Massive bowel resection upregulates the intestinal mRNA expression levels of cellular retinol-binding protein II and apolipoprotein A-IV and alters the intestinal vitamin A status in rats

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Abstract. Short bowel (SB) syndrome causes the malabsorption of various nutrients. Among these, vitamin A is important for a number of physiological activities. Vitamin A is absorbed by epithelial cells of the small intestine and is discharged into the lymphatic vessels as a component of chylomicrons and is delivered to the liver. In the present study, we used a rat model of SB syndrome in order to assess its effects on the expression of genes associated with the absorption, transport and metabolism of vitamin A. In the rats with SB, the intestinal mRNA expression levels of cellular retinol-binding protein II (CRBP II, gene symbol Rbp2) and apolipoprotein A-IV (gene symbol Apoa4) were higher than those in the sham-operated rats, as shown by RT-qPCR. Immunohistochemical analysis revealed that absorptive epithelial cells stained positive for both CRBP II and lecithin retinol acyltransferase, which are both required for the effective esterification of vitamin A. In the rats with SB, the retinol content in the ileum and the retinyl ester content in the jejunum were lower than those in the sham-operated rats, as shown by quantitative analysis of retinol and retinyl esters by high performance liquid chromatography. These results suggest that the elevated mRNA expression levels of Rbp2 and Apoa4 in the rats with SB contribute to the effective esterification and transport of vitamin A.

Introduction

Short bowel (SB) syndrome occurs as a consequence of malabsorption from an intestinal surface area insufficient to maintain growth and a normal nutritional status (1,2). Intestinal adaptation is a well-known phenomenon that increases the absorptive capacity following surgical resection of the small intestine. The most prominent feature of intestinal adaptation is the proliferation of intestinal cells that increases crypt depth and enlarges the length and width of the villi (3). It is known that micronutrients affect several aspects of intestinal adaptation. Wang et al (4) reported that intestinal adaptation was facilitated by retinoic acid, an active form of vitamin A. The authors demonstrated that the intravenous administration of retinoic acid exerted trophic effects in rats with SB by inhibiting apoptosis and stimulating crypt cell proliferation (4).

Vitamin A is a fat-soluble vitamin required for a number of physiological activities. Retinyl esters (REs), animal-derived forms of vitamin A, are hydrolyzed into retinol before entering the absorptive epithelial cells. By contrast, β-carotene, a vegetable-derived form of vitamin A, is converted into retinal after entering the absorptive epithelial cells and is then reduced to retinol within the cells (5). The retinol thus produced binds to cellular retinol-binding protein II (CRBP II, gene symbol Rbp2) in absorptive epithelial cells, forming a retinol-CRBP II complex. This complex is esterified to REs by lecithin retinol acyltransferase (LRAT, gene symbol Lrat). REs are then discharged basolaterally into the lymphatic vessels as a component of chylomicrons and are delivered to the liver through the general circulation (6). In hepatocytes, REs are hydrolyzed into retinol and complexed with retinol-binding protein (RBP, gene symbol Rbp4). The retinol-RBP complex is transferred to hepatic stellate cells (HSCs) where the retinol binds to CRBP I (gene symbol Rbp1) and is again esterified to REs by LRAT for storage (7,8).
CRBP I and II are members of the retinoid-binding protein family (9) which constitutes the calycin protein superfamily together with the fatty acid-binding proteins (FABPs) (10). In mammals, CRBP I is expressed ubiquitously (11,12), whereas CRBP II is expressed specifically in the intestine and the fetal liver (13). CRBP II expression increases perinatally, a change that is associated with the growth and differentiation of the intestine (13). CRBP II knockout mice have been shown to be more susceptible to vitamin A deficiency, suggesting that CRBP II plays an important role in vitamin A homeostasis (14). Takase et al (15) reported that jejunal bypass surgery led to a marked increase in the levels of CRBP II in the residual jejunal segment of rats. The upregulation of the Rbp2 gene in rats with SB was also reported by Dodson et al (16). It has been demonstrated that the upregulation of the mRNA expression of Rbp2 results in the growth of absorptive epithelial cells, presumably through increased vitamin A absorption by those cells (17).

As noted above, vitamin A is thought to be trophic to the bowel, and may therefore be important during adaptation in SB syndrome, and the deficiency of vitamin A may have negative consequences for adaptation. In the present study, we examined the effect of significant bowel resection on vitamin A absorption, transport and metabolism. For this purpose, we used a rat model of SB and analyzed vitamin A-related factors by reverse transcription-quantitative PCR (RT-qPCR), western blot analysis and immunohistochemistry, as well as measurements of vitamin A in tissues and cultured cells.

Materials and methods

Animals and surgical procedures. Protocols for animal experimentation were approved by the Animal Research Committee, Akita University Graduate School of Medicine, Akita, Japan. All animal experiments adhered to the ‘Guidelines for Animal Experimentation’ of the University. Male Sprague-Dawley rats (ages ranged from 8 to 11 weeks, 280-480 g body weight) were used in this study. Twelve hours after fasting, each animal was anesthetized by an intraperitoneal injection of pentobarbital. In the sham-operated rats, the gut was simply divided 10 cm distal to the ileocecal junction, resulting in a 75% resection of the bowel, and may therefore be important during adaptation in SB syndrome, and the deficiency of vitamin A may have negative consequences for adaptation. In the present study, we examined the effect of significant bowel resection on vitamin A absorption, transport and metabolism. For this purpose, we used a rat model of SB and analyzed vitamin A-related factors by reverse transcription-quantitative PCR (RT-qPCR), western blot analysis and immunohistochemistry, as well as measurements of vitamin A in tissues and cultured cells.

Western blot analysis. A total of 50 µg of each organelle fraction was separated by SDS-PAGE and transferred onto PVDF membranes (Atto, Tokyo, Japan). The membranes were incubated with primary antibodies against either CRBP I (1:200 dilution, sc-30106; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CRBP II (1:10 dilution, HPA035866; Sigma-Aldrich, St. Louis, MO, USA) or LRAT antibody (1:200 dilution; Immuno-Biological Laboratories, Gunma, Japan). A peroxidase-conjugated secondary antibody (1:5,000 dilution; Immuno-Biological Laboratories, Gunma, Japan) was applied. Bound antibodies were detected by ECL chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ, USA) and recorded on X-ray film (Fujifilm, Tokyo, Japan). As the positive controls, whole cell lysates from HEK293T cells transfected with Rbp1 or Rbp2 plasmids were used.

Immunohistochemistry and histochemistry. Paraffin-embedded ilea were sectioned at 3-5 µm intervals. To unmask the antigens, the specimens were boiled in citrate buffer (0.01 M, pH 6.0) for 25 min. For the inactivation of endogenous peroxidase activity, the slides were immersed in 1% H2O2 for 20 min. The specimens were incubated with either CRBP II antibody (1:10 dilution) or LRAT antibody (1:100 dilution) overnight at 4°C. The slides were treated with a 1:500 dilution of peroxidase-conjugated secondary antibody and incubated with DAB substrate (Roche Diagnostics). The nuclei were counterstained with Mayer’s hematoxylin solution (Wako Pure Chemical, Osaka, Japan). Hematoxylin and eosin (H&E) staining was also used.

RNA extraction and RT-qPCR. The frozen rat livers and intestines were dissolved in TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and total RNA was isolated. The cDNAs were synthesized from 5 µg of total RNA using SuperScript III Reverse Transcriptase (Life Technologies) using an oligo(dT)18 primer. The quantification of cDNA was carried out using the LightCycler 480 SYBR-Green I Master (Roche Diagnostics, Meylan, France). Transcript amounts were normalized against the glyceraldehyde 3-phosphate dehydrogenase (Gapdh) transcript. The nucleotide sequences used are summarized in Table I.

Organelle fractionation. Small sections of tissue (50-150 mg) were homogenized in 0.5 ml of cell disruption buffer (0.25 M sucrose, 20 mM HEPES, pH 7.5) using a Polytron CT 2100 homogenizer (Kinetica AG, Littauer-Luzern, Switzerland). The samples were further homogenized by 20 strokes using a Dounce tissue grinder (Wheaton, Millville, NJ, USA). The homogenates were centrifuged for 7 min at 1,100 x g. The supernatants were further centrifuged at 1,900 x g for 30 min. The pellets were set aside as mitochondrial fractions and the supernatants were again centrifuged at 105,000 x g for 1 h. The pellets were set aside as microsomal fractions and final supernatants were cytosoal fractions. The pelleted organelle fractions were dissolved in 100 µl of cell disruption buffer. Protein concentrations in all the organelle fractions were determined using the BCA protein assay reagent (Thermo Scientific, Rockford, IL, USA).

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performed for histological comparisons. The specimens were recorded digitally using the NanoZoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu, Japan).

Quantitative analysis of retinol and REs by high performance liquid chromatography (HPLC). Small sections of tissue (30-100 mg) were homogenized in 0.5 ml of cell disruption buffer (20 mM Tris-HCl, pH 8.0) using a Polytron CT 2100 homogenizer. Following the addition of 0.5 ml methanol and 1.75 ml dichloromethane, the homogenates were vigorously agitated with a Vortex mixer for 1 min. Subsequently, 0.5 ml of distilled water and 2.75 ml of hexane were added and agitated with a Vortex mixer for 5 sec, and the samples were then centrifuged for 5 min at 3,000 x g. The supernatants were set aside in a pear-shaped flask. The same procedures were carried out using the lower layer of the centrifuged samples without the addition of water. The dried supernatant was dissolved in 200 µl of N1 (mixture of 125 ml benzene, 25 ml tert-butyl methyl ether, 0.2 ml ethanol and 350 ml hexane) and assessed by HPLC (ELITE LaChrom; Hitachi, Tokyo, Japan) with a silica gel column (SL12S03-1506WT; YMC, Kyoto, Japan) for the quantification of retinol. The flow-through fraction containing REs was collected, dried and saponized in KOH (0.33 M in ethanol) for 30 min at 40˚C. Subsequently, 1.75 ml of dichloromethane and 2.75 ml of hexane were added to the saponized samples, and mixed for 30 sec. Distilled water (1 ml) was added and mixed for 30 sec and centrifuged for 5 min at 3,000 x g. The supernatant was set aside in a pear-shaped flask. The same procedures were repeated. The retinol contained in the extracts was analyzed by HPLC.

Plasmids. The cDNA for CRBP II (Rbp2) and LRAT (Lrat) was amplified from the isolated rat HSCs by reverse transcription-PCR using primers designed from the reported cDNA sequences (GenBank accession no. NM_012640 for Rbp2 and GenBank accession no. NM_022280 for Lrat) and inserted into the KpnI and EcoRI sites of pcDNA3.1 (Life Technologies). The primer pairs used for the cloning of Rbp2 and Lrat were 5’-GACTGGAATTCATGACGAAGACCAGATTGG-3’ and 5’-GGAATTCTCATCTTTTTTTGAACA

Table I. Primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Length (bp)</th>
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<tr>
<td>Gapdh</td>
<td>ACAGCAACTCCATCCATTCTTCC</td>
<td>TCCACACCCTGTGTTGCTGTA</td>
<td>118</td>
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<tr>
<td>Rbp1</td>
<td>AGGCATAGTGACCGCAAGT</td>
<td>TCATCACCTCAATCCACTG</td>
<td>117</td>
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<tr>
<td>Rbp2</td>
<td>GAAAACACCTGTTGTTGACG</td>
<td>GAACACTTGTGACCAGAACCT</td>
<td>122</td>
</tr>
<tr>
<td>Rbp4</td>
<td>GTTTTCTCGTGACCCCAATG</td>
<td>ACTGTTTCTTCTAGGGTCTG</td>
<td>139</td>
</tr>
<tr>
<td>Lrat</td>
<td>GCGAACAACCTTTGTGACCTAC</td>
<td>GACAGCTGAAGCGACAAC</td>
<td>119</td>
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<tr>
<td>Apoa4</td>
<td>TGAAGGTGTGCTGTCGTA</td>
<td>CTCCTTGCCATTTGTCG</td>
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<tr>
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<tr>
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<tr>
<td>Fabp2</td>
<td>TGATGGCAGCTTTGGAAGTAGAC</td>
<td>CTTCTCTGTGTTGCTGTCG</td>
<td>126</td>
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</table>

*Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Rbp1*, retinol-binding protein 1; *Lrat*, lecithin retinol acyltransferase; *Apoa4*, apolipoprotein A-IV; *Apob*, apolipoprotein B, *Fabp*, fatty acid-binding protein.

Transfection. Transfection of the plasmids into HEK293T cells was carried out using Lipofectamine 2000 reagent (Life Technologies). A total of 2 µg each of Rbp2 and Lrat cDNA was added to a 6-cm dish (n=8 for each condition). Eighteen hours after transfection, the cells were treated with 10 µM retinol (Sigma-Aldrich) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with fetal bovine serum (SAFC Bioscience, Lenexa, KS, USA) for 10 min. The cells were collected, quick-frozen in liquid nitrogen and stored at -85˚C until vitamin A quantification by HPLC.

Statistical analysis. Data are expressed as the means ± standard deviation (SD). The statistical significance of the differences was evaluated by an unpaired Student’s t-test for data shown in Figs. 2 and 5, and by ANOVA combined with Tukey’s test for data shown in Fig. 6. P-values <0.05 were considered to indicate a statistically significant difference.

Results

Upregulation of Rbp2 and apolipoprotein A-IV (Apoa4) mRNA expression in the intestines of rats with SB. We examined the mRNA expression levels of genes related to retinoid absorption, transport and metabolism (Fig. 2). Rbp1 mRNA was predominantly expressed in the liver and no significant changes were observed in its expression levels between the rats with SB and the sham-operated rats in either the liver or the intestine. The mRNA expression levels of Rbp4 in the ilea of rats with SB was specifically expressed in the intestines, and the mRNA expression levels of Rbp2 in the rats with SB were higher than those in the sham-operated rats in both the jejunum and the ileum.
Lrat mRNA coding for retinol-esterifying enzyme was expressed in both the liver and the intestine and did not show any significant differences between the rats with SB and the sham-operated animals. The intestinal mRNA expression levels of Apoa4 in the rats with SB were higher than those in the sham-operated rats in both the jejunum and the ilea (P<0.05).
The mRNA expression of Fabp1 in the jejuna of rats with SB was higher than that in the sham-operated rats (P<0.05).

Co-localization of CRBP II and LRAT in the intestinal absorptive epithelial cells of rats with SB. We analyzed the cellular locations of CRBP II and LRAT proteins in the small intestines of rats with SB and sham-operated rats. CRBP II was observed mainly in the absorptive epithelial cells in the ilea of rats with SB (Fig. 3A and C, arrows), while weak staining was observed in the ilea of the sham-operated rats (Fig. 3F and H). Immunohistologic staining of LRAT revealed its presence in the absorptive epithelial cells and lamina propria mucosae (Fig. 3B, D, G and I); the staining intensities seemed to be at the same level in the rats with SB and the sham-operated rats.

In the rats with SB, the absorptive epithelial cells were positive for both CRBP II and LRAT staining (Fig. 3C and D, arrows). Some fibroblast-like cells in the lamina propria mucosae seemed to be double-positive (Fig. 3C and D, closed arrowheads) and others to be positive only for LRAT (Fig. 3C and D, open arrowheads). No histological differences were observed between the intestines of the sham-operated rats and the rats with SB, as shown by H&E staining (Fig. 3E and J).

Subcellular localization of LRAT, CRBP I and CRBP II in the livers and intestines of rats with SB and sham-operated rats. We then analyzed the subcellular distributions of retinoid metabolism-related gene products in the liver and ileum. CRBP II was expressed in the cytosolic fraction of the ileum (Fig. 4A), but not in the liver (Fig. 4B). LRAT was expressed in both the liver and the ileum (Fig. 4), localized in the microsomal fractions. CRBP I was mainly expressed in the liver (Fig. 4B); its expression levels were higher in the cytosolic than in the mitochondrial and microsomal fractions.

Downregulation of retinol and REs in the intestines of rats with SB. We then used HPLC to quantify the amounts of retinol and REs in the livers and the intestines of the rats with SB and the sham-operated rats. The content of REs in the
jejuna of the rats with SB was lower than that of the sham-operated rats (Fig. 5A; P<0.05). The retinol content in the ilea of the rats with SB was lower than that of the sham-operated rats (Fig. 5B; P<0.05). There were no significant differences in the contents of retinol and REs in the livers of the rats with SB and the sham-operated rats (Fig. 5).

**Upregulation of REs in cultured cells overexpressing CRBP II and LRAT.** We wished to determine whether CRBP II affects the amount of REs produced by LRAT. Thus, we quantified REs in HEK293T cells overexpressing CRBP II and/or LRAT. The overexpression of LRAT in the HEK293T cells led to the accumulation of a large amount of REs in the cells within 10 min of the addition of retinol to the medium (P<0.05; Fig. 6). CRBP II overexpression enhanced the accumulation of REs in the LRAT-transfected HEK293T cells (P<0.05).

**Discussion**

In this study on SB syndrome, we used a rat model of SB to demonstrate that the intestinal mRNA expression levels of Rbp2 were significantly higher in the rats with SB than those in the sham-operated rats (Fig. 2). The esterification of vitamin A is regulated by the cooperative actions of LRAT and CRBPs (18,19). In accordance with this, we demonstrated that the overexpression of CRBP II and LRAT cooperatively increased the amount of REs in the HEK293T cells (Fig. 6). However, we observed a decrease in vitamin A content in the intestines of rats with SB (Fig. 5). The mRNA expression levels of Apoa4 in the intestines of rats with SB were significantly higher than those in the sham-operated animals (Fig. 2), as was previously reported by Rubin et al (20). Apolipoprotein A-IV is a component of chylomicrons (21) in which REs are transported (22). Thus, the upregulation of Apoa4 mRNA may lead to the enhanced transport of vitamin A from the absorptive epithelial cells to the lymphatic vessels, resulting in the reduction of vitamin A content in the intestines of rats with SB. In spite of the 75% reduction in the surface area of the intestine, our quantitative analysis revealed no significant differences in the contents of retinol or REs in the livers of rats with SB and the sham-operated rats (Fig. 5).
fibroblast-like cells in the lamina propria mucosae also seemed to be positive for both CRBP II and LRAT. We recently reported that vitamin A was absorbed and stored in the cells of the lamina propria mucosae of rat intestines and transmission electron microscopic analysis revealed that these vitamin A-storing cells in the lamina propria resembled extrhepatic stellate cells (EHSCs) (28). We speculate that the CRBP II- and LRAT-positive cells in the lamina propria represent EHSCs. If that is the case, EHSCs express CRBP II, differing from HSCs that express CRBP I. It would be intriguing to determine whether the differential expression of CRBPs in HSCs and EHSCs leads to functional differences between these cells.

SB syndrome is linked to the defective absorption of many types of nutrients, including carbohydrates, fats, amino acids and vitamins. The resultant malnutrition may lead to diseases in various organs. For example, it has been reported that arginine becomes an essential amino acid after massive bowel resection, because citrulline, a precursor of arginine, is synthesized in the small intestine (29). We previously reported that 90% resection of the rat small intestine caused an insufficiency of citrulline, leading to focal tubulointerstitial fibrosis in the kidney (30). In addition, liver fibrosis has been shown to occur after massive small bowel resection in the neonate piglet SB model (31). The increased esterification and transport of retinol in the absorptive epithelial cells of the intestines of rats with SB, which is suggested in this study, may contribute to prevent such fibrosis, as the suppressive effects of vitamin A on various types of fibrosis are known (32).

In conclusion, our results suggest that the esterification and transport of vitamin A are enhanced in the absorptive epithelial cells of the intestines of rats with SB through the upregulation of Rbp2 and Apoa4 mRNA expression.

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References


