Intestinal Microflora and Clinical Medicine

Kyotaro KANAZAWA*

Emeritus Professor of Surgery, Jichi Medical School, 3311–1, Yakushiji, Minami Kawachi, Tochigi 329–0498, Japan

Presented at the International Conference of Intestinal Bacteriology (ICIB2001), held in Tokyo, July 5–6, 2001. Received for publication, August 3, 2001

Germ-free animals retards in wound healing to their counterparts with normal intestinal flora. Intestinal microflora modify the wound healing process via activating cellular immune reactions of the host. Gnotobiota with either *Lactobacilli* or *Bifidobacteria* could restore the healing potential of germ-free animals to the control levels. Supportive effects of probiotics on wound healing may be modified by their nutritional actions as well. Intestinal flora are also implicated in carcinogenesis of various organs. Their effects on colorectal carcinogenesis has attracted deep interests naturally. In spite of some promising findings, no definite bacterial metabolites surely capable of inducing human colorectal cancer have not been demonstrated yet. Many studies have confirmed promoting effects of intestinal microflora on developing cancer of the large intestine. Normal intestinal microflora modify mammary carcinogenesis via the route of metabolizing phytoestrogens or manipulating the enterohepatic circulation of estrogen. Heated debates on *Helicobacter pylori* and gastric cancer problem have not been settled down yet, although the agreement that this microorganism can induce gastritis has been obtained. Cancer of the urinary bladder, bile ducts, etc., induced by parasitic infection can be explained by the deconjugating action of contaminating bacteria to liberate masked carcinogens. Intestinal microflora are also implicated in miscellaneous metabolic fields which are intimately related with clinical medicine offering endless interests.

Key words: wound healing; probiotics; carcinogenesis; cancer of the large intestine; mammary cancer; gastric cancer

INTRODUCTION

The clinical importance of intestinal microflora in maintaining the healthy state of the host has been well understood. Many studies have been done to elucidate the role of intestinal microflora implicated in nutritional benefit, vitamin production or deprivation, prevention of intracolonic putrefaction, stimulation of immunity, prevention of various infection, cholesterol metabolism, antihypertensive effect, sleep induction, etc.

Basing upon our data, the author should like to show the influence of intestinal microflora on wound healing and carcinogenesis.

Possible beneficial effects of probiotics in clinical practice are also briefly introduced.

1. WOUND HEALING AND INTESTINAL MICROFLORA

Believing that microflora exert deleterious effects on wound healing process, we surgeons have tried to keep the surgical field aseptic to achieve uneventful recovery after surgical interventions. It is true that contamination of wound by noxious bacteria results in impaired wound healing. Since massive combined administration of strong antibiotics can protect patients from lethal effects of extensive radiotherapy or antineoplastic

drugs, elimination of all microflora in the body has been thought as if it were the best way to offer good health to the patient.

Recent studies have demonstrated that contamination of wound by bacteria per se does not necessarily result in the disturbance of healing.

The author would like to show the effects of normal resident intestinal microflora on the healing of aseptic wounds.

Materials and Methods

IQI/jic germ-free mice, purchased from CLEA/Japan, were used. In addition to those kept in the germ-free state throughout the experiments, GF, their counterpart of the conventionalized group, CVZ, were produced by force-feeding them with 10 mg of fresh feces from healthy SPF mice, ICR CLEA/Japan, in 0.1 ml of normal saline. Gnotobiota were produced by force-feeding GF mice with 1×10^5 of one of the following: *Lactobacillus reuteri* (RCA 19-14), Lac; *Bifidobacterium longum* (JCM1217), Bif; *Escherichia coli* (RCA 19-4), Ecl; *Bacteroides vulgatus* (RCA 19-10), Bac; or *Clostridium perfringens* (JCM 1290), Clp. These bacteria used to produce gnotobiota were isolated from SPF mice, and were kindly supplied by Dr. Y. Benno, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Japan.

Only male mice were used for experiments at the age of 4 weeks and weighing 15 to 20 g. All mice in-

*Corresponding author. Mailing address: Emeritus Professor of Surgery, Jichi Medical School, The Director, Kurosu Hospital, 2650, Ujiie, Tochigi 329–1395, Japan. Phone: +81–28–682–8811. Fax: +81–28–682–9499.

cluding CVZ were kept in separate vinyl isolators, specific for each group, placed in the same room, and fed with sterile chow of the same batch (CL2 50KGY Radiation Sterile Diet, CLEA, Japan) and re-sterilized water ad libitum in order to keep environmental conditions exactly identical. Body weight checks were repeated periodically to compare the effects of flora on growth.

Conventionalization and inoculation of specific bacteria to produce gnotobiota were done at the age of 4 weeks, and after the mice were checked 2 weeks later to verify they were in the expected conditions, they were operated upon as will be mentioned later at the age of 7 weeks. GF mice were operated upon at the age of 7 weeks as well.

A longitudinal skin incision was made under intra-peritoneal injection of 50 mg/kg body weight of sodium pentobarbital. A slender perforated silicon tube containing polyvinyl alcohol sponge was implanted into the subcutaneous tissue lateral to the incision. The incisional wound was closed with interrupted stitches using 5-0 nylon monofilament thread.

Mice were sacrificed by ethyl ether, skin flaps were used to measure the break strength, and the contents of the polyvinyl alcohol sponge were used to measure hy-

droxyproline concentration.

Cecal content was analyzed to measure the amount of short-chain fatty acids (SCFAs) and lactic acid.

Results

There were no significant differences in the increase of body weight among all experimental groups (Fig. 1).

The break strength of the surgical wound deteriorated in GF compared to CVZ. Twice repeated experiments showed the same results. Lac and Bif groups showed the same break strength compatible with CVZ, on the other hand, Ecl, Bac, and Clp were at the same levels as GF (Fig. 2). The hydroxyproline level of each

Fig. 1 The rates of body-weight increase during the experiment. The values expressed are percentages of body-weight increase. GF, germ-free group; CVZ, conventionalized group; L, *Lactobacillus* group; E, *E. coli* group; B, *Bacteroides* group; C, *Clostridium* group; BB, *Bifidobacterium* group. N.S.: no significant difference.

Fig. 2 Difference of break strength among the germ-free, conventionalized, and various gnotobiota. GF, germ-free; CV, conventionalized.

Table 1. Hydroxyproline concentration in tissue fluid ($\mu\text{mol/l}$) mean $\pm$ SD.

Germ-free	17.7 $\pm$ 2.9
Conventionalized	22.0 $\pm$ 4.9*
<i>Lactobacillus</i>	21.2 $\pm$ 2.0*
<i>Bifidobacterium</i>	31.8 $\pm$ 7.9**
<i>E. coli</i>	17.9 $\pm$ 2.4
<i>Bacteroides</i>	17.8 $\pm$ 2.1
<i>Clostridium</i>	17.8 $\pm$ 2.6

* $p < 0.05$, ** $p < 0.01$ (compared to the germ-free).

Table 2. Amounts of organic acids in the caecum ($\mu\text{mol/g}$ wet weight) mean $\pm$ SD.

	Acetic	Propionic	Iso-butyric	Butyric	Iso-valeric	Valeric	Lactic
Germ-free	1.06 $\pm$ 0.08	0.04 $\pm$ 0.01	0.14 $\pm$ 0.07	0.29 $\pm$ 0.06	0.12 $\pm$ 0.05	0.14 $\pm$ 0.05	0.21 $\pm$ 0.57
Conventionalized	37.30 $\pm$ 4.02*	5.18 $\pm$ 0.54*	0.77 $\pm$ 0.05*	14.67 $\pm$ 1.20*	0.55 $\pm$ 0.06	1.09 $\pm$ 0.08*	3.49 $\pm$ 3.37**
<i>Lactobacillus</i>	1.84 $\pm$ 0.18	0.09 $\pm$ 0.03	0.07 $\pm$ 0.05	0.20 $\pm$ 0.07	0.21 $\pm$ 0.06	0.20 $\pm$ 0.06	6.29 $\pm$ 3.63**
<i>Bifidobacterium</i>	2.93 $\pm$ 0.13	0.20 $\pm$ 0.01	0.19 $\pm$ 0.07	0.42 $\pm$ 0.06*	0.18 $\pm$ 0.05	0.19 $\pm$ 0.06	6.17 $\pm$ 2.33**
<i>E. coli</i>	2.53 $\pm$ 0.19	0.09 $\pm$ 0.02	0.11 $\pm$ 0.08	0.26 $\pm$ 0.08	0.06 $\pm$ 0.04	0.30 $\pm$ 0.07	0.46 $\pm$ 0.11
<i>Bacteroides</i>	2.55 $\pm$ 0.17	6.45 $\pm$ 0.49*	0.56 $\pm$ 0.01*	0.31 $\pm$ 0.07	0.46 $\pm$ 0.05	0.28 $\pm$ 0.09	0.81 $\pm$ 1.01
<i>Clostridium</i>	2.82 $\pm$ 0.17	0.13 $\pm$ 0.02	0.00 $\pm$ 0.00	0.66 $\pm$ 0.02*	0.33 $\pm$ 0.05	0.30 $\pm$ 0.05	0.46 $\pm$ 0.99

* $p < 0.05$, ** $p < 0.01$ (compared to the germ-free).

group corresponded well with the break strength (Table 1).

Compared to CVZ, other groups showed diminished concentrations of SCFAs in the cecal content. Lactic acid concentrations were more elevated in both Lac and Bif groups compared to CVZ, and other groups left behind showed far lower lactic acid concentrations than those 3 groups (Table 2) (16).

Discussion

The gastrointestinal tract serves as the entrance of various antigens, and continuous stimulations of antigens related to intestinal bacteria or virus enhance the action and number of immunocompetent cells in the body, and antigenic stimulation of these cells results in the liberation of interferon- γ , which causes loosening of epithelial tight junctions to allow more influx of antigens to reenforce immunological processes (1). Such an immunological stimulation being less prominent in the germ-free state, the number of immunocompetent cells in the body, including the *Lamina propria* mucosae, is reduced in the germ-free state (13). Complicated cytokine cascades are involved during the course of the wound healing process: injury to the vascular endothelia results in the aggregation of platelets to the damaged part which release TNF- β to stimulate macrophages. Stimulated macrophages activate T lymphocytes by releasing IL-1. Activated T lymphocytes release TGF- β , which stimulates proliferation of fibroblasts and their collagen production. Activated T lymphocytes produce IL-2 which also stimulates proliferation of fibroblasts (26, 28). These immunocompetent cells being scarce in germ-free animals, one can easily understand that the wound healing process is retarded in this state.

The other mechanism is nutritional support derived from intestinal microflora: urea excreted into the gastrointestinal tract is metabolized to ammonia by intestinal microflora, and ammonia is used by other bacteria to synthesize amino acids, which can be utilized by the

host as well. This beneficial effect of urea has already been adopted in agricultural practice to promote feeding efficiency. Bacterial fermentation of dietary fibres produces SCFAs and lactic acid. Lactic acid is absorbed from the gut and transported to the liver where it is used to synthesize glucose. Dietary fibres are fermented by intestinal bacteria to form butyric acid, acetic acid, etc., which are utilized by colonic epithelial cells as an energy source (24).

Knowing about the impaired healing of infected wound by bacterial contamination, we have been very much keen on keeping the patient as aseptic as possible to get satisfactory wound healing. Actually these procedures have been adopted to protect patients from heavy exposure to very strong anticancer drugs or irradiation (4, 5, 29). So far as the local change at the wounded site is concerned, contamination of the wound by microorganisms per se does not necessarily result in the retardation of the healing process. Incisional wounds to the tongue heal equally between conventional and germ-free animals (25). The same finding is observed at the anastomotic site of the colon (22). Furthermore, there is a report that contamination of the wound with *Staphylococcus aureus* enhances the healing process (15).

Summary

The existence of intestinal microflora is thus intimately implicated with the wound healing process by modulating the immunological and nutritional conditions of hosts. Some intestinal bacteria exert beneficial effects on wound healing by enhancing these reactions. So far as we studied, there were no bacteria which play an actively inhibitory role in this field.

2. CARCINOGENESIS AND INTESTINAL MICROFLORA

Citing our results, the author would like to discuss the influence of intestinal microflora on carcinogenesis in a couple of organs.

a) *Cancer of the Large Intestine*

The mucous membrane of the large intestine being in close contact with a tremendous amount of intestinal bacteria, cancer of the large intestine has been assumed to develop due to various actions of intestinal microflora. Carcinogenic substances produced by intestinal microflora have been investigated. Starting from the classic study of Druckrey stating the possible production of *E. coli*, isolated from a patient with colon cancer, of sarcomogenic substance to the mouse subcutaneous tissue (6), nucleotoxic substances such as streptolindol (23), cholesterol metabolite (27), fecapentaenes (2), etc., have been isolated as candidates for colorectal carcinogens, although their carcinogenic action on the human large intestine has not definitively been confirmed yet.

Intestinal microflora are implicated in producing promoters of colorectal carcinogenesis, the main interest having been focussed on the metabolism of bile acids. Recent progress in this field is the demonstration of bacterial production of diacyl glycerols, which are stimulants of protein kinase C to promote cell growth (18, 19).

Some intestinal microflora are involved in inhibiting colorectal carcinogenesis by binding carcinogens, such as heterocyclic amines, to sweep them out from the intestinal lumen or inhibiting the action of alkylating agents to form DNA adducts.

Some intestinal bacteria, such as lactobacilli, can modify the process of colorectal carcinogenesis, probably by enhancing the immunosurveillance in the gastrointestinal system (11, 12).

b) *Carcinoma of the Mammary Gland*

Intestinal microflora are involved in the metabolism of phytoestrogens, and may influence the incidence of mammary carcinoma. Our experimental studies have shown that intestinal microflora can suppress the incidence of mammary carcinoma of GR mice via the metabolism of flavonoids of soy beans (17). Intestinal microflora are also capable of producing estrogenic substances or nucleotoxic cholesterol metabolites which may be related to mammary carcinogenesis.

c) *Carcinoma of the Stomach*

Decrease in gastric acid secretion results in an increased number of bacteria in gastric juice in parallel with an increase in the amount of mutagenes (20). Gnotobiotic mice with *Helicobacter pylori* per se could not induce gastric cancer so far as our studies are concerned. The bacteria may be involved in gastric carcinogenesis

by reducing gastric acid secretion after inducing gastritis.

d) *Other Cancers*

Bacterial metabolites of tryptophane are nucleotoxic and involved in the carcinogenesis of the urinary tract (3). Bacteria are also important for hydrolyzing detoxified carcinogens to liberate active substances; this mechanism is related to carcinogenesis in the urinary tract or biliary tract (8, 9).

3. CHOLESTEROL METABOLISM AND INTESTINAL MICROFLORA

Intestinal microflora can reduce serum cholesterol levels by following mechanisms: taking up luminal cholesterol to compose their cellular constituents, precipitating cholesterol together with calcium ion and organic acids which they produced or with free bile acids which they liberated. Intestinal microflora also inhibit hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (7).

4. ANTIHYPERTENSIVE SUBSTANCES PRODUCED BY INTESTINAL MICROFLORA

Some lactobacilli can produce angiotensin I-converting enzyme antagonists. These are tripeptides and can reduce elevated blood pressure (21, 30).

Intestinal microflora are also involved in corticosteron metabolism by hydrolyzing its glucuronide (10).

5. SLEEP AND INTESTINAL MICROFLORA

Muramyl peptides produced by intestinal microflora are immunopotentiators, pyrogens and hypnotics, causing deep slow wave sleep. Muramyl peptides stimulate macrophages to release IL-1, which is the causal element of the phenomena (14).

Thus intestinal microflora are involved in regulating miscellaneous functions in the host. Much more thorough studies in these fields are cordially invited to benefit human lives.

REFERENCES

- (1) Adams RR, Planchon SM, Roche JK. 1993. INF- γ modulation of epithelial barrier function. Time course, reversibility and site of cytokine binding. *J Immunol* **150**: 2356-2363.
- (2) Bruce WR, Baptista J, Che T, et al. 1982. General structure of "Fecapentaenes," the mutagenic substances in human feces. A preliminary report. *Naturwissenschaften* **69**: 557-558.
- (3) Chung KT, Fulk GE, Slein MW. 1975. Tryptophanase of fecal flora as a possible factor in the etiology of colon can-

- cer. *J Natl Cancer Inst* **54**: 1073–1078.
- (4) Dietrich M. 1975. Gnotobiotics in hematology: Improvement of treatment of acute leukemia. *Eur J Cancer* **11** (Suppl): 49–55.
- (5) Donati RM, McLaughlin MM, Stromberg LR. 1973. Combined surgical and radiation injury. The effect of the gnotobiotic state on wound closure. *Experientia* **29**: 1388–1390.
- (6) Druckrey H, Richter R, Vierthaler R. 1941. Zur endogenen Entstehung krebserzeugender Stoffe beim Menschen. *Klin Wschr* **20**: 781–785.
- (7) Fujishima M, Nakano M. 1995. The effect of probiotic on faecal and liver lipid classes in rats. *Br J Nutr* **73**: 701–710.
- (8) Hicks RM. 1983. The canopic worm; role of *Bilharziasis* in the aetiology of carcinoma of human bladder. *J Roy Soc Med* **76**: 16–22.
- (9) Hirono I. 1982. Carcinogenicity examination of inflorescence of *Zingiber mioiga* Roscoe. *Cancer Lett* **15**: 203–208.
- (10) Honour J. 1982. The possible involvement of intestinal bacteria in steroidal hypertension. *Endocrinol* **110**: 285–287.
- (11) Horie H, Kanazawa K, Okada M, Narushima S, et al. 1999. Effects of intestinal bacteria on the development of colonic neoplasm: an experimental study. *Eur J Cancer Prev* **8**: 237–245.
- (12) Horie H, Kanazawa K, Kobayashi E, Okada M, et al. 1999. Effects of intestinal bacteria on the development of colonic neoplasm. II. Changes in the immunological environment. *Eur J Cancer Prev* **8**: 533–537.
- (13) Joshi JH, Entringer MA, Robinson WA. 1979. Bacterial stimulation of serum colony-stimulating activities and neutrophil production in germ-free mice. *Proc Soc Exp Biol Med* **162**: 44–47.
- (14) Krueger JM, Toth LA, Floyd R, Fang J, et al. 1994. Sleep, microbes and cytokines. *Neuroimmunomodulation* **1**: 100–109.
- (15) Levenson SM, Kan-Gruber D, Gruber C, et al. 1983. Wound healing accelerated by *Staphylococcus aureus*. *Arch Surg* **118**: 310–320.
- (16) Liu Q, Okada M, Koshizuka S, Kanazawa K, et al. 1996. The influence of intestinal bacteria on wound healing in mice. *Bioscience Microflora* **15**: 85–92.
- (17) Mizunuma H, Kanazawa K, Ogura S, et al. 2002. Anticarcinogenic effects of isoflavones may be mediated by genistein in mouse mammary tumor virus-induced breast cancer. *Oncology* **62**: 78–84.
- (18) Morotomi M, Guillem JG, Weinstein IB. 1990. Production of diacylglycerol, an activator of protein kinase C, by human intestinal microflora. *Cancer Res* **50**: 3595–3599.
- (19) Morotomi M, Logerfo P, Weinstein BI. 1991. Fecal excretion, uptake and metabolism by colon mucosa of diacylglycerol in rats. *Biochem Biophys Res Commun* **181**: 1028–1034.
- (20) Morris DL, Youngs D, Muscroft TJ, et al. 1984. Mutagenicity in gastric juice. *Gut* **25**: 723–727.
- (21) Nakamura Y, Yamamoto N, Sakai K, Takano T. 1995. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitor to angiotensin I-converting enzyme. *J Dairy Sci* **78**: 1253–1257.
- (22) Okada M, Bothin C, Kanazawa K, Midtvedt T. 1999. Experimental study of the influence of intestinal flora on the healing of intestinal anastomoses. *Br J Surg* **86**: 961–965.
- (23) Osawa T, Namiki M. 1983. Structure elucidation of streptindole, a novel genotoxic metabolite isolated from intestinal bacteria. *Tetrahedron Lett* **24**: 4719–4722.
- (24) Roediger WEW. 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology* **83**: 424–429.
- (25) Rovin S, Costich ER, Fleming JE, et al. 1965. Healing of tongue wounds in germ-free and conventional mice. *Arch Pathol* **79**: 641–643.
- (26) Sasaki H, Pollard R, Schmitt D, et al. 1992. Transforming growth factor- β in the regulation of the immune response. *Clin Immunol Immunopathol* **65**: 1–9.
- (27) Suzuki K, Bruce WR, Baptista J, et al. 1987. Characterization of nucleotoxic steroids in human feces: Do they cause colorectal cancer? *Cancer Lett* **33**: 307–316.
- (28) Wahl SM. 1992. Transforming growth factor β in inflammation A cause and a cure. *J Clin Immunol* **12**: 61–74.
- (29) Wilson R, Barry TA, Bealmear PM. 1970. Evidence for a toxic substance of bacterial origin in the blood of irradiated mice. *Radiat Res* **41**: 89–103.
- (30) Yamamoto N, Akino A, Takano T. 1994. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J Dairy Sci* **77**: 917–922.