume, the rats were normally fed before surgery. The rats to be implanted with the fistula and to have the sham operation, were anaesthetized by an intraperitoneal injection of sodium pentobarbital (45 mg/kg body weight i.p.).

Before surgery, all fistula parts were sterilized in ethylene oxide gas overnight, and two silicon rings (Fig. 1-I) were attached to the brimmed side. After anaesthesia, the rats were bound in the supine position to a paper sheet and the abdominal wall was opened by making a 40 mm-long midline incision. The cecum was exposed and carefully lifted through the incision with sterile swabs. At first, a small incision was made in the serosal layer of the cecum, which has fewer blood vessels. This was enlarged to 10 mm with forceps (Fig. 2-a). The inner flange of the silicone ring was inserted into the opening, and the serosal layer was held from both sides by means of silicone rings (Fig. 2-b). In this way the main body (Fig. 1-II) was fixed in the cecum. Next, a small incision to penetrate the skin and the muscle layer of the main body, was made in the left or right flank. The main body was held with pincettes and carefully extracted from the abdomen through the flank incision (Fig. 2-c). Simultaneously, the cecum was carefully returned to its original position. In order to prevent the main body from falling from the cecum, the main body was fixed in the muscle layer; the fixing plate was placed under the muscle layer and ligated to it with 3 sutures (Fig. 2-d). A stainless steel screw cap (Fig. 1-IV) and an acrylic-

Fig. 2. Surgical procedure to implant the fistula. a) A small nick was made in the serosal layer of the cecum, which has fewer blood vessels than other parts of the cecum, and then enlarged to 10 mm with forceps. b) The inner flange of the silicone ring was inserted into the opening. c) Extraction from the inside of the abdomen through the flank incision. d) The main body was positioned in the muscle layer. The fixing plate was then placed under the muscle layer and ligated to the muscle layer with 3 sutures.

plastic stick (Fig. 1-III) were then attached to the main body. The abdominal wall was then closed by stitching the muscle layer with a continuous suture and the skin with a simple interrupted suture. We did not administer the rats with antibiotics to prevent infection after surgery, because antibiotics would have significantly changed the composition of the intestinal microflora.

Sham operation

The procedure for surgery was almost the same as that described above for implantation of the fistula, but the serosal layer of the cecum was opened once, and then closed with 2 sutures.

Biochemical blood analysis

Four weeks after surgery, the blood sample was collected from the aorta ventralis under pentobarbital anaesthesia (40 mg/kg body weight i.p.). The hemat-

ocrit values and the pH of the blood were measured with an automatic electrolyte analyzer CRT-8 (Nova biochemical, Waltham). Total protein, albumin, total cholesterol, triglyceride, free fatty acids, glutamic oxalotic transaminase (GOT), glutamic pyruvic transaminase (GPT), and alkaline phosphatase (ALP) activity of the blood were measured with an automatic analyzer 7170 (Hitachi Ltd., Tokyo).

Bacteriologic analysis

The bacterial analysis method for the intestinal flora in this study was essentially the same as that of Kaneko [3]. After 0.1 g of cecal sample was suspended in 5 ml of anaerobic trypticase soy broth (TS broth). The anaerobic TS broth (pH 7.2) contained the following components (g/l): trypticase peptone, 17; phytone peptone, 3; NaCl, 5; K_2HPO_4 , 2.5; Na_2CO_3 , 0.84; agar, 0.5; L-cysteine.HCl.H₂O, 0.5. In addition, serial 10-fold

dilutions from 10^{-1} to 10^{-8} were prepared in an anaerobic chamber (N_2 - CO_2 - H_2 [8:1:1]). Aliquots (0.05 ml each) of the serial dilutions from 10^{-3} to 10^{-8} were spread on four selective agar media [3]. After incubation, preliminary identification of bacterial groups was done by colonial and cellular morphologies, Gram-stain, and aerobic growth [8, 9]. The results were expressed as $\log_{10}$ colony counts per gram(wet weight) of cecal contents.

SCFA

The SCFA were quantified by the method developed by Kaneko [4]. Samples were frozen in liquid nitrogen and stored in a freezer at -80°C until analysis.

Statistics

The data in the tables show the mean value with pooled SEM, whereas the data in the figures are expressed as means $\pm$ SEM. A one-way ANOVA with a subsequent Bonferroni/Dunn test [6] was done at each point to test for significant differences in the mean values for the experimental groups ($p < 0.05$).

Results

Observation and macroscopic inspection

Five fistula-implanted rats were used in this experiment, excluding two fistula-implanted rats which died within one week after surgery.

Within six days after surgery, the body weight of the fistula-implanted rats and sham-operated rats decreased at the early days and next constantly increased (Fig. 3). They remained in normal condition during the postoperative period. Upon postmortem examination, a new connective tissue around the fistula was observed in all fistula-implanted rats, but no abnormal adhesions within the fistula, the cecum and the surrounding tissues were observed. Upon visual observation, there was no inflammation in the cecum or the surrounding tissues, but tylosis was seen at a section of the liver under the mid-line in three fistula-implanted rats and one sham-operated rat.

Biochemical blood indices

All biochemical indices are shown in Table 1. The serum albumin concentration and the ratio of albumin to globulin (A/G ratio) in the fistula implanted rats were

Fig. 3. Body weight of fistula-implanted rat.

significantly lower than those in the sham-operated rats ($p < 0.05$, $p < 0.05$) and the control rats ($p < 0.01$, $p < 0.01$). The serum triglyceride and free fatty acid concentration in the fistula-implanted rats were slightly lower than those in the sham-operated rats and the control rats. There were no significant differences in total protein, GOT, GPT, ALP activities and pH. The hematocrit values in the fistula-implanted rats and the sham-operated rats were significantly lower than those in the control rats ($p < 0.01$, $p < 0.01$).

SCFA, pH and oxidation-reduction potential (ORP) in cecal contents

The SCFA concentration, pH and ORP values in cecal contents are shown in Table 2. Among the three groups, there were no significant differences in the acetic acid, propionic acid and n-butyric acid concentration in cecal contents, pH and ORP values.

The composition of cecal microflora

The composition of the cecal microflora in the three groups is shown in Table 3. Among the three groups, there were no significant differences in the numbers of total bacteria, anaerobes (Bacteroidaceae, lactobacilli, eubacteria, clostridia, and Gram-positive cocci) and aerobes (Enterobacteriaceae and streptococci). We could not detect bifidobacteria in the sample of three groups.

Table 1. Biochemical indices of the blood of fistula-implanted rats

		fistula [5] ^a	sham [7]	control [6]
Total protein	(g/dl)	5.9 ± 0.1	6.1 ± 0.1	6.2 ± 0.1
Albumin	(g/dl)	3.7 ± 0.1*	4.1 ± 0.1	4.2 ± 0.1
A/G rate		1.6 ± 0.1*	2.1 ± 0.1	2.1 ± 0.1
Free fatty acids	(mEq/dl)	1.05 ± 0.15	1.37 ± 0.19	1.70 ± 0.12
Triglyceride	(mg/dl)	88 ± 18	115 ± 15	149 ± 22
GOT	(IU/l)	97 ± 17	92 ± 13	82 ± 4
GPT	(IU/l)	39 ± 4	45 ± 3	46 ± 2
ALP	(IU/l)	479 ± 17	519 ± 18	487 ± 15
pH		7.43 ± 0.03	7.46 ± 0.04	7.50 ± 0.01
Hematocrit value	(%)	45 ± 1*	45 ± 1*	49 ± 1

Values are means ± SEM. ^a Number of rats examined. *p < 0.05 in comparison with the control values.

Table 2. Short-chain fatty acid composition, pH and ORP in the cecum of fistula-implanted rats

		fistula [5] ^a	sham [7]	control [7]
Acetic acid	(mM)	110.94 ± 7.40	93.03 ± 6.57	99.02 ± 2.44
Propionic acid	(mM)	25.26 ± 2.24	22.25 ± 1.76	23.94 ± 0.45
N-butyric acid	(mM)	53.24 ± 5.89	50.33 ± 4.67	54.01 ± 2.93
pH		6.34 ± 0.09	6.42 ± 0.06	6.50 ± 0.06
ORP	(mV)	-130 ± 13	-139 ± 4	-142 ± 7

Values are means ± SEM. ^a Number of rats examined.

Table 3. Composition of microflora in the fistula-implanted cecum of the rats

	fistula [5] ^a	sham [6]	control [6]
Total bacteria	9.26 ± 0.09	9.28 ± 0.08	9.36 ± 0.11
Bacteroidaceae	8.94 ± 0.04 (80) ^b	8.55 ± 0.15 (100)	8.56 ± 0.20 (100)
Lactobacilli	8.99 ± 0.10 (100)	9.06 ± 0.06 (100)	9.06 ± 0.15 (100)
Eubacteria or Clostridia	8.48 (20)	8.51 ± 0.09 (50)	8.40 ± 0.10 (50)
Gram (+) cocci	7.65 ± 0.35 (100)	7.24 ± 0.24 (33)	7.63 ± 0.63 (33)
Enterobacteriaceae	6.21 ± 0.26 (100)	5.59 ± 0.31 (83)	5.74 ± 0.20 (100)
Streptococci	7.91 ± 0.35 (100)	7.96 ± 0.30 (100)	8.40 ± 0.13 (100)

Values are means ± SEM of log₁₀ bacterial counts/g of cecal contents. ^a Number of rats examined.

^b Frequency of occurrence (%).

The frequency of occurrence of Gram-positive cocci in the fistula-implanted rats was lower than those in the another two groups.

Discussion

The cecum is an important organ in rats for the fermentation of food ingredients. In this study, we created a method for implanting a cecal fistula to estimate the

exact changes in microflora composition and the production of metabolites in the gastrointestinal tract.

Several studies so far have considered of the fistula rat in the cecum. A cecostomy model with an ileal fistula was designed by Sakata [10]. In this case, the ileum of a rat was cut at 5 cm oral to the ileo-cecal junction. Next the aboral cut end of the ileum was passed through a hole made in the abdominal wall and was sutured to the abdominal wall. This model rat

could digest and absorb material from the mouth, because intestinal continuity was restored by an end-to-side anastomosis of the oral cut, the ileal segment between the suture to the abdominal wall and the ileo-cecal junction. But this fistula was so fine that the cecal contents could not be taken through it. The second model [7] was a cecal fistula made of silicon rubber and stainless steel. The surgery method is similar to ours, but there was no bleeding from the suture incision in the model rat developed in this study. This is because the serosal layer of the cecum is held by the silicon rings instead of sutures to fix the fistula in the cecum.

We examined the microflora composition, SCFA concentrations, pH and ORP values in the cecal contents, and biochemical blood indices of all the groups (fistula, sham and control) to determine the characteristics of this model animal. There was no difference in body weight between the fistula group and the sham group. This result indicate that both fistula-implanted rats and sham-operated rats probably suffered the similar degree of stress due to surgery, though there were some differences in the biochemical indices between the fistula group and the other groups. The hematocrit values of the fistula group and the sham group were significantly lower than those of the control group. These results also suggested that the fistula group had the similar stress due to surgery to the sham group, but different from the control group. The serum albumin concentration and the A/G ratio in the fistula group were significantly lower than those in the other groups ($p < 0.05$, $p < 0.05$). Furthermore, the serum triglyceride and free fatty acid concentration in the fistula group was slightly lower than those in the control group. Therefore, it is thought that fistula-implanted rats were in poor nutritional condition at the early stage, and the physical condition recovered later along with body weight recovery [1]. There was no difference in the microflora compositions, SCFA, pH and ORP in the cecal contents among the three groups. The above finding make it obvious that the cecal fistula prepared in this study is valuable for investigating the change of microflora component and metabolites production in the cecum. This model animal is also useful for collecting the cecal contents through the fistula, for injecting the identified materials directly into the cecum,

and for measuring environmental indices such as pH, temperature and ORP in the cecum. Additionally, the cecal contents are taken from the fistula-implanted rat, without the stress due to anaesthetization and restriction. This is an another benefit because excess stress causes the change of intestinal microflora [2, 11]. In conclusion, this model rat developed in this study would provide a tool for studying the effects of food and medicine on the intestinal environment.

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