Table 1 The expression of fibrotic/cirrhotic markers on the HSCs activation in various human pathologies and cirrhotic models

	Human hepatic fibrosis	Human hepatic cirrhosis	Rat BDL model	Rat CCl_4 model	Rat BDL model (ours)	Rat CCl_4 model (ours)
KGF[a]	++	++			++	±
α-SMA[b]	+	+++	++	+	++	++
GFAP[b]	++	+	+++	+++	+++	

Our results of immunohistochemistry in the damaged liver induced by BDL were similar to those from previous reports

[a] Steiling et al. [17] reported KGF was expressed in human chronic liver

[b] Cassiman et al. [13] demonstrated α-SMA and GFAP were expressed not only in rat models, but also in human hepatic fibrosis and cirrhosis

Electron microscopy revealed that lipid droplets were almost depleted in BDL as well as CCl_4 model, which were recovered to some extent by administration of vitamin A. This suggests that vitamin A administration restored vitamin A level in HSCs, which may contribute to histological repair of the liver from the surgical and medical insults. Some reports have mentioned the anti-oxidant effects of vitamin A [15, 16], and others suggested vitamin A stored in HSC prevents HSC itself from activating [7, 8].

Subsequently, we tried to investigate the effectiveness of anti-fibrotic changes derived from vitamin A. There are many markers indicating HSC activation. KGF [17] and α-SMA [18–20] are definitive markers of hepatic fibrosis. GFAP is a protein that originates in the glia cells of the central nervous system, and is acknowledged as a detective marker of degenerative diseases such as multiple sclerosis [21]. It has been reported that GFAP increases in hepatic fibrosis, in a similar way to desmin and nestin [22]. It is generally accepted that GFAP expression is seen in human hepatic fibrosis in its early phase. After that, KGF and α-SMA are found while fibrosis is progressing [23]. Steiling et al. reported that KGF strongly expressed in human chronic liver disease (Table 1) [17], and Cassiman et al. [13] demonstrated that α-SMA and GFAP are highly expressed in human hepatic fibrosis and cirrhosis (Table 1). Our results that expression of all three fibrotic markers was enhanced in the rat BDL model were consistent with those clinical observations. On the other hand, vitamin A administration reduced the expression of those three markers, and immunohistochemical electron microscopy using anti-GFAP antibody with morphometric analysis showed that the expression of GFAP in the BDL + vitamin A group was significantly lower than that of the BDL-treated alone. These results indicate that vitamin A suppresses the expression of GFAP in the HSCs in the BDL rat model. Taken together with the histological findings, vitamin A administration may improve the histological damages and fibrosis in the BDL-treated liver by restoring vitamin A in the HSCs.

With regard to GFAP expression, there are a number of paradoxes. GFAP expression has been reported in normal livers, but rarely in fibrotic livers [23]. Conversely, in genome assay, Maubach et al. [24] reported that GFAP expression was upregulated in accordance with the HSCs activation. One reason for this apparent contradiction could be that the former findings came from in vivo experiments, while the latter from in vitro. In vivo experiments may allow interaction between HSCs and surrounding cells. Minicis et al. [25] determined gene expression changes in different models, and found that in culture- and in vivo-activated HSCs displayed a significantly different gene expression patterns. Our results suggest that GFAP expression was enhanced by activation of HSCs in vivo.

There are some limitations in this study to make a conclusion of anti-fibrotic efficacy of vitamin A administration on BDL-treated rat liver as a model of BA. First, most of this study is a qualitative observation with only one quantitative element. Hence, we should perform more quantitative analyses concerning chemical mediator of hepatic fibrosis such as TGF-beta/Smad pathway, common markers of fibrosis such as pro-collagen, and serum level of hyaluronic acid in the future. Also, further morphometric study should be required to elucidate the role of vitamin A in development of hepatic fibrosis. Second, the BDL model is an acute cholestatic model that is not equivalent to clinical situation of BA, which is rather chronic. Third, the expression of GFAP in electron microscopy showed distinct localization in the nuclei of HSCs. The reason of this localization is not clear. Garcia et al. [26] reported GFAP and nuclear lamins share an epitope recognized by the same monoclonal antibody. It may be possible that nuclear lamins sharing an epitope recognized by the same anti-GFAP antibody were detected in this experiment.

Despite of these limitations, this is the first study to investigate the effect of vitamin A on the damaged liver induced by BDL as a rat model of BA. Our results suggest that vitamin A administration may ameliorate hepatic fibrosis by restoring vitamin A level in the HSCs, and supplement of vitamin A in the clinical setting may contribute not only to improvement of the nutritional status but also to prevention of progressive hepatic fibrosis in BA patients.

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Leukocytapheresis in Pediatric Patients With Ulcerative Colitis

*Takeshi Tomomasa, †Hitoshi Tajiri, ‡Seiichi Kagimoto, §Toshiaki Shimizu, ||Atsushi Yoden, ¶Kosuke Ushijima, #Keiichi Uchida, **Hiroaki Kaneko, ††Daiki Abukawa, ‡‡Mutsuko Konno, §§Shun-ichi Maisawa, ||||Takao Kohsaka, ¶¶Akio Kobayashi, and the Japanese Study Group for Pediatric Ulcerative Colitis

ABSTRACT

Objective: Leukocytapheresis (LCAP) is a nonpharmacologic therapy that has recently been used to treat ulcerative colitis (UC). This multicenter open-label study prospectively assessed the efficacy and safety of LCAP in pediatric patients with UC.

Patients and Methods: Twenty-three patients ages 8 to 16 years with moderate (n = 19) to severe (n = 4) steroid-resistant UC were enrolled. One of 2 LCAP columns with different volumes (model EX and the half-volume model EI) was selected, according to body weight. LCAP was performed once per week for 5 consecutive weeks. Clinical and laboratory data were collected at predetermined time points. The primary endpoint was decreased stool frequency/hematochezia score, and secondary endpoints were clinical, laboratory, and endoscopic improvements.

Results: The stool frequency/hematochezia score decreased significantly from 4.5 ± 1.2 before treatment to 1.6 ± 1.9 after the fifth treatment. Clinical parameters, including stool frequency, presence of visible blood, abdominal pain, and body temperature, were significantly improved. Fecal calprotectin decreased significantly. Endoscopic findings evaluated using Matts score also improved ($P < 0.01$). The steroid dose decreased from 1.1 ± 0.4 mg/kg before treatment to 0.8 ± 0.5 mg/kg after treatment. There were no significant differences in changes between the EX and EI columns. The incidence of adverse effects was 61%, although none was serious. The most common adverse effects were decreased hematocrit and hemoglobin concentration.

Conclusions: The present study showed that LCAP was well tolerated in children with UC, mostly moderate, and was as effective as in adults. The types of pediatric patients best suited to LCAP remain to be determined.

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From the *Departments of Pediatrics, Gunma University Graduate School of Medicine, Gunma, the †Osaka General Medical Center, the ‡Saitama Children's Medical Center, the §Juntendo University School of Medicine, the ||Osaka Medical College, the ¶Kurume University Medical Center, the #Departments of Gastroenterology and Pediatric Surgery, Mie University Hospital, the **Mirai Children's Clinic, the ††Miyagi Children's Hospital, the ‡‡Departments of Pediatrics, Sapporo Kosei General Hospital, the §§Morioka Children's Hospital, the ||||Departments of Pediatrics, Tokyo West Tokushukai Hospital, and the ¶¶Showa University, Toyosu Clinic, Japan.

Address correspondence and reprint requests to Takeshi Tomomasa, MD, Department of Pediatrics, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan (e-mail: tomomasa@gunma-u.ac.jp).

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Leukocytapheresis (LCAP) has recently been developed in Japan as a possible treatment for active ulcerative colitis (UC). It is a form of extracorporeal therapy that removes leukocytes and blood platelets, which may be involved in inflammation, from the peripheral blood, by passage through a column housing a polyester fiber nonwoven fabric cloth rolled up into a cylindrical shape. The leukocyte adsorption principle of LCAP is attributed to the cells' adhesiveness and a sieving effect. It has been reported that 1 to 1.5×10^{10} leukocytes (granulocytes, monocytes, lymphocytes) and approximately 5×10^{11} blood platelets are removed during apheresis of 3 L of blood (1). LCAP has been shown to suppress inflammation by removing activated leukocytes that cause inflammation (2), normalizing platelet function by removing activated platelets (3,4), reducing the number of reactive oxygen-species–producing granulocytes (5), improving the effector T-cell/suppressor T-cell balance (6), and inhibiting proinflammatory cytokines (7,8).

In adult patients, LCAP has been reported to be more effective than increasing the steroid dose as a treatment for steroid-resistant disease (9–12), and may also be effective in steroid-free patients (13). The Japanese guidelines for the management of UC (14), which were developed by the Research Group for Intractable Inflammatory Bowel Disease, funded by the Japanese Ministry of Health, Labour, and Welfare, indicate LCAP for patients who fail to respond to 1 to 2 weeks of prednisolone (PSL) treatment at an adequate dose (30–40 mg/day).

Only a few retrospective case reports have been published on LCAP therapy in children (15,16), and its usefulness and safety in such patients are thus largely unknown. An additional problem is that the available columns are unsuitable for the treatment of pediatric patients with low body weights because of the large volume of extracorporeal circulating blood. A new column (model EI), which uses the same material as the EX column but with only half the volume, has recently been developed and is expected to increase the availability of LCAP for children. In the present study, we assessed the efficacy and safety of LCAP in pediatric patients with UC using the standard EX and the reduced-volume EI columns.

PATIENTS AND METHODS

Study Design

The present study was a prospective open trial conducted at 21 participating institutions. The study protocol was reviewed and approved by the institutional review board of each participating

institution. Age-appropriate patient consent or assent was obtained, in addition to informed parental consent.

Patient Selection

The present study enrolled subjects between the ages of 8 and 16 years with steroid-resistant moderate-to-severe UC, including 1 fulminant case. UC was diagnosed on the basis of standard clinical and endoscopic criteria. A patient was regarded as steroid-resistant when the decrease in stool frequency/hematochezia score was ≤ 1 after at least 2 weeks of oral or intravenous steroid therapy (PSL $\geq 1\,mg \cdot kg^{-1} \cdot day^{-1}$ or $\geq 40\,mg/day$), or if the score had increased by ≥ 1 at the end of the first week. Continuous use of preceding drugs (mesalazine, salazosulfapyridine, and azathioprine) was permitted on the condition that they had already been used for at least 2 weeks and that the dose was not changed or reduced during the period of the trial. Patients who were being treated with cyclosporine or steroid pulse therapy or who had participated in another clinical trial in the last 6 months were excluded.

Patients with the following conditions were excluded from the present study: toxic megacolon, serious kidney dysfunction or circulatory disorders, currently pregnant, currently breast-feeding, serious infection, a history of shock during extracorporeal circulation, a history of nafamostat mesilate hypersensitivity, prescribed an angiotensin-converting enzyme inhibitor, a blood platelet count $<100,000/\mu L$, and a white blood cell count $<3000/\mu L$.

LCAP Treatment

During LCAP treatment, blood was continuously drawn from a cubital or femoral vein into the extracorporeal circuit by a blood pump, passed through the column, and then returned to a cubital or femoral vein on the opposite side of the inlet. In the present study, 2 types of columns with different volumes were used. The EX column used in clinical practice has a volume of 170 mL, whereas the recently developed EI column has a volume of 90 mL, approximately half the volume of the EX. The choice between the EX and EI columns depended on the patient's body weight. The EI column was used for patients with a body weight of 20 to 30 kg, and the EX column was used for patients weighing ≥ 40 kg. The investigator in charge decided which column to use for patients who weighed between 30 and 40 kg, based on each patient's general condition.

The volume of blood processed during a single treatment with either column was 30 to 50 mL/kg. Treatment was performed at a blood flow rate of 30 to 50 mL/min with the EX and 15 to 25 mL/min with the EI column. Nafamostat mesilate was used as the anticoagulant.

Study Protocol

LCAP therapy was performed once per week for 5 consecutive weeks in moderate and severe cases. In the fulminant case, LCAP was performed twice during the first week, and then once per week for the next 4 weeks. LCAP therapy could be performed once per week for an additional 5 consecutive weeks from the sixth week onward, at the discretion of the investigator in charge.

The primary endpoint was a decrease in baseline (pretreatment) stool frequency/hematochezia score after 5 treatments. Presence or absence of remission, stool frequency before treatment and after 5 treatments, hematochezia, body temperature, abdominal pain, steroid dose, serum C-reactive protein level, erythrocyte sedimentation rate, hemoglobin concentration, fecal calprotectin, Lichtiger clinical activity index (CAI) (17), and Matts endoscopic score (Appendix A) (18) were assessed as secondary endpoints.

The stool frequency/hematochezia score was calculated as the sum of the diarrhea frequency score and visible fecal blood score using Lichtiger CAI. Severity of UC was defined according to the diagnostic criteria for Crohn disease and UC, modified Truelove criteria, published by the Ministry of Health, Labour, and Welfare (Appendix B) (14). Remission was defined as no fever, no abdominal pain, no blood in the stools, a normal frequency and consistency of bowel movements, and normal erythrocyte sedimentation rate (≤ 30 mm/h) and C-reactive protein (≤ 1.0 mg/dL) values (19).

In addition, we assessed the number of patients with improvements, defined as reductions in stool frequency/hematochezia score ≥ 2 points or reduction in Matts endoscopic score ≥ 1 grade. We also evaluated the decrease in PSL dose after LCAP therapy.

Most patients enrolled in the present study had moderate disease and only 4 had severe/fulminant disease as described below; thus, the effect of disease severity on the efficacy of treatment was not clearly demonstrated. We retrospectively classified the patients into 2 groups according to disease severity and PSL administration: group 1 ($n = 15$) included patients with moderate disease treated with oral prednisone and group 2 ($n = 8$) included patients with severe/fulminant disease ($n = 4$) or patients treated with intravenous steroids ($n = 4$), and compared stool frequency/hematochezia scores before and after LACP treatment between these 2 groups.

Statistical Analysis

Last observation carried forward was used as a substitute for the next measurement after 5 treatments; however, if no supplementary data other than baseline data existed because of discontinuation or dropping out, the worst value of all of the subject's data was substituted. Numerical data were analyzed using Wilcoxon signed rank-sum test, and categorical data were analyzed using the Fisher exact test. Values of $P < 0.05$ were considered to be significant.

RESULTS

Twenty-three patients ages 8 to 16 years were enrolled. Their backgrounds are shown in Table 1, and the patient disposition is shown in Figure 1. Eleven patients were treated using an EI and 12 using an EX column. LCAP treatment was discontinued and switched to another form of treatment in 1 patient because the abdominal pain and fever associated with UC became more severe during the first course of treatment.

Clinical Outcome

The stool frequency/hematochezia score decreased significantly, from 4.5 ± 1.2 before the series of LCAP therapy to 1.6 ± 1.9 after LCAP therapy ($P < 0.01$). The remission rate was 9/23 (39%) patients, and clinical improvements were noted in 19/23 (83%) patients. Stool frequency, hematochezia, and abdominal pain improved in 74%, 78%, and 63% of patients, respectively. There was no change in body temperature after treatment. Lichtiger CAI score also decreased significantly, from 9.9 ± 4.1 after the first LCAP to 5.4 ± 4.5 after the fifth LCAP ($P < 0.01$). Matts endoscopic score improved significantly from 0/0/12/4 (grade 1/2/3/4) before therapy to 4/9/2/1 (grade 1/2/3/4) after therapy ($P < 0.01$). The PSL dosage decreased in 18/23 (78%) patients, from

TABLE 1. Patient characteristics

Factor	Category classification	Data	%
Sex	Male	16	70
	Female	7	30
Age, y	13.2 ± 2.3		
Body weight, kg	38.2 ± 11.4		
Duration of illness, mo	18.1 ± 23.2		
Pathological type	Pancolitis type	15	65
	Left-sided colitis type	8	35
Severity	Moderate	19	83
	Severe	3	13
	Fulminant	1	4

Data are numbers with percentages in parentheses, or mean ± SD.

1.1 ± 0.4 mg/kg before therapy to 0.8 ± 0.5 mg/kg after therapy. Fecal calprotectin and the leukocyte count were also lower after therapy than before therapy (Table 2).

The changes in stool frequency/hematochezia score and Lichtiger CAI score are shown in Figure 2. Both scores decreased in a similar manner with each treatment, with a correlation coefficient of $r = 0.8986$ (Fig. 3).

The changes in stool frequency/hematochezia score between baseline and after treatment using the EX and EI columns are shown in Figure 4. The stool frequency/hematochezia score decreased significantly, from 4.8 ± 1.3 before treatment to 1.5 ± 2.1 after treatment with EI, and from 4.3 ± 1.1 before treatment to 1.8 ± 1.9 after treatment with EX. Matts endoscopic score improved in both groups after treatment.

The stool frequency/hematochezia scores before and after LACP treatment in group 1 were 3.9 ± 1.4 and 0.7 ± 1.7, and those in group 2 were 5.8 ± 1.0 and 2.0 ± 2.4, respectively.

Adverse Effects

Adverse effects were reported in 14 of the 23 patients (61%): 5/11 (45%) with EI and 9/12 (75%) with EX. The most common adverse effects were a decrease in hematocrit in 6 patients (26.1%), pain at the infusion site in 5 patients (21.7%), and a decrease in red blood cell count in 3 patients (13.0%). None of the adverse effects were considered to be serious, and none necessitated discontinuation or interruption of treatment.

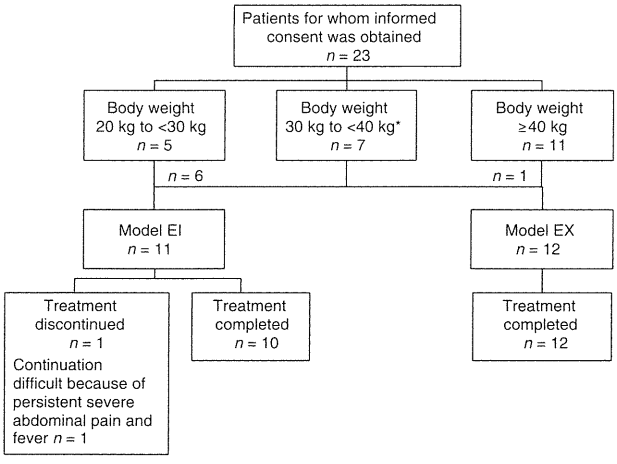

FIGURE 1. Patient disposition. *Decided by physician based on the patient's conditions.

DISCUSSION

Two types of cytapheresis therapy, LCAP and granulocyte/monocyte apheresis (GMA), have recently been developed for UC in Japan. Circulating granulocytes, monocytes, and lymphocytes are removed by LCAP using a Cellsorba column (Asahi Medical, Tokyo, Japan), whereas granulocytes and monocytes are removed by GMA using an Adacolumn (JIMRO, Takasaki, Japan), with little effect on lymphocytes. Cytapheresis therapy has been used to treat many adult patients with UC (9–13,20), and its efficacy and safety have been reported. Although a few reports have also suggested its usefulness in children (15,16,21–23), these were case reports with small numbers of patients, and most of them used GMA (21–23).

The present study was performed to determine the efficacy and safety of LCAP in steroid-refractory pediatric patients. The data showed a significant decrease in the stool frequency/hematochezia score, from 4.5 ± 1.2 before a series of LCAP therapy, to 1.6 ± 1.9 after LCAP therapy. Improvement, defined as a decrease in stool frequency/hematochezia score by ≥1 or a decrease in Matts endoscopic score by ≥1 grade, was achieved in 19 out of 23 patients (83%). In addition, laboratory parameters such as fecal calprotectin and white blood cell count were reduced after therapy. The results of a randomized multicenter trial in adult patients with moderate and severe steroid-resistant UC showed an improvement rate of 74% in the LCAP group, which was significantly higher than the 38% improvement rate in the intensive PSL therapy group, in which the dose of PSL was increased (9). Another double-blind study using sham columns found an improvement rate of 80% in the LCAP group, which was significantly higher than the 33% in the sham-column group ($P < 0.05$) (10). The improvement rates in children in response to LCAP shown in the present study suggest that this treatment has an efficacy similar to that demonstrated in adults.

Some previous reports have suggested that some adverse effects of steroids are more serious in children than in adults (24,25). In the present study, the steroid dose was reduced in 18/23 (78%) patients, compared with the dose before LCAP therapy, suggesting that LCAP may be helpful in allowing a reduction in steroid dose in pediatric UC, thus mitigating the adverse effects of steroids.

The present study was performed as an uncontrolled study because the use of sham columns is ethically unacceptable in clinical trials involving children. Some reports, however, have shown improvements in the natural course of UC (26), and the results of the present study may therefore have included the effects of natural changes in the course of the disease. It should also be noted that the long-term effects of this therapy were not determined in the present study, and further studies are needed to explore this aspect.

In the present study, the effect of disease severity on the efficacy of LCAP was not clear. Previous studies have reported reduced or delayed efficacy of LCAP therapy in patients with deeply penetrating ulcers or in steroid-dependent patients, suggesting that not all patients with UC benefit from LCAP (12). Further clinical studies are needed to confirm the usefulness of LCAP therapy in children and to determine the type of pediatric patients in whom LCAP is indicated.

TABLE 2. Clinical test values before and after treatment

Parameter	Overall, n = 23		
	Pre	6W*	Results of the statistical analysis**
Fecal calprotectin	6636.2 ± 13,667.9	2568.1 ± 3564.3	$P < 0.05$
RBC count, $\times 10^4/\mu L$	431.0 ± 55.2	422.7 ± 66.8	NS
WBC count, /μL	14,181.7 ± 7951.8	9745.2 ± 4982.1	$P < 0.01$
Platelet count, $\times 10^4/\mu L$	40.0 ± 22.2	34.7 ± 14.4	NS
Hemoglobin, g/dL	11.6 ± 2.2	11.5 ± 2.0	NS
CRP, $mg \cdot dL^{-1} \cdot qualitative^{-1}$	1.1 ± 1.6	0.7 ± 1.5	NS
ESR, mm/h	32.5 ± 27.3	20.5 ± 16.6	NS

All data are mean ± SD. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; NS = nonsignificant; RBC = red blood cell; WBC = white blood cell.

* RBC count, WBC count, platelet count, hemoglobin, and CRP data at the time that treatment was stopped were used as the posttreatment data for the patient whose treatment was discontinued after the first week. Fecal calprotectin and ESR were not measured after treatment, and the worst data from all of the patients (calprotectin: 11,195.9 μg/g; ESR: 52.0 mm/h) were substituted.

** *P* values according to Wilcoxon signed rank-sum test.

The usual recommended volume of extracorporeal circulating blood is 10% of the patient's total blood volume. The volume of extracorporeal circulating blood using the EX column is the sum of the column volume (170 mL) and the volume of the circuit. The volume in patients whose body weight is <30 kg may thus exceed 10%, making the treatment difficult to perform. In the present study we used the newly developed EI column (90 mL), the volume of which is half that of the EX. The flow rate of circulating blood also can be reduced with the EI to half that required with the EX. The results showed an improvement in 10/12 (84%) patients with UC, younger than 18 years of age, weighing 38 to 60 kg, and treated with EX, and in 9/11 (82%) patients younger than 18 years of age, weighing 21 to 36 kg, and treated with EI. This suggests that LCAP using a column with a smaller volume can be an effective treatment in patients with low body weight.

Adverse effects occurred in 14/23 (61%) patients. The nature of the adverse effects was similar to that previously reported for other types of apheresis treatment, and none of them were serious. An iron preparation was used in some patients to treat the most common adverse effect, anemia, manifested by a low hematocrit, and all of these patients recovered. The next most common adverse effect was pain at the infusion site. It is possible that the reported frequency of this effect was high because the study subjects were children, who are more afraid and less tolerant of injection pain.

Although several different types of CAI score (17,27,28) have been used as evaluation indices for UC in adult patients, we developed stool frequency/hematochezia score as a primary endpoint for use in the present study. The previous scores include several items that require subjective judgment, such as abdominal pain and feelings of well-being; however, we considered that the primary endpoint should not involve highly subjective items because the open-label nature of the study meant that the impressive appearance of an apheresis device may be associated with a considerable placebo effect. The Pediatric UC Activity Index (29) was not available at the start of this trial. We therefore

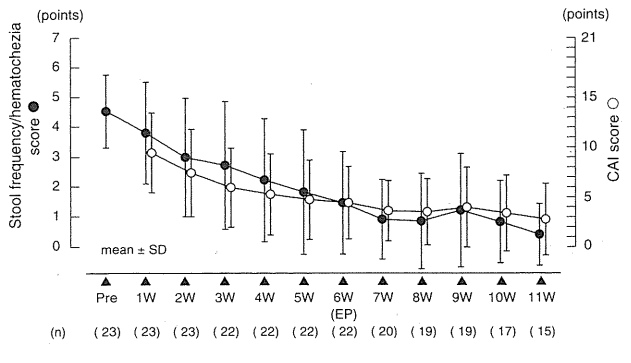

FIGURE 2. Changes in stool frequency/hematochezia score and CAI score. Treatment of 1 patient was discontinued after 1 week. After the endpoint at 6 weeks of treatment, observation was continued until 11 weeks, and observations ended when any treatment besides LCAP was added. CAI = clinical activity index, EP = endpoint.

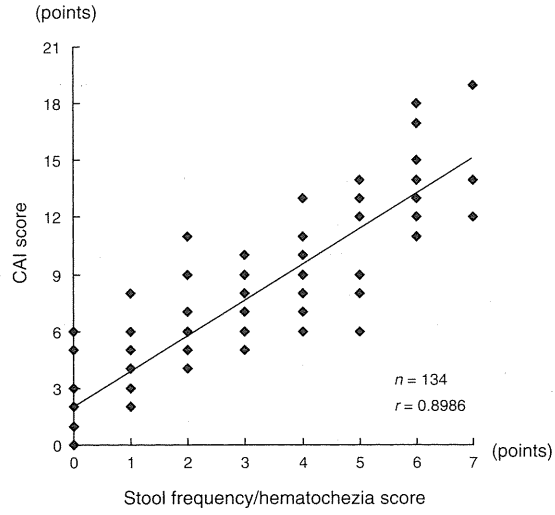

FIGURE 3. Correlation between stool frequency/hematochezia score and CAI score. CAI = clinical activity index.

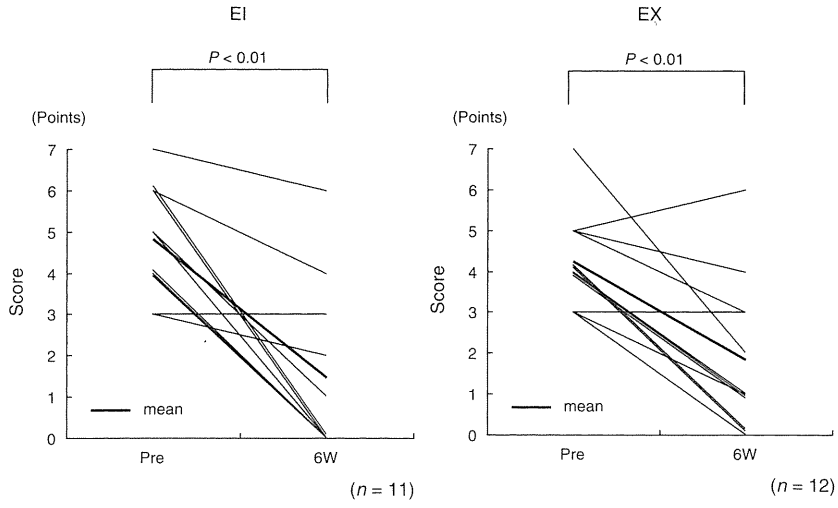

FIGURE 4. Changes in stool frequency/hematochezia score (before/after treatment). Data at the time treatment was discontinued were used as the posttreatment data for the patient whose treatment was discontinued after 1 week. *P* values according to the Wilcoxon signed rank-sum test.

developed a special score using the 2 main items from Lichtiger CAI, to produce a score that was as simple and objective as possible. This score was highly correlated with Lichtiger CAI ($r = 0.8986$).

In summary, by selecting an appropriate column for the patient's body weight, LCAP could be performed safely and effectively in pediatric patients with UC, despite a wide range of ages and body weights. The efficacy of the procedure was comparable to that performed in the adult population. The results of the present study suggest that LCAP can be a useful medical procedure for pediatric patients in the active phase of UC, and further studies are required to determine the types of pediatric patients best suited to LCAP therapy.

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APPENDIX A
MATTS SCORE[18]

Score	Description
1	Normal
2	Mild granularity of the mucosa, with mild contact bleeding
3	Marked granularity and edema of the mucosa, contact bleeding, and spontaneous bleeding
4	Severe ulceration of mucosa with hemorrhage

APPENDIX B
CLASSIFICATION OF UC BY SEVERITY[14]*

Variable	Severe	Moderate	Mild
(1) Frequency of defecation	≥6 times	Intermediate between severe and mild	≤4 times
(2) Apparent melena	(+++)		(+) to (−)
(3) Fever	≥37.5°C		Absent
(4) Tachycardia	≥90/min		Absent
(5) Anemia	Hb ≤10 g/dL		Absent
(6) Erythrocyte sedimentation rate	≥30 mm/h		Normal

Rated as "severe" when criteria (1) and (2) and 1 of the systemic symptoms (3) or (4) are satisfied, and at least 4 of the 6 criteria are satisfied. Rated as "mild" when all of the 6 criteria are satisfied. Among patients with "severe" disease, those showing extremely severe symptoms are classified as having "fulminant" disease, and, depending on the rapidity of the disease onset, "fulminant" disease is subdivided into "acute fulminant" and "relapsing fulminant" disease. Criteria for the diagnosis of fulminant UC: patients satisfying all of the following criteria are classified as having "fulminant" disease:

(1) Satisfying the criteria for "severe" disease.
(2) Bloody stools occurring at a frequency of about 15 times/day or more.
(3) Persistent high fever (38°C or higher).
(4) Increase of the leukocyte count to 10,000/mm^3 or more.
(5) Severe abdominal pain.

Hb = hemoglobin; UC = ulcerative colitis.
*Modification of Truelove criteria.

ラット小腸移植における ischemic preconditioning および remote ischemic preconditioning の有用性

佐伯　勇　松浦俊治　林田　真
柳　佑典　田口智章

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東京医学社　〒113-0033 東京都文京区本郷 3-35-4
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■ 特集 小児移植医療―最近の話題

ラット小腸移植における ischemic preconditioning および remote ischemic preconditioning の有用性

佐伯 勇* 松浦俊治 林田 真
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はじめに

小腸は虚血再灌流により障害を受けやすい臓器であり，障害を受けた小腸は，粘膜障害から bacterial translocation を引き起こし，急性肺障害や敗血症の原因となる[1,2]。しかし，小腸の虚血再灌流障害のメカニズムは未だに明確にはわかっておらず，それを防ぐ有効な手段もないのが現状である。

1986 年に Murry ら[3]は，イヌの心筋において 5～6 回の 5 分間の虚血と再灌流のサイクルが，その後の長時間の虚血再灌流障害から心筋を保護する効果があると報告した。Ischemic preconditioning（IPC）とよばれるその効果は，その後小腸を含む多くの臓器で有効性が証明され，そのサイクルも，10～15 分の 1 回の虚血と再灌流を行うだけで防御効果があることが示されている[4～6]。現在諸外国ではすでに，臓器移植に対する IPC の臨床研究が行われている[7,8]。

また，IPC はそれを行った臓器だけでなく，そのほかの臓器にも防御効果をもたらすことが知られており，remote ischemic preconditioning（RIPC）とよばれている[9～11]。RIPC は IPC と異なり，複数回の短時間の虚血再灌流を行ったほうが，効果があるとされている。RIPC は IPC と比べ安全で容易に施行することができることから，とくに心血管領域において臨床研究が進んでおり[12]，2010 年に Bøtker ら[13]は，急性心筋梗塞患者で，搬送から経皮的冠動脈血管形成施行までのあいだに，血圧計のマンシェットを用いて四肢の RIPC を行い，リスクの高い梗塞巣の回復率を高めることができたと報告している。現在までに，心血管系以外の多くの臓器でも RIPC の有効性が示されているが，小腸冷虚血再灌流障害に対する RIPC の効果は未だ示されていない。

IPC，RIPC の機序にはメディエーター説，神経伝達説などさまざまな説があり，熱蛋白の一種である heme oxygenase-1（HO-1）や，NF-κB，NO などの関与が示唆されている[11]。HO-1 はヘムをビリベルジンと CO へと酸化する律速酵素であり，ビリベルジンはさらにビリルビンへと代謝され，CO とともに強い抗酸化作用を有する（**図 1**）[14]。小腸の熱障害やエンドトキシンショック，炎症性腸疾患においても HO-1 の発現が認められており，防御効果をもたらしていると考えられている[15～17]。

本稿では，ラット小腸移植モデルを用いて，小腸の冷虚血再灌流障害に対する IPC と RIPC の効果を検討し，またその防御効果への HO-1 の関与の検討を行った。

Ⅰ．対象と方法

200～300 g のオスの Lewis ラットをドナーおよびレシピエントとして用い，ラット同系異所性小腸移植を施行した。ドナーラットより Treitz 靭帯から 30 cm の空腸を採取し，レシピエントの下大動脈とグラフト上腸間膜動脈（SMA），下大静脈とグラフト門脈をそれぞれ端側吻合で吻合した。小腸冷虚血時間は 3 時間とし，4℃のラクテー

Isamu Saeki Toshiharu Matsuura Makoto Hayashida Yusuke Yanagi Tomoaki Taguchi
* 九州大学大学院医学研究院小児外科学分野
〔〒812-8584 福岡市東区馬出 3-1-1〕

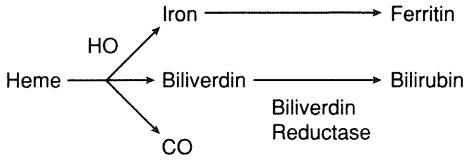

図 1　Heme Oxygenase System
(Nakao ら[14], 2008 より引用一部改変)

トリンゲル液で冷保存を行った。

小腸移植のみ行ったコントロール群，グラフト採取前に SMA の 10 分クランプ 30 分オープンを行った IPC 群，グラフト採取前に大動脈下部の 15 分クランプ 15 分オープンを 3 サイクル行った RIPC 群の 3 群に分け，検討を行った（各群 n＝20，**図 2**）。

移植後 3，6，12，24 時間で犠死させて標本を採取し，血液生化学的検討および病理学的検討を行った。また，ELISA を用いて血中 HO-1 の測定を行った。

II．結　果

ラット同系異所性小腸移植の手術時間は各群ともにドナーラットが 90〜110 分，レシピエントラットが 55〜65 分で差は認められず，グラフトの温虚血時間も 22〜30 分と各群で差はなかった。

小腸移植後 3 時間でのグラフト腸管の組織所見を**図 3** に示す。コントロール群（**図 3A**）では虚血再灌流障害に伴う粘膜の脱落が認められるが，IPC 群（**図 3B**）と RIPC 群（**図 3C**）では粘

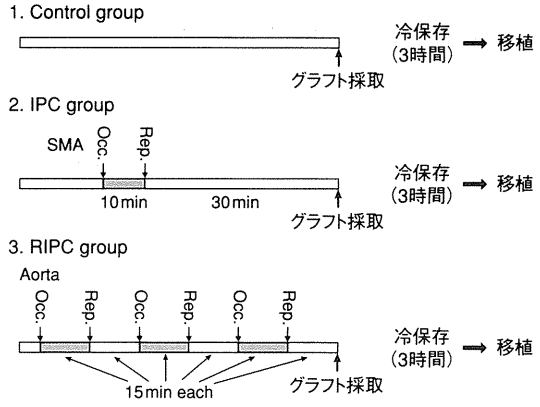

図 2　ラット小腸移植実験プロトコールとグループ分類
IPC 群では 10 分間の SMA の虚血 30 分の再灌流を 1 回施行。RIPC 群では下大動脈の 15 分間の虚血，15 分間の再灌流を 3 サイクル施行した。
Occ.：クランプ　Rep.：再灌流

膜がほぼ正常に保たれている。粘膜障害度を Park 分類[18]によって数値化し，経時的変化をみたものを**図 4** に示す。IPC 群と RIPC 群では，移植後 3 時間後，6 時間後で有意に粘膜障害度が軽度であった。しかし，移植後 12 時間後や 24 時間後ではコントロール群とのあいだに差はなくなっていた。

血液生化学的所見においては，LDH が小腸の粘膜障害の指標となるとされているが[4,19]，LDH は小腸移植後 3 時間で IPC 群および RIPC 群ではコントロール群と比べ有意に低値を示していた（**図 5A**）。また，AST 値および ALT 値も同様に，小腸移植後 3 時間で IPC 群および RIPC 群では

図 3　小腸移植 3 時間後のグラフトの組織所見（HE 染色）
A．コントロール群，**B**．IPC 群，**C**．RIPC 群。
コントロール群では粘膜の脱落が認められるが，IPC 群と RIPC 群では粘膜が保たれている。

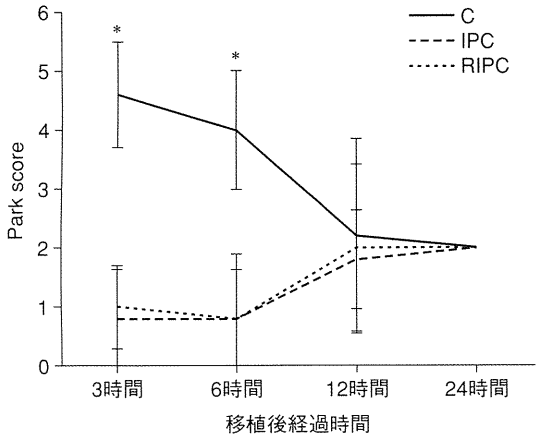

図 4　移植後の小腸グラフトの粘膜障害度（Park 分類）の経時的推移
移植後 3〜6 時間では，コントロール群に比べ，IPC 群，RIPC 群で有意に粘膜障害が軽減している。

コントロール群と比べ有意に低値を示していた（**図 5B, C**）。

ELISA を用いて測定したレシピエントラット血中の HO-1 濃度は，移植後 3 時間で IPC 群および RIPC 群ではコントロール群と比べ有意に高値を示していた（**図 6**）。

Ⅲ. 考　察

この研究において，IPC および RIPC が小腸の早期の虚血再灌流障害に対して，著明な抑制効果をもたらすことが示された。小腸の障害を示すマーカーとして用いられる LDH は，IPC および RIPC を施行した群では，コントロール群に比べて有意に低下していた。また組織学的評価においても，IPC や RIPC を施行することで粘膜障害度が有意に低下していた。

IPC は最初に心筋においてその効果が認められ[3]，その後小腸を含む多くの臓器でその有用性が証明されているが[6,7,20]，小腸の冷虚血に対する効果を報告したものは少なく，IPC のサイクルも一定したものはない[21]。しかし，ラット小腸の温虚血に対する IPC の有用性を報告したものの多くは 10 分の虚血とその後の 30 分の再灌流というプロトコールを用いており，今回の冷虚血に対する実験でも，同様のプロトコールを用い，効果的であった。

RIPC の報告に関しては，ラット小腸の冷虚血再灌流障害に対する RIPC の効果をみた報告はこれまでにない。Vlasov ら[20]は 1 サイクルの後足の 15 分の虚血と 30 分の再灌流を，長時間の小腸温虚血再灌流の前に施行したが，十分な防御効果はなかったと報告している。しかし現在，多くの臓器で RIPC は 1 サイクルよりも複数サイクルの虚血再灌流を行ったほうが有効であるとされており[9]，Ren ら[10]はラットの脳の温虚血に対して，後足の 15 分の虚血と 15 分の再灌流を 3 サイクル施行したほうが，2 サイクル施行した群よりも防御効果が高かったと報告している。このことから，本研究においても，RIPC として下大動脈の 15 分の虚血と 15 分の再灌流を 3 サイクル施行するプロトコールを用いることで，IPC と同等の効果を得ることが可能であった。

IPC と RIPC のメカニズムに関しては未だ明確にはわかっていないが，IPC や RIPC を施行することにより，血中に NF-κB，NO，熱蛋白などが

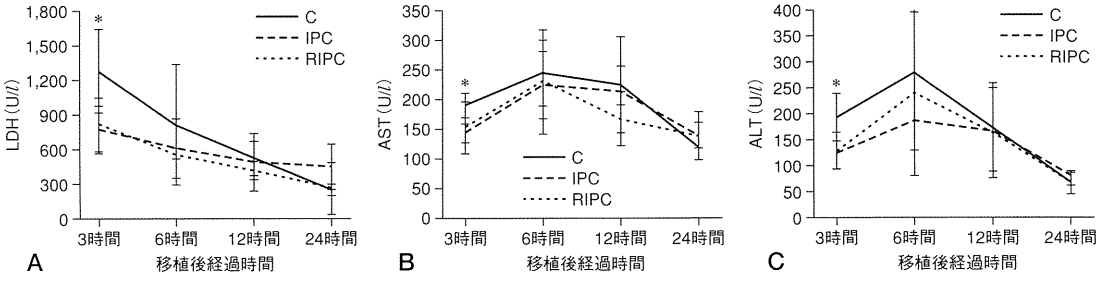

図 5　血液生化学所見
A. LDH, **B**. AST, **C**. ALT。
小腸移植後 3 時間ではコントロール群に比べ IPC 群，RIPC 群で有意に LDH, AST, ALT が低値となっている。

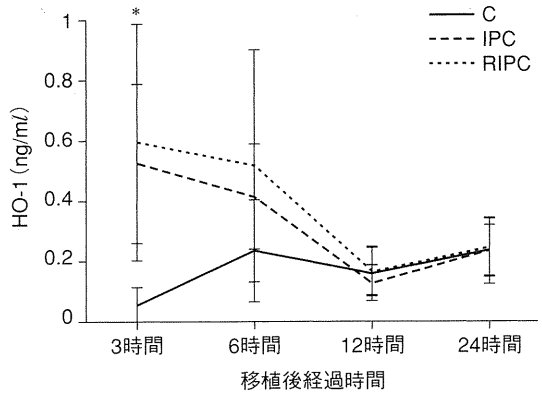

図 6 血中 HO-1
移植後 3 時間ではコントロール群に比べ，IPC 群，RIPC 群で有意に HO-1 の発現が認められる。

放出されていると報告されている[5~9]。Mallick ら[4]は，小腸温虚血再灌流障害に対する IPC の有効性の報告を行い，そのなかで IPC を施行した群では血中の HO-1 濃度がコントロール群に比べ，有意に上昇していたと報告している。また，Lai ら[11]はラットの肝臓の虚血再灌流障害に対して後足での RIPC を行い，RIPC の防御効果が肝臓内での HO-1 発現と関連していたと報告している。本研究においても，IPC 群，RIPC 群ともに血中の HO-1 濃度は移植後 3 時間でコントロール群と比べ有意に上昇していた。

今回の検討では，ドナーラットに IPC もしくは RIPC をすることで，レシピエントラットの血中 LDH や肝逸脱酵素が低下しており，防御効果が認められた。また，レシピエントラットの血中の HO-1 が上昇しており，IRI に対する防御効果をもたらしていると考えられた。移植されたグラフトや体液を通じて防御効果がもたらされることは，以前にも報告されている。Dickson ら[22]は，IPC を施行したラットの冠状動脈の浸出液を別のラットに投与することで，心筋 IRI からの防御効果がもたらされるとしており，Oltean ら[23]はタクロリムスを投与したドナーラットから肝臓を移植したレシピエントラットで，サイトカインの放出が抑えられ，肝障害が軽減すると報告している。本研究においても，IPC や RIPC を施行されたドナーラットからグラフト小腸が移植されることにより，レシピエントラットにおいて HO-1 の活性化が引き起こされ，防御効果をもたらしたという仮説が考えられる。

IPC によってもたらされる防御効果は大きく 2 つの phase があるとされている。IPC を施行して 1～3 時間の比較的早期の early phase と，IPC 後 24 時間から 3 日ほど続くとされている late phase である[7,8]。この時間は臓器によって異なり，片方の phase が認められない臓器もあると報告されている。本研究では，IPC および RIPC の効果は 3～6 時間で消失しており，early phase の防御効果として矛盾しない結果であった。小腸粘膜上皮の turn over は非常に早く，3 時間の冷虚血での虚血再灌流障害では，コントロール群の小腸粘膜障害も 24 時間後にはわずかなものとなっているため，小腸における IPC および RIPC の late phase の検討には，プロトコールの変更が必要であると考えられた。

おわりに

IPC や RIPC は特殊な手技や薬剤を用いることなく安全に施行することが可能である。とくに RIPC は，ターニケットや血圧計マンシェットを用いて四肢の短時間の虚血再灌流を行うことで効果を得ることができることから，非常に安全かつ簡便に施行することができる[13]。今回の検討により，小腸の冷虚血再灌流障害に対して RIPC が IPC と同等の防御効果をもたらすことを証明した。今後の小腸移植において RIPC が虚血再灌流障害に伴うさまざまな合併症を減少させるのに有用な手段となっていくことが期待される。

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■ 特集 小児における消化管機能障害の診断と治療

Hirschsprung 病の組織診断

田口智章* 手柴理沙 佐伯 勇 家入里志

はじめに

Hirschsprung 病（H 病）の診断は注腸造影，直腸肛門内圧検査，粘膜生検が 3 種の神器とされてきたが，注腸造影と内圧検査は偽陽性や偽陰性の率がある程度高いので確定診断までにはいたらない。もちろん直腸肛門反射が陽性に出れば H 病が否定できるし，病変範囲を推定するには注腸造影が必要である。しかし H 病を確定診断するには粘膜生検標本のアセチルコリンエステラーゼ（AchE）染色がもっとも正診率が高く，H 病の診断には必要不可欠である。また組織診断として，直腸全層生検や術中迅速診断，さらに H 病類縁疾患（H 類縁）の組織診断も重要なテーマである。これらについて筆者らの経験および文献的知見について述べる。

Ⅰ．直腸粘膜生検の AchE 染色

H 病の無神経節腸管では通常であれば外来性の神経線維が神経節細胞にシナプスをつくるべきなのが，神経節細胞が存在しないために，粘膜下層，粘膜筋板，粘膜固有層に無制限に伸びている。これは「変電所がないために高圧電線が直接家庭に配線される」とたとえられる。この性質を利用して，この神経線維を Karnovsky & Roots の AchE 染色で観察する方法である。この方法が H 病の診断に応用できることを Meir-Ruge により提唱され，正診率が高いため広く用いられるようになった。諸家の報告をまとめると sensitivity 100%，specificity 91～96%となっている[1]。

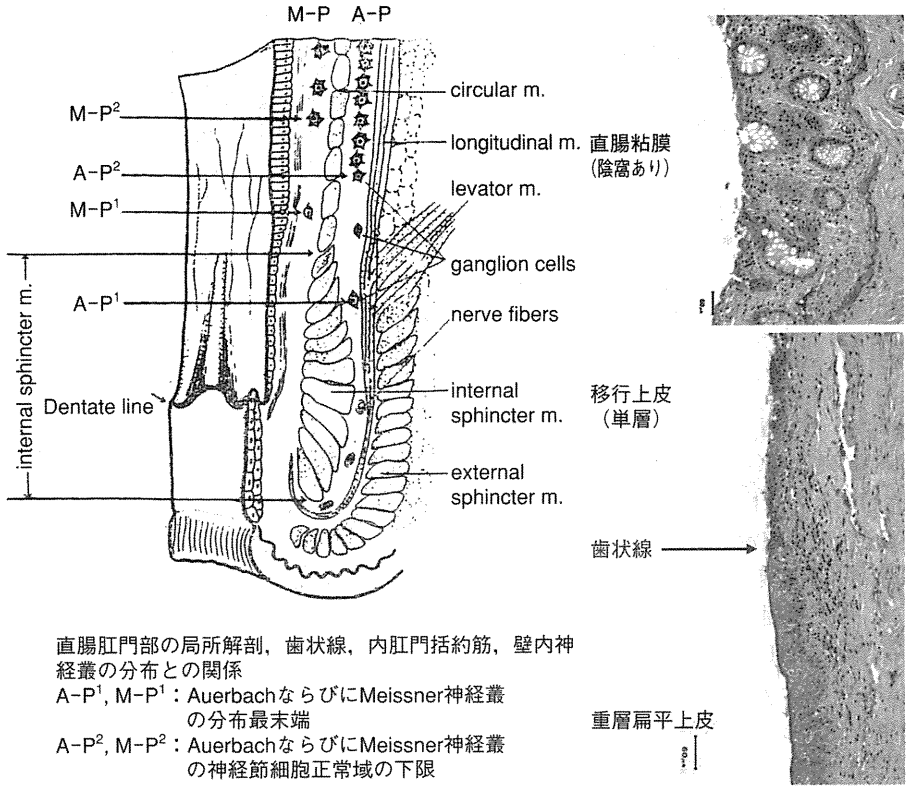
Tomoaki Taguchi Risa Teshiba Isamu Saeki Satoshi Ieiri
* 九州大学大学院医学研究院小児外科
〔〒812-8582 福岡市東区馬出 3-1-1〕

1．標本採取法

標本の採取は吸引生検や punch 生検が行われている。われわれはプラスチックの透明な試験管に穴をあけ粘膜がもりあがってきたところを耳鼻科用の生検鉗子で粘膜生検している[2]。生検に際しては，深さと部位が重要である。まず深さは粘膜下層まで採取する。部位は直腸粘膜の陰窩が存在する部位で採取する。歯状線よりも外側は重層扁平上皮，歯状線は単層の移行上皮，直腸になると陰窩を有する円柱上皮になる（**図 1**）。標本としてこの直腸粘膜が採取されていることが最低必要条件である。さらに神経節細胞が存在する下限を認識しておく必要がある。これは採取した標本に神経節細胞が存在しない場合にそれが正常か判断するのに必要な情報である。教室の久米[3]が小児の剖検例で詳細な検討を行った結果，粘膜下神経叢（Meissner 神経叢）の分布は新生児では歯状線から 1 cm 以内にも存在するが，乳児では 1～1.5 cm 以内には存在せず，幼児学童では 1.5 cm 以内には存在しないことが証明された（**図 2A**）。したがって新生児では歯状線より 1 cm 程度上方でもよいが（**図 3**）[4]，乳幼児以上はさらに上方で採取しないと神経節細胞が入ってなくても正常ということになる。実際に吸引生検や punch 生検を行う場合は，歯状線は確認できず肛門縁からの距離で粘膜生検を行う。その場合に推奨される肛門縁からの距離は年齢で異なる（**表 1**）。これは肛門縁から歯状線までの距離も成長に伴って伸びるためである。

2．染色法

染色法は，まず標本を凍結し，それをクリオスタットで 10 μm に薄切し，スライドグラス上に進展乾燥。その後染色液の Karnovsky 液（淡緑色液）



直腸肛門部の局所解剖，歯状線，内肛門括約筋，壁内神経叢の分布との関係
A-P¹, M-P¹：Auerbachならびに Meissner神経叢の分布最末端
A-P², M-P²：Auerbachならびに Meissner神経叢の神経節細胞正常域の下限

図 1 直腸肛門部の病理 （久米[3]，1971）

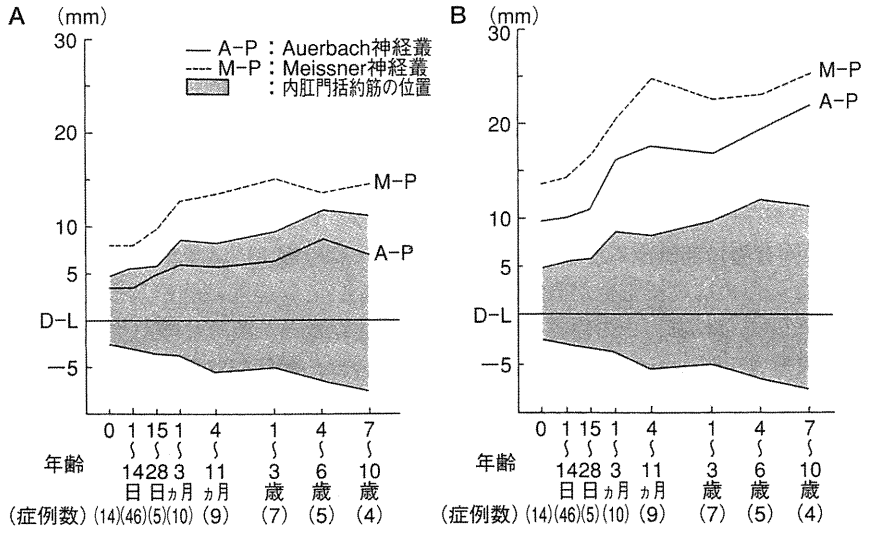


図 2 小児の直腸肛門部における正常神経節細胞の分布

A．Auerbach および Meissner 神経叢の分布下限
B．正常の Auerbach および Meissner 神経叢の分布下限 （久米一弘[3]，1971 より改変）

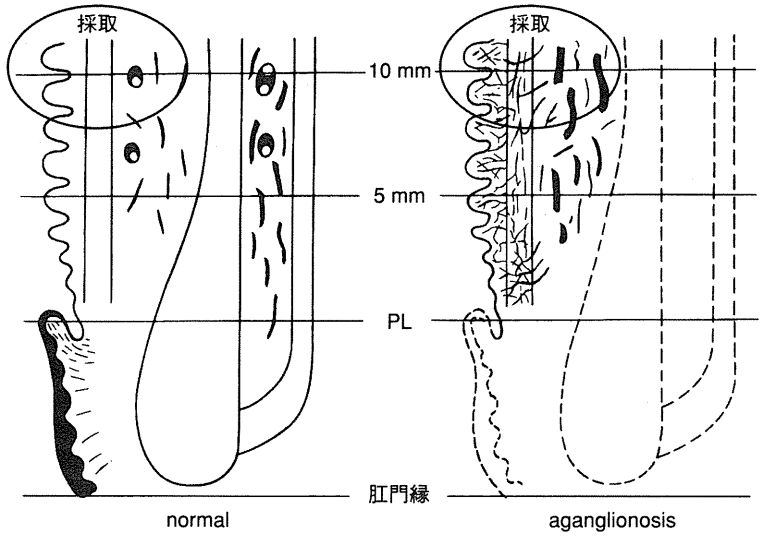


図 3 AchE 陽性線維の分布様式（新生児）
（渡辺ら[4]，1977 より引用）

表 1 直腸粘膜生検の採取部位

年齢	肛門縁からの距離
新生児	1.5 cm
乳児	2.0〜2.5 cm
1〜3 歳	2.5〜3.0 cm
年長児	3.0 cm

表 2 AchE 染色（ルベアン酸増強法）

1．10 μm 厚で，凍結切片作成
2．A 液（15〜20 分）
① sodium hydrogen maleate buffer（pH6）：6.5 ml（0.1 mol/l）
② クエン酸ナトリウム：0.5 ml（0.1 mol/l）
③ 硫酸銅：1 ml（30 mmol/l）
④ フェリシアン化カリウム：1 ml（5 mmol/l）
⑤ 水：1 ml
⑥ ヨウ化アセチルコリン：10 mg
3．B 液（5〜10 分）
① ルベアン酸：10 mg
② 酢酸ナトリウム：6.55 g
③ 100％エタノール：10 ml
4．脱水，封入

につける。われわれはさらにルベアン酸（透明液）で増強している（**表 2**）。標本の色は Karnovsky 液で茶色に染まりルベアン酸で黒色に変わる。

3．組織診断

神経節細胞は胞体は染色されるが核は抜けて見える。正常では粘膜下層に AchE に染まる神経節細胞が存在し，神経線維は粘膜筋板や粘膜固有層にはみられない。一方 H 病では粘膜下層に太い神経線維束（bundle）が観察され粘膜筋板および粘膜固有層に線維（fiber）が増生している（**図 4**）。われわれの現在用いている診断基準は**表 3** に示す[5]。例をあげると**図 4B** では＋＋＋と判定される。

AchE 染色で診断に際して問題になるのは新生児例である。新生児早期では H 病でも粘膜固有層に線維の増生がみられない場合がある（**図 5**）[5]。こういった症例では陰性と診断されることがあり偽陰性となる。例えば**図 6** の症例は 8 生日で H 病の症例である。粘膜筋板と粘膜固有層にごく一部しか増生がみられないが粘膜下層の bundle の状態から H 病と診断した症例である。この症例では時間をおいて再検してみると粘膜固有層に増生が認められ 2 カ月で＋＋＋となっている。このように新生児では粘膜固有層に増生がみられなくても臨床症状が続く場合は 1 カ月以上経過して再検することが勧められる。これが AchE 染色の specificity が 100％にならない理由と考えられる。

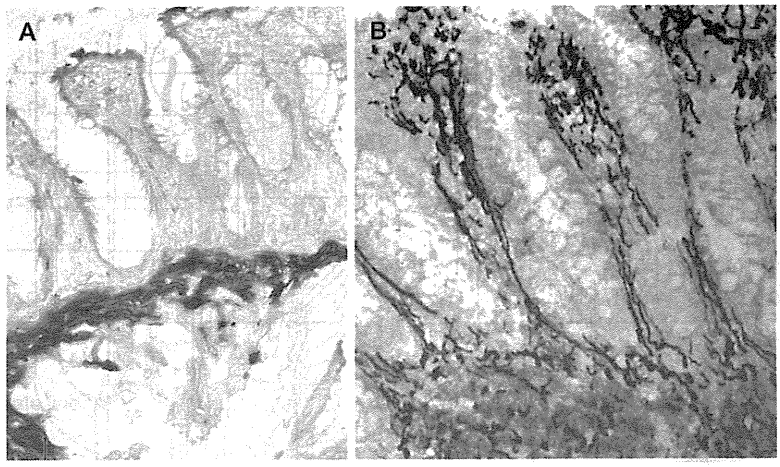


図 4 AchE 染色
A. 正常, B. H 病。

表 3 AchE 染色の診断基準

−	増殖なし
±	わずかの細い線維が粘膜固有層の基部
+	明らかな線維が粘膜固有層の下部
++	明らかな線維が粘膜固有層の先端まで
+++	線維が横行したりネットワーク形成

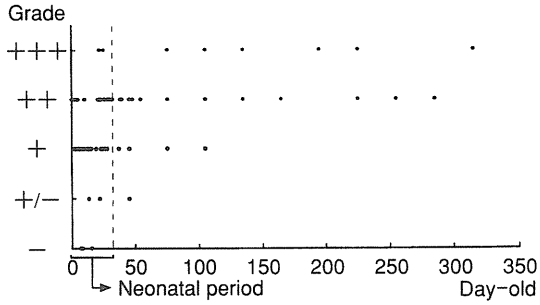


図 5 AchE 染色と日齢の関係
(Nakao ら[5], 2001)

4. intestinal neuronal dysplasia (IND)

粘膜生検の AchE 染色で議論になるのが IND である。この概念は H 病の診断に AchE 染色が有用であることを示した Meier-Ruge ら[6]により提唱されたもので, Type A と Type B に分類されているが, ほとんど (95%以上) が Type B で, 臨床的に H 病との鑑別が問題となるのは Type B である。IND Type B は症状や年齢は H 病に類似し, 組織学的には神経節細胞が存在するのに AchE 陽性線維が増生しているという病理学的な特徴を有する。

最近の文献では Type B の診断基準 (AchE 染色) は, ① giant ganglia (Meissner 神経叢 1 個あたりの ganglion cell が 7 個以上), ② AchE 陽性線維増生が粘膜固有層にみられる, ③ AchE 陽性線維増生が粘膜下層の血管周囲にみられる, ④ 粘膜固有層に異所性 ganglion cell あり, の 4 項目で, すべての所見を満たすものを Severe IND, ① と ②〜④ のどれかがみられるものを mild IND としている[7]。

われわれの経験した症例は, 1 カ月女児, 出生体重 3,352 g 37 週 4 日にて出生。20 生日より腹満出現。浣腸にて排便, 排ガスあるも腹満が続くため当科紹介された。腹単では腸管の拡張像と骨盤内のガス像の欠如, 注腸造影にて S 状結腸やや長く caliber change 様にもみえる (**図 7**)。直腸粘膜生検の AchE 染色では粘膜下層に giant ganglia を認め, 粘膜固有層にさほど太くはないが先端まで達する AchE 陽性線維の増生を認めた (**図 8**)。病理像より mild IND と診断した。H 病は否定されたため保存的治療を施行。治療は浣腸を 1 日 1 回継続したところ 1 カ月程度で自然排便がみられた。1 歳 9 カ月まで follow し, 2 日に 1 回自然排便あり, 腹満なく成長発達良好である。

IND の臨床経過について Puri らのグループ[8]

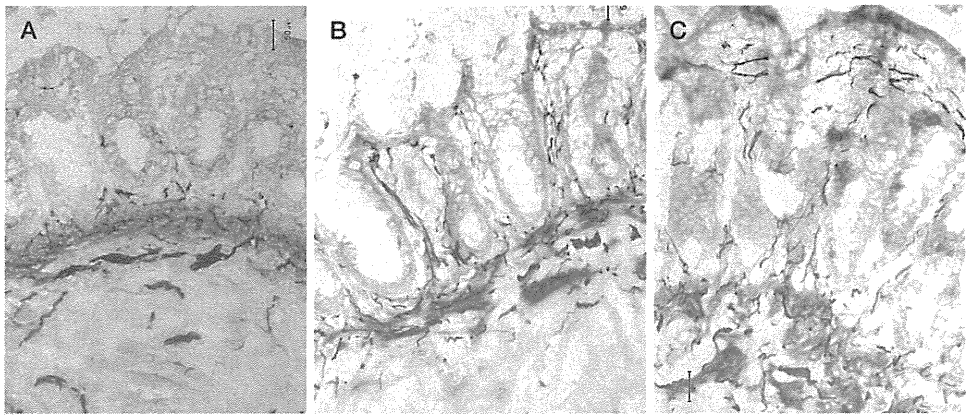


図 6 新生児の経時的変化
A. 8 生日男児（±～＋），B. 15 生日男児（＋＋），C. 2 カ月男児（＋＋＋）。

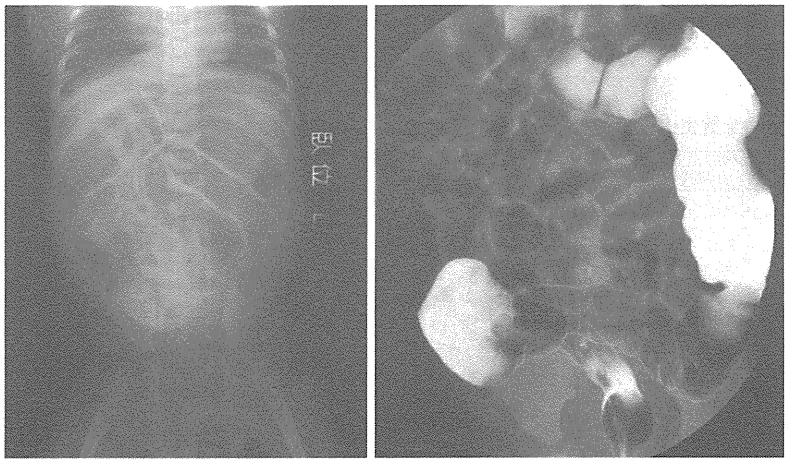


図 7 IND の X 線像（自験例）
1 カ月女児（YK），3,352 g，37 週 4 日にて出生。20 生日より腹満。浣腸にて排便，排ガスあるも腹満が続くため当科紹介。左：腹単では腸管の拡張像と骨盤内のガス像の欠如，右：注腸造影にて S 状結腸やや長く caliber change 様にもみえる。

からの報告によると，1992 年から 1998 年までの間に 418 例生検したうち 33 例（7.8%）が IND と診断され，そのうち 21 例（64%）は保存的治療に良好に反応し正常排便が得られるようになったが，12 例（36%）は内肛門括約筋切開術を施行し，うち 7 例は現在正常排便，2 例は浣腸にてコントロール可能となったが，3 例は拡張 S 状結腸切除術を施行し正常排便を得たとしている。つまり 36%はなんらかの外科的治療を必要としたということになる。

IND に関しては本当に存在する疾患なのか便秘による二次的な変化なのか議論がある[9]。また IND には isolate なものと H 病の合併例があったり，保存的治療でかなりの割合が治癒することから，独立した疾患概念としてとらえるべきか検討が必要である。しかし IND の動物モデルの存在や，2004 年の国際シンポジウムでの議論では isolated entity として認められている[10]。

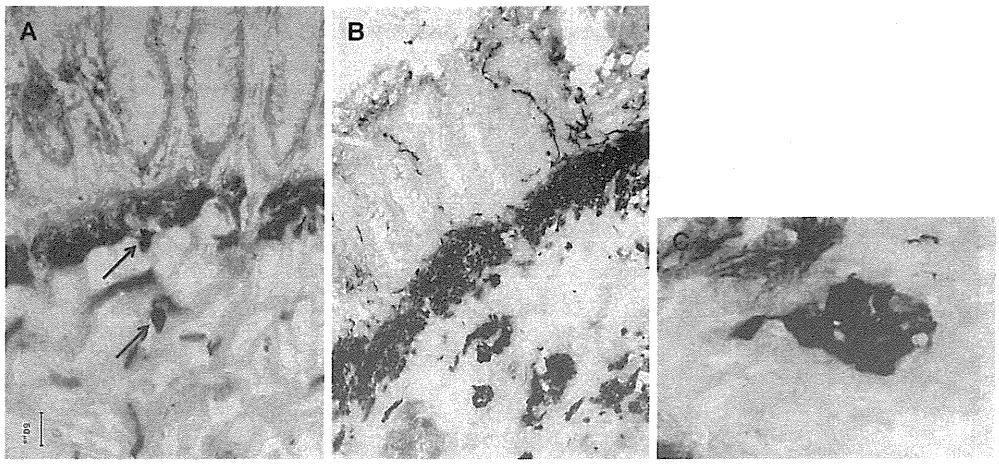


図 8 正常と IND（自験例）の比較

A. 正常，B. IND，C. IND の giant ganglia（強拡大）。
矢印は正常の Meissner 神経叢。IND では粘膜下層に giant ganglia（ganglion cell が 7 個以上）がみられ粘膜固有層には AchE 陽性線維が先端まで伸びている。

表 4 直腸全層生検の採取部位

年齢	歯状線からの距離
新生児	1.5 cm
乳児	2.0 cm
1〜3 歳	2.5 cm
4〜10 歳	3.0 cm

Auerbach 神経叢を対象とした距離

II. 直腸全層生検

これは全身麻酔下に固有筋層を採取し，Auerbach 神経叢を観察する目的で行われる。H 病の診断は直腸粘膜生検で十分であるので適応は限られるが，H 病が否定されたのにもかかわらず臨床症状が続き結腸直腸の拡張像が継続する場合など H 類縁疾患を疑い生検する場合や，肛門管静止圧が高い場合に治療をかねて肛門括約筋切開を行う際に標本採取することが多い。この場合も標本の採取部位が問題になる。つまり歯状線から一定距離は神経節細胞が存在しないのが正常である。**図 2B** に示すように Auerbach 神経叢に正常に神経節細胞が存在する分布下限は新生児では歯状線から 10 mm，幼児以上では 20 mm であり，歯状線から距離をおいて生検する必要がある。乳幼児では肛門管が狭いので視野が不良でなかなか正確な位置から生検するのは困難なことが多い。**表 4** は久米[3]が剖検例から検討し Auerbach 神経叢を対象とする標本採取部位を提唱したものである。

全層生検の手術手技であるが，全麻下に肛門管を肛門鏡で十分に開き，歯状線を確認，歯状線から適切な位置に 2 本支持糸をかける。その口側にさらに 2 本支持糸をかけ，その中間に糸をかけその部分から固有筋層を含む標本を採取する（**図 9**）[3]。

不適当な標本の例をあげると，採取標本に移行上皮や重層扁平上皮が含まれていると歯状線近傍から採取されていることになる。この部位では正常でも神経節細胞が存在しないので診断的価値がない。

III. 術中迅速生検

最近 H 病の根治手術は開腹しない endorectal pull-through が主流となり，術中生検の重要性が増してきた。本術式では結腸の全貌が観察できないため，正常神経節部の確認が肉眼的に不明瞭になる場合があり，術中迅速全層生検診断が必要である（**図 10**）。術中迅速診断でも標本の採取部位が重要である。正常神経節部から無神経節部に移行する移行部は腸間膜付着部のほうが口側によっていることが古くから報告されている（**図 11**）[11]。