Fat Absorption and Morphological Changes in the Small Intestine in Model Mice with Hyperlipidemia (Apo E Deficiency)

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ABSTRACT

In addition to abnormalities in lipid metabolism, the small intestine, which absorbs fat, plays an important role in the pathogenesis of hyperlipidemia. Using hyperlipidemia/arteriosclerosis models (apo E deficient mice), we measured morphological changes in the small intestinal villi, and investigated fat absorption using a $^{13}$C-trioctanoin breath test. In the apo E deficiency groups, the total cholesterol and triglyceride concentrations were higher than the values in the corresponding control groups. The height of the small intestinal villi was increased, showing a histological hyperplastic change. The concentrations of $\Delta^{13}$CO$_2$ were significantly increased, suggesting the enhancement of fat absorption or an increase in the total volume of absorption.

In apo E deficient mice, hyperplasia of the small intestinal villi and the enhancement of fat absorption were involved in the pathogenesis of hyperlipidemia. Excessive fat absorption in the small intestine may be one of the etiological factors for hyperlipidemia.

Introduction

Increased consumption of Western food is associated with lifestyle-related diseases such as hyperlipidemia, obesity, and diabetes mellitus, raising an important social issue. These lifestyle-related diseases are frequently comorbid conditions, and may be closely involved in the deterioration of arteriosclerosis, causing cardiovascular diseases including ischemic heart disease 1). Research on hyperlipidemia/arteriosclerosis with respect to endocrine/metabolic functions has markedly advanced; however, few studies have reported dietary fat absorption. As an increase in dietary fat intake is closely associated with an increase in blood cholesterol 2), the role of the small intestine, which absorbs fat, may be important for lipid metabolism.

Using mouse hyperlipidemia/arteriosclerosis models (apoprotein E(apo E) deficient mice), we investigated hyperlipidemia-related morphological changes in the small intestinal villi and changes in...
fat absorption in a $^{13}$C-trioctanoin breath test to clarify the pathogenesis of hyperlipidemia with respect to digestion/absorption.

Materials and Methods

1. Materials

We used 16-week-old (16w group, 6 animals), 24-week-old (24w group, 6 animals), and 32-week-old (32w group, 6 animals) female apo E deficient mice (Apo E deficiency group) and control C57BL/6J mice (control group: 16w group, 6 animals; 24w group, 6 animals; and 32w group, 6 animals respectively). These mice were housed in the animal room (6 per cage) at room temperature (20 to 25°C), and the lighting cycle was 12 hours. In the apo E deficiency group and the control group, food (MM-3, Funabashi Farm, Chiba) and water were given ad libitum.

We obtained apo E deficient mice from Department of Vascular Medicine, Graduate School of Medicine, Tokyo Medical and Dental University (Prof. Kentaro Shimokado).

This study was approved by the Animal Research Committee of Osaka Medical College and the animals received care according to the guidelines of this committee.

2. Methods

1) $^{13}$C-trioctanoin breath test (fat absorption test)

After a 24-hour fasting, each mouse was placed in a sealed chamber (capacity: 2.3 L, METABOLICA TYPE MM, Sugiyama Gen Iriki, Tokyo) for 30 minutes. After the air in the Chamber was stirred well in an air tight condition, 20 ml of air (pre-expiration) was collected and the baseline value was determined. After ventilation of the chamber, $^{13}$C-trioctanoin (trioctanoin-1, 1, 1-$^{13}$C; Cambridge Isotope Laboratories, Andover, MA) was administered into the stomach of mice through a gastric probe. After the mice were placed in the sealed chamber for 30 minutes, 20 ml of chamber air (expiration after 30 minutes) was similarly collected. Subsequently, expiration was collected 4-times at 30 minute intervals until 120 minutes after administration. After each sample was collected, the chamber was ventilated before the subsequent measurement. We measured the expiration level of $^{13}$CO$_2$ using an isotope ratio mass spectrometer (Tracer MAT; Finnigan MAT, San Jose, CA). The data 30, 60, 90, and 120 minutes after administration were calculated as the rate of increase in comparison to the basic value before administration of $^{13}$C-trioctanoin ($\Delta$ $^{13}$CO$_2$: %o=posttreatment value - pretreatment value), and regarded as 30-minute, 60-minute, 90-minute, and 120-minute values.

The initial dose of $^{13}$C-trioctanoin was 25 mg. The dose was increased by 25 mg until steatorrhea related to excessive fat loading occurred, with a decrease in the sum of 30-minute, 60-minute, 90-minute, and 120-minute values. The dose of $^{13}$C-trioctanoin immediately before steatorrhea and the decrease in the sum of $\Delta$ $^{13}$CO$_2$ values was regarded as the maximum loading dose. We confirmed steatorrhea by Sudan III staining 34).

2) Hematology and blood biochemistry

After the breath tests, under ether anesthesia, blood was collected percutaneous by cardiac puncture, and the animals were exsanguinated. We measured the concentrations of total cholesterol (enzyme method) and triglyceride (enzyme method: glycerol blanking method).

3) Histological examination

After exsanguination, the entire small intestine was removed immediately, and the length between the pyloric ring and the terminal ileum was measured. A 2-cm area of the upper small intestine 4-cm distant from the pyloric ring was resected, and washed in physiological saline several times. The specimen was fixed in 20% buffered formalin for 24 hours, and paraffin-embedded to prepare 3-µm paraffin sections. Hematoxylin and eosin (H&E) staining was performed. In histopathological specimens, the length (villus height) between the villous end and an area immediately above the muscularis mucosae was measured by light microscopy using a video micrometer (VM-30, OLYMPUS, Tokyo). In each specimen, measurement was randomly performed at 3 time points, and the mean was employed.

4) Statistical analysis

Data are expressed as the mean ± standard error (SE). Statistical analysis was performed using Student’s t-test. P<0.05 was regarded as significant.
Fat absorption in apo E deficient mice

Results

1. **Body weight**

In the 16w, 24w, and 32w apo E deficiency groups, the body weights of mice were 21.30±0.68, 23.06±0.79, and 24.45±0.32 g respectively. In the 16w, 24w, and 32w control groups, weights were 21.23±0.48, 23.18±0.19, and 24.16±0.33 g respectively. There were no significant differences at any time point between the apo E deficiency groups and the control groups (Fig. 1).

2. **Hematological and biochemical data**

1) **Total cholesterol**

Total cholesterol concentrations in the 16w, 24w, and 32w apo E deficiency groups were 447.66±27.88, 601.83±26.48, and 584.16±22.30 mg/dl respectively; those in the 16w, 24w, and 32w control groups were 77.83±5.42, 72.50±2.56, and 79.66±2.26 mg/dl respectively. Each apo E deficiency groups showed a significantly higher total cholesterol concentration than the corresponding control group (Fig. 2).

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**Fig. 1** Body weight in each control group and the apo E deficiency group

**Fig. 2** Total cholesterol concentration in each control group and apo E deficiency group

**,** P<0.01

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2) Triglyceride

Triglyceride concentrations in the 16w, 24w, and 32w apo E deficiency groups were 40.83 ± 10.06, 42.50 ± 2.32, and 43.50 ± 4.26 mg/dl respectively; those in the 16w, 24w, and 32w control groups were 14.50 ± 2.83, 22.66 ± 3.21, and 25.33 ± 2.47 mg/dl respectively. Each apo E deficiency groups showed a significantly higher triglyceride concentration than the corresponding control group (Fig. 3).

3. Histological findings

1) Total lengths of the small intestine

Total lengths of the small intestine in the 16w, 24w, and 32w apo E deficiency groups were 34.5 ± 1.0, 38.3 ± 1.2, and 38.3 ± 0.2 cm respectively; those in the 16w, 24w, and 32w control groups were 36.6 ± 1.4, 40.1 ± 0.7, and 41.1 ± 0.7 cm respectively. In the 32w control group, the small intestine was significantly longer than that in the 32w apo E deficiency group (Fig. 4).

![Fig. 3 Triglyceride concentration in each control group and apo E deficiency group](image3)

*: P<0.05, **: P<0.01

![Fig. 4 Total lengths of the small intestine in each control group and apo E deficiency group](image4)

**: P<0.01
2) Small intestinal villous heights

Small intestinal villous heights in the 16w, 24w, and 32w apo E deficiency groups were 349.50±7.09, 438.52±10.81, and 457.71±16.94 μm respectively; those in the 16w, 24w, and 32w control groups were 336.73±10.95, 335.81±11.34, and 328.59±6.25 μm respectively (Fig. 5). Villi in the 24w and 32w apo E deficiency groups were significantly higher than that in the corresponding control groups (Fig. 6A), showing hyperplastic changes (Fig. 6B).

4. $^{13}$C-trioctanoin breath test

We compared the maximum load of $^{13}$C-trioctanoin, the peak $\Delta^{13}$CO$_2$ value, and the sum of $\Delta^{13}$CO$_2$ values among the groups. In the 16w, 24w, and 32w apo E deficiency groups, the interval until the $\Delta^{13}$CO$_2$ value reached a peak were 60±0.0, 75±6.7, and 70±6.3 minutes respectively. In the 16w, 24w, and 32w control groups, the values were 70±6.3, 55±5.0, and 80±6.3 minutes respectively.

In the 16w, 24w, and 32w apo E deficiency

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Fig. 5 Small intestinal villous heights in each control group and apo E deficiency group

**: P<0.01

Fig. 6 Microscopic findings of small intestinal villi in each 32w control mouse (A) and 32w apo E deficient mouse (B), (H&E staining, ×100)
groups, the maximum load of $^{13}$C-trioctanoin were 91.66±5.27, 108.33±5.27, and 116.66±5.27 mg respectively; those in the 16w, 24w, and 32w control groups were 41.66±5.27, 41.66±5.27, and 33.33±5.27 mg respectively (Fig. 7).

In the apo E deficiency groups, the peak $\Delta^{13}$CO$_2$ values were 4, 466.11±217.82, 5, 174.79±222.92, and 4, 998.17±203.16 % in the 16w, 24w, and 32w groups, respectively. In the control groups, the values were 3, 496.16±139.77, 2, 928.92±123.02, and 3, 325.74±99.56 % respectively (Fig. 8).

In the apo E deficiency group, the sum of $\Delta^{13}$CO$_2$ values was 13, 909.73±439.92, 16, 516.78±729.29, and 16, 134.22±712.06 % in the 16w, 24w, and 32w groups, respectively. In the control group, the values were 9, 907.17±537.63, 7, 926.79±327.26, and 9, 045.88±712.26 % respectively (Fig. 9).

At all time points, the maximum load of $^{13}$C-trioctanoin, the peak $\Delta^{13}$CO$_2$ value, and the sum of $\Delta^{13}$CO$_2$ values in the apo E deficiency group were significantly higher than the control values.
Discussion

Apo E deficient mice have been created by homologous recombination in ES cells. A standard diet was given ad libitum after birth to induce hyperlipidemia, and arteriosclerosis was confirmed at 8 weeks of age. Arteriosclerosis was localized at the bifurcation of the main vessel of the aorta, which resembled the localization of arteriosclerosis in humans. The blood sugar and insulin levels were within the normal ranges. Therefore, these mice are used as a hyperlipidemia/arteriosclerosis model. Inbred C57BL/6J mice are routinely employed in studies on cancer and immunity, and in the toxicological and microbiological fields. In these mice, a standard diet given ad libitum does not induce hyperlipidemia or arteriosclerosis. We prepared an apo E deficiency model using C57BL/6J mice. Therefore, in this study, we used apo E deficient mice aged 16 to 32 weeks and C57BL/6J mice of the same age as controls.

In histological examination of the upper small intestine, the height of the small intestinal villi in the apo E deficiency group significantly increased with age in comparison to the control group, showing hyperplastic changes. Fat is mainly absorbed in the upper small intestine, and the intestinal tract absorption area may be involved in fat absorption in addition to the function of absorbing cells. An increase in the height of the small intestinal villi in this area indicates an increase in the absorption area. In apo E deficient mice, fat absorption may be enhanced, or the total volume of absorption may be increased.

Fat absorption tests include the Sudan III staining method, fat balance study method, 131I-triolein absorption test, and 14CO2 breath test with radioisotopes. However, there are limitations such as complexity and exposure. Recently, as a simple less invasive test, breath tests using a non-radioactive stable isotope, 13C-compounds, have been performed to evaluate digestion and absorption. 13C-compounds are present at a ratio of 1.1% in nature, and there is no risk of exposure due to the absence of nuclear decay. Therefore, 13C-compounds can be used in infants and pregnant women.

In breath tests with 13C-labelled substances, 13CO2 excreted in expired air after absorption of 13C-labelled substances in the small intestine, metabolism, and decomposition, is measured. This parameter accurately reflects absorption, as a final metabolite, 13CO2, is serially measured after administration. However, breath tests are an indirect method for measuring final products after several processes, that is, digestion, absorption, metabolism, and excretion of 13C-labelled substances, and may be influenced by various factors. In this breath/absorption test, we used trioctanoin, which is hydrolyzed and absorbed by the small intestinal villi without being influenced by peptic juice such as bile acid, as a 13C-labelled substance. Furthermore, to investigate the influence of other factors in the process of metabolism/excretion, we confirmed the absence
of rough lesions in the stomach, liver, and lung at autopsy. In addition, using another experimental system, we confirmed that gastric emptying was not delayed in diabetes or hyperlipidemia animals of the same age.

In the small intestine, 97% of fat is absorbed, and only a small amount of fat is excreted in stool. However, excessive loading leads to excretion of steatorrhea. It has not been determined whether fat absorption was reduced in apo E deficient mice. Therefore, the maximum fat load that does not cause steatorrhea must be calculated, and compared between the apo E deficiency group and the control group. At all ages, the maximum fat load in the apo E deficiency group was significantly higher than that in the control group, suggesting that fat absorption allowance is increased in apo E deficient mice, enhancing fat absorption.

Chylomycin, which is synthesized in the small intestine, and very low density lipoprotein (VLDL), which is synthesized in the liver, lose a portion of triglycerides embedded by lipoprotein lipase in blood, functioning as a remnant (chylomycin remnant, VLDL remnant). Apo E is a ligand necessary for the binding of these remnants to receptors on the hepatocellular membrane surface. When the binding of apo E to lipoprotein receptors is affected, or when apo E deficiency occurs, the remnants are not absorbed in the liver, and are increased in blood, causing hyperlipidemia.

Trioctanoin is medium-chain triglycerides, with a carbon count of 8. It is hydrolyzed in the intestinal mucosal cells by mucosal lipase, resulting in medium-chain fatty acid (MCFA). The greater portion of MCFA absorbed in the small intestine are transported via the portal vein into the liver, where they are metabolized. In apo E deficient mice, the greater portion of 13C-trioctanoin are absorbed and metabolized in the liver as MCFA. 13C-lipids, which are metabolized from 13C-trioctanoin in the liver, are bound to VLDL, and taken into VLDL remnant via metabolism. However, because apo E deficiency occurs, VLDL remnant are inhibited absorption into liver. Therefore, expired air excretion of 13CO2 related to 13C-trioctanoin metabolism may be decreased in apo E deficient mice. However, in the Apo E deficiency group, the expired air concentration of 13CO2 was significantly higher than that in the control group, suggesting that 13C-trioctanoin metabolism is more marked in apo E deficient mice, increasing the total volume of lipid absorption.

Some studies have reported that cholesterol absorption in the small intestine depends on the phenotype of apo E. An increase in the intestinal absorption area may be a feedback resulting from apo E deficiency-related abnormalities in lipid metabolism. In the future, the association between fat absorption related to excessive ingestion of fat and morphological changes in the small intestinal villi should be investigated.

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