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Dramatic Changes of the Gut Flora Immediately After Severe and Sudden Insults

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Abstract

Background The gut flora is crucially involved in host homeostasis. However, the changes in the gut flora during the early phase of a critical illness are unknown.

Aims We investigated the changes in the gut flora at an early phase of severe insult in critically ill patients.

Methods Fifteen patients who experienced a sudden and severe insult were studied, along with 12 healthy volunteers as the control group. Fecal samples were acquired from the subjects by swabs of the rectum within 6 h after admission to the emergency room (day 0). Samples were serially collected from patients until day 14. Samples were also collected from control subjects.

Results On day 0, total bacterial counts were decreased to one-thousandth the number of the control subjects, in particular, obligate anaerobes and *Lactobacillus* were significantly decreased. In addition, on day 0, the major short-chain fatty acids of the patients were significantly lower than those of the control subjects. The gut flora and the concentrations of major short-chain fatty acids did not recover to normal levels. In contrast, *Enterococcus* and *Pseudomonas* increased during the study period.

Conclusions The gut flora in critically ill patients changed immediately after a severe insult. The concentrations of the three major short-chain fatty acids were immediately decreased in tandem with the destruction of the gut flora. The gut flora and the concentration of major short-chain fatty acids did not improve during the first 2 weeks after hospital admission. At the same time, the number of harmful bacteria gradually increased.

Keywords Critical illness · Bacterial translocation · Short-chain fatty acid · Probiotics · Prebiotics · Synbiotics

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Introduction

Multiple organ dysfunction syndrome (MODS) is a central cause of death of critically ill patients in intensive care units (ICU). For several decades, it has been hypothesized that the gut may be the "motor" of MODS [1–4]. This hypothesis proposes that several insults promote the translocation of intestinal bacteria or their toxins into the systemic circulation, resulting in the release of proinflammatory cytokines, and inducing systemic inflammatory response syndrome (SIRS) and MODS [3, 4]. Many previous reports have described the mechanism by which the translocation of intestinal bacteria or their toxins occurs [5, 6]. MacFie et al. [5, 6] indicated that bacterial species obtained in the gut, mesenteric lymph nodes, and postoperative septic focus matched in abdominal septic patients. De-Souze et al. [7] demonstrated that an increase of intestinal permeability induced bacterial and toxin translocation from the intestinal lumen to the systemic circulation, causing sepsis and MODS in critically ill patients. Furthermore, several recent reports have indicated that the interactions between the commensal bacteria and host also

have an important role in the induction of gut-derived sepsis [2–4].

The gut flora of the intestinal tract, which includes several hundred grams of bacteria, is crucial for host homeostasis because these bacteria have metabolic, trophic, and protective activities that are active in the host [8]. The gut flora ferments non-digestible dietary residue and produces short-chain fatty acids and vitamins, controls the proliferation and differentiation of intestinal epithelial cells, and protects host from pathogenic bacteria [8]. Clark et al. [2] and Alverdy et al. [3] demonstrated that intestinal crosstalk with commensal bacteria has an important role in preventing gut-derived sepsis and MODS in critically ill patients. Recently, several reports indicated that the normalization of gut flora by prebiotic, probiotic, and symbiotic treatments reduced infectious complications in critically ill patients [9–12]. Nonetheless, it has never been elucidated how and when the gut flora change in critically ill patients. Kanazawa et al. [12] reported that the gut flora had already been destroyed 1 week after hepatectomy. Similarly, Shimizu et al. [13] demonstrated that the gut flora was affected 1–2 weeks after admission to the ICU. However, the condition of the intestinal lumen at an early phase of severe insult has not been examined, although the early phase is the most important period in critically ill patients.

In the present study, we investigated the changes of gut flora and the intestinal environment at an early phase of severe and sudden insult in critically ill patients.

Methods

The present study was approved by the Institutional Review Board of Hokkaido University Hospital. Informed consent was obtained from each patient's family. The present study included patients who experienced a sudden and severe insult, including trauma, out-of hospital cardiac arrest, and cerebral vascular disease, who had all been in good health just before their admission, and who were directly admitted to the emergency department from their normal daily life. Patients were excluded if they were under 18 years of age, had a terminal illness, chronic disease, drug abuse, alcoholism, or malnutrition, or had been living in a nursing home. Patients who were taking steroids or antibiotics were also excluded. Twelve healthy volunteers served as the control group.

Two fecal samples (for bacteriological analysis and organic acid analysis) were acquired from the subjects by swabs of the rectum within 6 h after admission to the emergency department (day 0) and before the first administration of antibiotics. Samples were serially collected on days 1, 3, 5, 7, 10, and 14. Samples were also collected from control subjects. Samples with cotton applicator were put

into test tubes containing 1 ml RNAlater (Ambion, Inc., Austin, TX, USA), a ribonucleic acid (RNA) stabilization solution prior to bacteriological analysis. Samples with cotton applicator were put into test tubes containing 1 ml of 1% perchloric acid prior to fecal organic acid analysis. The samples were stored at −20°C until analysis. Two fecal samples (for the bacteriological analysis and organic acid analysis) were also obtained from the healthy volunteers.

The samples for bacteriological analysis were incubated for 5 min at room temperature. RNA was isolated using the method described elsewhere [14, 15]. Finally, the nucleic acid fraction was suspended in 1 ml nuclease-free water. A standard curve was generated with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (using the threshold cycle [C_T] value, the cycle number when the threshold fluorescence was reached) and the corresponding cell count, which was determined microscopically with 4,6-diamidino-2-phe-nylindole (Vector Laboratories, Burlingame, CA) staining for dilution series of the standard strains as described elsewhere [14, 15]. For determination of the type of bacteria present in the samples, three serial dilutions of an extracted RNA sample were used for RT-qPCR, and the C_T values in the linear range of the assay were applied to the standard curve to obtain the corresponding bacterial cell count in each nucleic acid sample. These data were then used to determine the number of bacteria per sample. The specificity of the RT-qPCR assay using the group-, genus- or species-specific primers was determined as described previously [14, 15].

The samples used for the fecal organic acid analysis were homogenized and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was put in a glass tube, and allowed to stand at 4°C for 12 h. The suspension was then passed through a filter with a pore size of 0.45 μm (Millipore Japan, Tokyo). The sample was analyzed for organic acids by high-performance liquid chromatography, and the concentrations of organic acids were calculated with the use of external standards, and the reproducibility and stability of these measurements were described previously [16].

Unless otherwise indicated, all measurements are expressed as the median (interquartile range 25–75%). The SPSS 15.0 J statistical software package (SPSS Inc., Chicago, Illinois) was used for all statistical analyses. Comparisons between the two groups were made using either the Mann–Whitney U test or the chi square test. The Jonckheere-Terpstra test was used for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant.

Results

Fifteen critically ill patients who were admitted to the hospital following sudden and severe insults were included

in this study. Table 1 presents the characteristics of the patients. No differences in age or sex were observed between the healthy volunteers and the patients. All patients needed mechanical ventilation. The median values of ventilator days were 5 days (25–75% points, 3–10 days) during the first 2 weeks. Antibiotics were administrated intravenously for the patients during 5 days (25–75% points, 3–7 days) during the first 2 weeks. Cefazolin, ceftriaxone, sulbactam/cefoperazone, cefmetazole, piperacillin, meropenem, clindamycin, and amikacin were intravenously administered. Oral antibiotics and selective digestive decontamination were not used for any patients. Normal enteral feeding was performed for all patients. Prebiotic and probiotic therapies were not administered to any of the patients. Abdominal surgical treatment was not performed to any patients.

The fecal flora of the control subjects and the critically ill patients on the day of admission (day 0) are presented in Table 2. In the critically ill patients on day 0, the total bacterial counts, especially the various obligate anaerobes and *Lactobacillus,* significantly decreased to one-thousandth in comparison to those of the control subjects.

Table 3 presents the fecal organic acid levels of the control subjects and the critically ill patients on day 0. The total organic acid levels of the patients were significantly lower than those of the control subjects, particularly the levels of three major short-chain fatty acids (acetic acid, propionic acid, and butyric acid).

Total bacterial counts did not recover to normal levels during the study period. The obligate anaerobe counts of the patients did not improve until day 14. *Lactobacillus* counts were one-thousandth those of the control subjects upon admission, and increased gradually thereafter ($P = 0.027$), but did not attain the levels found in controls within the study period. The counts of *Enterococcus* and *Pseudomonas,* which are pathogenic bacteria, increased in the critically ill patients during the observation period ($P = 0.011$, and 0.024, respectively). The total organic acid, acetic acid, propionic acid, and butyric acid levels of the critically ill patients continued to be lower than the level seen in controls until the end of the study period (day 14). However, these changes in the bacterial counts and organic acid concentrations from day 1 to day 14 were affected by various treatments, which included antibiotics, enteral nutrition, and parenteral nutrition.

Discussion

Destruction of the gut flora in critically ill patients has often been reported. However, precisely when and how the destruction of the gut flora was induced remains to be elucidated. In the present study, we clarified several points: (1) The gut flora is destroyed dramatically and immediately after severe insult, (2) the concentrations of major short-chain fatty acids in the intestine decrease immediately after the severe insult, accompanied by the destruction of the gut flora, (3) the gut flora and the short-chain fatty acids concentrations do not improve for at least 2 weeks after the severe insult, and (4) the number of pathogenic bacteria gradually increases in the intestine after the severe insult.

Our findings indicate that the gut flora change dramatically and immediately in critically ill patients after sudden and severe insults. The total bacterial counts were only one-thousandth those of the control subjects, especially obligate anaerobes and *Lactobacillus* significantly decreased, although the critically ill patients had been in good health just before their admission. Shimizu et al. [13] reported alterations of the gut flora in patients with severe SIRS. However, they investigated the gut flora of the patients 9.0 ± 7.4 days after an admission to ICU. It is unclear when the gut flora changed in these patients [13]. Eaves-Pyles et al. [17] reported that gut barrier function was impaired as early as 5 min after thermal injury in mice. However, they did not study the alternation of gut flora [17]. There has been no report describing the changes in gut flora immediately after insult. This is therefore the first demonstration that the gut flora is destroyed just after severe insult in critically ill patients.

Several reports have shown changes in the gut flora of critically ill patients [11–13]. For example, Kanazawa et al. [12] reported that *Bifidobacterium* and *Lactobacillus* decreased, while the number of pathogenic bacteria increased 1 week after hepatectomy. Shimizu et al. [11, 13] reported that obligate anaerobes and *Lactobacillus*

Characteristics	Control subjects ($n = 12$)	Patients ($n = 15$)	P value
Age (year)	35 (29–46)	40 (28–62)	ns
Sex (male/female)	11/1	11/4	ns
APACHE II	–	21 (18–26)	
Disease			
Out-of-hospital cardiac arrest	–	5	
Multiple trauma	–	8	
Cerebral vascular disease	–	2	

Table 1 Characteristics of the healthy volunteers and patients

APACHE acute physiology and chronic health evaluation, *ns* not significant

Measurements were presented as frequencies or median (interquartile range 25–75%)

Table 2 Fecal flora on day 0

Flora	Control subjects ($n = 12$)	Patients ($n = 15$)	P value
Total bacteria	10.1 (9.8–10.4)	7.5 (6.7–8.0)	<0.001
Obligate anaerobes			
Clostridium coccoides group	9.4 (9.0–9.7)	6.1 (5.4–7.0)	<0.001
Clostridium leptum subgroup	9.2 (9.0–9.7)	6.5 (6.1–7.3)	<0.001
Bacteroides fragilis group	9.4 (9.0–9.8)	6.8 (5.9–7.6)	<0.001
Bifidobacterium	9.2 (8.9–9.4)	6.0 (<5.0–6.9)	<0.001
Atopobium cluster	9.0 (8.5–9.5)	6.0 (<5.1–7.1)	<0.001
Prevotella	8.8 (8.5–9.1)	<5.0 (<5.0–6.0)	<0.001
Clostridium perfringens	<3.5 (<3.5–<3.5)	<3.5	ns
Facultative anaerobes			
Lactobacillus	5.7 (4.5–6.9)	<2.5 (<2.5–2.7)	<0.001
Enterobacteriaceae	5.9 (5.5–6.4)	5.5 (<5.4–6.4)	ns
Enterococcus	5.2 (4.3–5.8)	4.6 (<2.9–5.5)	ns
Staphylococcus	3.8 (3.4–4.5)	<3.0 (<3.0–4.4)	ns
Obligate aerobes			
Pseudomonas	<3.0	<3.0	ns

ns not significant

All measurements were presented as log10 counts/g and median (interquartile range 25–75%)

Table 3 Fecal organic acid concentrations on day 0

Organic acids	Control subjects ($n = 12$)	Patients ($n = 15$)	P value
Total organic acids(μmol/g)	90.1 (83.1–94.2)	41.3 (16.2–65.6)	<0.001
Acetic acid (μmol/g)	64.4 (60.4–67.6)	35.7 (12.1–58.4)	0.002
Propionic acid (μmol/g)	11.7 (9.6–14.8)	1.4 (0.5–6.1)	<0.001
Butyric acid (μmol/g)	10.3 (7.6–10.5)	<0.55 (<0.55–4.4)	<0.001
Succinic acid (μmol/g)	0.5 (<0.075–1.9)	<0.075	ns
Formic acid (μmol/g)	0.4 (<0.05–1.27)	0.4 (<0.05–0.8)	ns
Lactic acid (μmol/g)	<0.2 (<0.2–<0.2)	<0.2 (<0.2–<0.2)	ns
Isovareric acid (μmol/g)	<0.8	<0.8	ns
Valeric acid (μmol/g)	<0.65	<0.65	ns

ns not significant

All measurements were presented as median (interquartile range 25–75%)

decreased, and pathogenic bacteria increased, 1–2 weeks after admission to the ICU. It is clear that the total bacterial counts considerably decreased as a result of the reduction of obligate anaerobes and *Lactobacillus* after various insults, based on both the present and previous studies [11–13].

Lactobacillus and obligate anaerobes, especially *Bifidobacterium*, have several important roles in the intestine [8]. These commensal bacteria are an important line of resistance to colonization by pathogenic microbes [8]. Therefore, they are related to the prevention of pathogen invasion of the intestinal tissues [8]. These beneficial bacteria compete with pathogenic bacteria for attachment sites in the brush border of intestinal epithelial cells, and prevent entry of enteroinvasive bacteria into the intestinal epithelial cells [8, 18]. They are able to inhibit pathogenic bacterial adherence to the intestinal epithelium by inducing increased production of mucins [19]. In addition, these beneficial bacteria inhibit the growth of pathogenic bacteria in the intestine by secretion of bacteriocins, which are antimicrobial substances [3, 8, 18]. For these reasons,

pathogenic bacteria may gradually overgrow after the decrease of beneficial bacteria induced by various insults [8]. In the present study, although the counts of *Enterococcus* and *Pseudomonas* were the same as those of the control subjects just after the insults, the number of pathogenic bacteria gradually increased during the observation period. Of course, antibiotic treatments and nutritional therapies after admission might have some influence on the overgrowth of the pathogenic bacteria in the intestine. However, the overgrowth of such pathogenic bacteria may be one reason for the observed underlying bacterial translocation and MODS [1, 8].

Lactobacillus and obligate anaerobes have other important roles. These commensal bacteria have enzymes and can use biochemical pathways not present in mammals that can ferment non-digestible dietary residue and produce short-chain fatty acids [8]. Short-chain fatty acids, especially acetate, propionate, and butyrate, are the principal products of fermentation in the large intestine [8]. In the present study, the dramatic decline in the concentrations of

the short-chain fatty acids, especially butyrate, apparently resulted from the reduction of obligate anaerobes and *Lactobacillus* in the critically ill patients just after the insults. Butyrate plays several important roles in the maintenance of intestinal integrity [8, 20]. Epithelial cells of the intestinal mucosa consume a major portion of the butyrate generated by the commensal bacteria, and the butyrate provides a major source of energy for colon cells [8, 20]. Furthermore, all three short-chain fatty acids stimulate proliferation and differentiation of the intestinal epithelial cells [8, 20]. However, in the present study, the concentrations of all three short-chain fatty acids did not improve during the first 2 weeks after the dramatic decline induced by the sudden insults. Such an effect may further impair the recovery of these patients.

In the present study, we elucidated that obligate anaerobes and *Lactobacillus* decreased dramatically and immediately after severe insult. However, the mechanism underlying this apparently immediate destruction of the gut flora is unclear. We have two hypotheses regarding this mechanism as follows: (1) intestinal hypoperfusion may play a role in this flora destruction and (2) the toxicity of the high concentration oxygen that was used in the resuscitation of the patients may have annihilated the anaerobes. However, further studies are necessary to elucidate this point. Regardless of the mechanism, sudden changes in the environment in the intestine after insults may induce a significant decrease in the obligate anaerobes and *Lactobacillus*.

In conclusion, both obligate anaerobes and *Lactobacillus* decreased dramatically and immediately after severe insults. The concentration of the three major short-chain fatty acids produced by these bacteria immediately decreased in tandem with the gut flora destruction.

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