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Two Siblings of Lewis Rats with a Congenital Homozygous Mutation of Gene Encoding the Large Subunit of Liver-Specific Microsomal Triglyceride Transfer Protein (MTP), Resulting in Pure Red Cell Aplasia and One Sibling with the Heterozygous Genetic Mutation of the Liver MTP Gene

Tadako NAKATSUJI

Department of Transfusion, Hamamatsu University School of Medicine, Hamamatsu, Japan

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Summary. **A homozygous sequence mutation of microsomal triglyceride transfer protein (MTP) was studied in three Lewis rat siblings. Two of the three siblings had a homozygous sequence mutation in the exon-1 of the large subunit gene of liver-specific MTP and one of the three siblings had a heterozygous mutation of the large subunit gene. The two rats with a homozygous mutation were confirmed to have pure red cell aplasia at the age of 130 days when they were sacrificed because of weakness. The findings of the two rats were as follows: 1) Body weights were decreased to 65% of normal rats. 2) Plasma apolipoprotein B (apoB) was 0.54-0.60 g/L. 3) A homozygous sequence mutation of the large subunit gene of liver-specific MTP was confirmed by polymerase chain reaction (PCR). The specific products of genomic liver DNA were a small amount in the PCR. 4) Acanthocytes constituted 7-14% of the red cells in the peripheral blood (PB). 5) The myeloid to erythroid cell ratio was 10: 1 in the bone marrow (BM). The BM tended to hypoplasia. 6) Peritubular damage and reduced erythropoietin (epo) secretion were found in the kidney. Amyloid degeneration of the tubules and perivasucular fibrosis were found in the male and the female sibling, respectively. 7) Thymus atrophy with a low % of CD8a+ cells was observed. The silent large subunit gene of genomic lymphocyte DNA was amplified actively in the PCR. 8) Iron was deposited in the small sized hepatocytes with a homozygous MTP gene mutation.**

In conclusion, silent MTP gene expression and tissue apoptotic changes developed with age in rats with a homozygous liver-specific MTP mutation.

Key words—rat, apolipoprotein B, microsomal triglyceride transfer protein, acanthocyte, pure red cell aplasia.

INTRODUCTION

Microsomal triglyceride transfer protein (MTP) is a heterodimer composed of protein disulfide isomerase (PDI) and a large (97-kDa) subunit[1,2]. MTP is required for the assembly and secretion of apolipoprotein B100 (apoB100)-containing triglyceride (TG) rich lipoproteins. A MTP defect results in a blocked assembly and secretion of very low-density lipoprotein (VLDL) from the liver[3]. In humans, at least 36 MTP mutations have been reported, in which 34 MTP mutations had abnormalities in the exon-2 of the large subunit gene[4]. The large 97-kDa subunit of MTP is synthesized in the liver and intestine of mammalian animals – including rats – at high levels[5]. Although embryonic mice did not express MTP in the heart[6], studies using human apoB transgenic mice[7,8] showed that apoB and MTP were produced in cardiac myocytes.

Correspondence: Tadako Nakatsuji MD, Department of Transfusion, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan.

This study describes two siblings of Lewis rats with a homozygous sequence mutation in the exon-1 of the large subunit gene of liver-specific MTP and one sibling with the heterozygous mutation of the large subunit gene. The rats with a homozygous defect of the gene looked healthy, but both succumbed to pure red cell aplasia at the age of 130 days. This is the first report of a homozygous MTP mutation of Lewis rats in which several complications developed with age.

MATERIALS AND METHODS

Animals

Sibling rats No. 1, 2 and 3 were born from the Lewis (LEW/Sea) strain of rats maintained in the animal colony at the Hamamatsu University School of Medicine. Rats No. 1 and 3 were male and Rat No. 2 was female. The three siblings were sacrificed at the age of 130 days. The parental rats (father and aunt) were sacrificed at the age of 6 months. Gene-mutated Rats No. 1, 2 and 3 were produced as the result of repeated inbreeding.

Plasma analyses

The plasma samples used in this study were obtained from Rats No. 1, 2 and 3 and the two parental rats at the time of sacrifice, and frozen at −80℃ for 1 to 2 months. Plasma apoB was measured using a single radical immunodiffusion (SRID) technique. APO B PLATE 'DAIICH' and APO B STANDARD SERUM were used in the SRID assays (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan). Rat apoB100 and apoB48, which are cross-reactive to human anti-apoB100 antibody, were determined by comparison with a calibration curve prepared using three standard human sera[9,10]. In Rat No. 1 and the male parent, plasma erythropoietin (epo) levels were measured by the method of a radioimmunoassay based on the 2-antibody method (Japan DPC Co., Tokyo, Japan).

Histopathological analyses

Peripheral blood (PB) was smeared a few minutes after blood was drawn from a tail vein. The PB smears were stained with May-Gruenwald-Giemsa. Bone marrow (BM), liver, spleen and thymus were fixed in 20% formalin to make tissue sections. All the tissue sections were stained with hematoxylin-eosin (H-E). Liver, spleen and kidney sections were also stained with Berlinerblau, azan and Congo red. Liver and spleen sections which were embedded in a Tissue-Tek O.S.T. medium (Miles Scientific, Naperville, IL, USA) were stained with Sudan III. Liver and kidney sections stained with H-E were used for photomicrographic image analyses. The photomicrographic images of the liver and kidney sections were analyzed using Adobe Photoshop (R) 4.0 software in a Mac OS system Ver. 7.5.3 (Apple Japan, Inc., Tokyo, Japan). To calculate the area of one unit hepatocyte, the gross area (23048 square μm) of the photomicrograph was divided by the total number of hepatocyte nuclei in the photomicrograph. The software program of Mac SCOPE Ver. 2.51 was applied for the image processing of the nuclear areas (Apple Japan, Inc.). Further, for each rat, five renal cortical areas were analyzed using Adobe Photoshop (R) 4.0 software. The mean number of glomerulus in an area of 677 × 867 square μm was calculated.

The spleen of Rat No. 1 was fixed with 2% glutaraldehyde for transmission electron microscopic analysis. After 2 hours, the fixed pieces of spleen were post-fixed with osmium tetroxide and embedded in epoxy resin. The sections were observed using a JEM 1220 transmission electron microscope (JEOL, Tokyo, Japan).

Flow cytometry (FCM) analyses

Lymphocytes separated from the PB, mesenteric lymph nodes (MLN) and thymus were analyzed using an EPICS (R) Profile II FCM (Coulter, Miami, FL, USA). One million cells were incubated with either 0.5 μg of mouse anti-rat CD4 IgG1 conjugated with fluorescein isothiocyanate (FITC) or 0.7 μg of mouse anti-rat CD8a IgG1 conjugated with FITC for 30 min at 4℃. Both types of FITC-labeled cells were then fixed with 1% paraformaldehyde overnight. After washing with phosphate-buffered saline (PBS), the percentage of FITC-positive cells was counted.

Genomic DNA analyses

Genomic DNA was extracted from the liver and MLN of Rats No. 1, 2 and 3 and the control rats, for polymerase chain reaction (PCR) analyses. A QIAamp DNA Kit (QIAGEN Inc., Valencia, CA, USA) was used for the DNA extraction. The primers used in the PCR were as follows: MTP primer set 1 was 5' primer, 5'-CCTACCAGGCTCATCAAGACA-AAG-3'[3] and blocking 3' primer, 5'-CCTAAATGC-TGGGGTTTAGT-3'[11]. MTP primer set 2 was 5' primer, 5'-CTGCAAGACAGCGTGGGCTA-3'[11] and blocking 3' primer, 5'-TTTTGATATGAGTAG-AACTC-3'[11]. In MTP primer set 3, 5' primer was the same as the 5' primer of set 2. The blocking 3' primer

of set 3 was 5'-TCATCGTTATTTGGATCAAC-3'[11]. To examine further the upstream of the large subunit gene, in MTP primer set 4, 5' primer, 5'-TCTGTT-AAAGGTCACACAAC-3'[11] was used together with the blocking 3' primer of set 3. Nagoya Katayama Chemical Co., Ltd., Japan synthesized all these primers. PCR consisted of 2 cycles of incubation at 96℃ for 1 min and 60℃ for 4 min, 35 cycles of incubation at 94℃ for 1 min and 60℃ for 2.5 min, and 1 incubation at 70℃ for 10 min. To estimate the sizes of specific products, a GeneRuler™ 100bp DNA Ladder (Fermentas Ltd., Graiciuno, Vilnius, Lithuania) was separated on the same electrophoresis gel as the specific products.

RESULTS

Observational findings

The mother of Rats No. 1, 2 and 3 killed three other siblings by herself suddenly 3 weeks after their birth. Though these three rats survived and looked healthy, Rats No. 1 and 2 became weak at the age of about 115 days. At the age of 130 days, they were sacrificed because of weakness. During these two weeks, Rats No. 1 and 2 lost body weight and the respective body weights of Rats No. 1, 2 and 3 were 170 g, 126 g and 265 g. The body weights of Rats No. 1 and 2 were about 65% of normal rats.

Plasma findings

The respective levels of plasma apoB were 0.66, 0.54 and 0.60 (g/L) in Rats No. 1, 2 and 3. The apoB levels of the father and aunt were 0.66 and 0.48 (g/L). Plasma apoB was not defective in any of the siblings. The levels of plasma apoB in Rats No. 1 and 2 were slightly higher than those in the other rats of the same sex. Plasma epo levels of Rat No. 1 and the father rat were 5.8 mU/mL and 8.0 mU/mL, respectively. The plasma epo level of Rat No. 1 was lower than that of the father.

Histopathological findings

In the PB smears of Rats No. 1 and 2, 7% and 14% of the red cells were acanthocytes, respectively. The normocellular BM of Rats No. 1 and 2 showed markedly decreased erythropoiesis as shown in Fig. 1. The myeloid (M) to erythroid (E) ratios of Rats No. 1 and 2 were both 10 : 1. Pure red cell aplasia was diagnosed in the two rats. However, the absolute numbers of immature myeloid cells later tended to

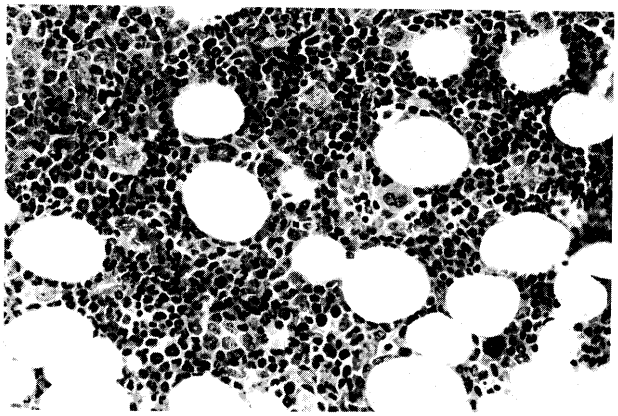

Fig. 1. The BM of the female Rat No. 2. Metamyelocytes, band neutrophils, and segmented neutrophils are the most abundant cell types. The M: E ratio was 10 : 1. Megakaryocytes and metamegakaryocytes are seen. (H-E stain, × 320).

Table 1. The areas per hepatocyte and per nucleus, ratio of nuclear area to hepatocyte area, and numbers of glomerulus in Rats No. 1, 2 and 3 and the parental rats.

Rat No. (Sex)	The area of one hepatocyte (H) (μm^2) M*±SD	The area of one nucleus (N) (μm^2) M±SD	N/H ratio	The number of glomerulis per 586959 μm^2 M±SD
Sibling-1 (M)	374±30	33±2	0.088	11±1
Sibling-2 (F)	335±32	36±4	0.107	14±1
Sibling-3 (M)	545±22	38±4	0.070	10±2
Father (M)	624±17	42±3	0.067	8±1
Mother's sibling aunt (F)	531±19	41±3	0.077	9±1

*Mean±SD.

decrease. The M: E ratios of Rat No. 3 and of the father and the aunt were 3.3 : 1, 3.4 : 1 and 3.0 : 1, respectively. Spleen extramedullary erythropoiesis was not found in Rats No. 1 and 2.

Small sized hepatocytes were observed in Rats No. 1 and 2. Very small iron deposits were stained positively with Berlinerblau in the hepatocytes of these rats. Their hepatocytes were not stained with Sudan III. Fatty liver was neglected in the two rats. As shown in Table 1, the hepatocyte sizes of Rats No. 1 and 2 were 60% to 63% of the parental hepatocytes. The hepatocyte to nucleus ratio was also high in these rats, as was the number of glomerulus in their renal cortex. Focal amyloid degeneration stained with Congo red was seen in the collecting tubules of Rat No. 1 as shown in Fig. 2A. Generally, the spots of amyloid degeneration were observed piecemeal. Collagen fibers stained with azan had proliferated around the vessels of the renal cortex of female Rat No. 2 as shown in Fig. 2B. At the marginal zones of the spleen white pulp, macrophages with large peroxisomes had proliferated abnormally in the male (Rat No. 1), which was confirmed by the electron micrograph of a spleen section. The male spleen showed a small amount of iron deposits. In contrast, a large amount of iron deposits was observed in the female spleen (Rat No. 2). Red cells were actively destroyed only in the female spleen.

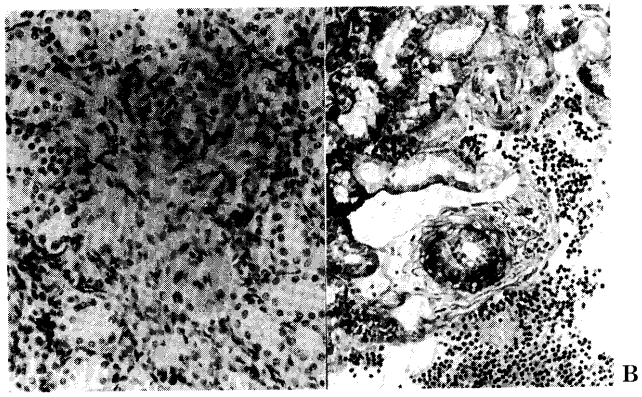

Fig. 2. The kidneys of the male Rat No. 1 (**A**) and the female Rat No. 2 (**B**). (**A**): Amyloid degeneration stained positively with Congo red is shown. The amyloid degeneration is observed in the collecting tubules (Congo red stain, ×218). (**B**): Perivascular fibrosis in the cortex is shown. The fibrous areas stained blue appear like as the areas stained weakly in the picture (azan stain, ×218).

FCM analyses

Table 2 shows the results of FCM analyses, in which CD8a+ and CD4+ cell percentages were measured in the PB, MLN and thymus of Rats No. 1, 2 and 3. The CD8a+ cell % was decreased in the atrophic thymus of Rat No. 2. Rat No. 1 also had an atrophic thymus. The CD4+ cell % of the MLN lymphocytes was high in all three siblings.

Genomic DNA analyses

Fig. 3 shows the specific PCR products for the large subunit gene of liver-specific MTP. In Fig. 3, the specific PCR products were amplified from genomic liver DNA. In the PCR system, only 5' primers reacted to the genomic liver DNA with the sequence specificities of the large subunit gene. Blocking 3' primers accelerated the gene amplification of 5' primers. All the primer sequences were in the exon-1 of bovine large subunit gene. MTP primer set 1 amplified the specific product of 90-bp. MTP primer sets 2 and 3 amplified the specific product of 138-bp. In the PCR of primer sets 2 and 3, the liver DNA of Rat No. 3 actively amplified the four products of

Table 2. The CD8a+ and CD4+ cell % measured in the lymphocytes of the PB, MLN and thymus

Rat No.	Sex	PB Positive cell %		MLN Positive cell %		Thymus Positive cell %	
		CD8a	CD4	CD8a	CD4	CD8a	CD4
Sibling-1	M	18.7	66.4	24.4	65.5	-	-
Sibling-2	F	27.9	55.0	22.1	66.2	64.9	81.7
Sibling-3	M	20.8	58.2	27.0	60.0	87.5	87.5
Cont. (n=5-8)	M	22±4*	63±5	16±3	41±4	92±5**	90±5
Cont. (n=4-5)	F	23±3	66±2	20±3	53±4	95±1**	85±5

*Mean±SD. **Anti-rat CD8 monoclonal antibody was used.

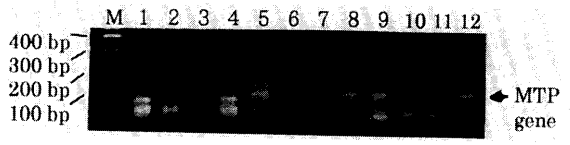

Fig. 3. The PCR results of Rats No. 1, 2 and 3. Genomic liver DNA was used in the PCR. In all the three blocks of lanes 1 to 4, lanes 5 to 8 and lanes 9 to 12, Rats No. 3, 1 and 2 and the control male DNA was applied in this order. In lanes 1 to 4 of the MTP primer set 1, the 90-bp specific product of the large subunit gene was amplified. In lanes 5 to 8 of the MTP primer set 2 and lanes 9 to 12 of the MTP primer set 3, the 138-bp specific product of the large subunit gene was amplified. In lanes 5 and 9 of Rat No. 3 DNA, the products of 138-bp, 155-bp, 220-bp and 300-bp were amplified.

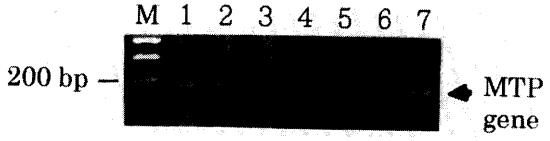

Fig. 4. The PCR results of the genomic liver and MLN lymphocyte DNA of Rats No. 1, 2 and 3. In lanes 1 to 4 of the MTP primer set 4, the liver DNA of Rats No. 3, 1 and 2, and the control male was applied in this order. In lanes 5 to 7 of the MTP primer set 4, the lymphocyte DNA of Rats No. 3, 1 and 2 was applied in this order. The specific product of the large subunit gene was 190-bp. The liver DNA of Rats No. 1 and 2 did not sufficiently amplify the specific product, but the lymphocyte DNA of Rats No. 1 and 2 adequately amplified the specific product of 190-bp.

138-bp, 155-bp, 220-bp and 300-bp. The liver DNA of Rats No. 1 and 2 amplified only a small amount of the specific product in the PCR. As shown in Fig. 4, primer set 4 amplified the specific product of 190-bp in the PCR of liver and lymphocyte DNA. The amplified specific product from liver DNA was slight in Rats No. 1 and 2. In contrast, the PCR of genomic MLN lymphocyte DNA sufficiently amplified the specific product of 190-bp in Rats No. 1 and 2. The PCR product of 190-bp was amplified in both the liver and MLN DNA of Rat No. 3. The MLN DNA of the control female amplified a small amount of 190-bp product. All the MLN DNA of Rats No. 1, 2 and 3 adequately amplified the specific product of 90-bp in the PCR of primer set 1 (data not shown). It was concluded that Rats No. 1 and 2 had a homozygous mutation of the large subunit gene of liver-specific MTP, while Rat No. 3 had a heterozygous mutation of liver-specific MTP gene. The MTP mutation was in the exon-1 of the large subunit gene. A silent MTP gene of lymphocytes was expressed actively in the PCR of the three siblings.

DISCUSSION

Two sibling rats with a homozygous sequence mutation of liver-specific MTP and one sibling with a heterozygous mutation have been described here. Parents of the three siblings were strongly suspected to have the heterozygous mutation of the large subunit gene. As the rats with the heterozygous MTP mutation were healthy and fertile, the heterozygous MTP mutation is thought to have been relatively common over the three generations in the rats studied here. However, the two siblings in question were the first rats with a homozygous mutation of liver-specific MTP gene among all the rats maintained in this colony.

MTP is essential for transferring the bulk of TG into the lumen of the endoplasmic reticulum (ER). In the MTP complex of the large subunit, PID, apoB and lipids, the amino-terminal region of the large subunit (residues 22-303) interacts with apoB. The α-helical domain of the large subunit (residues 297-603) interacts with PDI and the lipid-associated domain of the large subunit (residues 604-894) interacts with lipids[4,12]. Among the 36 human mutants of the large subunit, the 34 mutants are in the α-helical domain which interacts with PDI[4]. Among the 34 mutations of human MTP, in which MTP activity has been measured, only 13 mutations had low MTP activities ($< 50\%$ of wild type). In this study, MTP activity seemed to be kept in to the liver of the affected two rats, because no fatty liver was observed in the two rats. Plasma apoB levels were rather slightly higher in the two rats with a homozygous MTP mutation than in the same sex rats with a heterozygous MTP mutation. As the rat liver physiologically has high levels of apoB48, which is a difference between humans and rats, a high amount of apoB48 must be secreted from the liver to compensate apoB100 reduction[13]. The assembly and secretion of apoB48-containing lipoproteins are different from those of apoB100 lipoproteins which need MTP for the assembly and secretion. ApoB48 is translocated into the microsomes by binding newly synthesized phosphatidylcholine[14]. ApoB48 translocation was active in the presence of co-translational lipid synthesis without MTP. A larger amount of apoB48 seemed to be contained in the apoB values of the two affected rats. A sequence mutation in the exon-1 of the large

subunit gene of liver-specific MTP was concluded from the results of PCR. The abnormal sequences of the exon-1 seemed to be a sequence similar to the large subunit gene of bovines, because a small smount of specific products was observed in all the liver PCR of rats with a homozygous MTP mutation. A heterozygous mutation of liver-specific MTP was determined by the activated amplification of one normal MTP gene.

The two rats with a homozygous MTP gene mutation exhibited pure red cell aplasia at the age of 130 days. In the process of pure red cell aplasia, there were some different pathological findings between the affected male and female. The numbers of acanthocytes observed in the PB were higher in the female than in the male. Acanthocytes were degraded actively in the spleen of the affected female, but not in the affected male. The difference in the red cell morphologies seemed to depend on the lower level of plasma apoB100 in the female than the male. On the other hand, many macrophages with the proliferation of large peroxisomes, which dissociate apoB48 from lipoprotein particles, were observed in the affected male spleen, but not so often in the affected female spleen. As peroxisome proliferation is evoked by hypolipidemia[15], the hypolipidemic reactions were more clear in the male spleen. Renal amyloid degeneration was found in the collecting tubules of the affected male, while renal fibrosis around the vessels was prominent in the affected female. Lipid peroxidation via free radical injury plays an important role in the amyloid formation process[16]. Lipid peroxidation mediates the stimulation of collagen gene transcription[17]. The renal changes in the two rats must have been induced by activated lipid peroxidation. Lipid peroxidation occurred more strongly in the male kidney than the female.

MTP secretion from the kidneys of rats is not known. However, in the state of liver-specific MTP mutation, MTP and apoB might be expressed and lipoproteins might be secreted from the kidney as has been recognized in the heart of human apoB gene transgenic mice[7,8]. Pathological secretions of lipoproteins must lead to focal amyloid degeneration based on lipid peroxidation. The decreased epo secretion from the kidney of the affected rats must have been caused by damaging the fibroblast-like cells in the renal interstitium[18]. If the pure red cell aplasia observed in this study was not related to the kidney disturbance, epo levels would be high in pure red cell aplasia. However, the epo level was low in the affected rat. The large subunit gene was amplified actively in the PCR of the genomic lymphocyte DNA of the rats with a homozygous MTP mutation. Silent MTP gene of lymphocytes was expressed actively in those rats with a liver-specific MTP mutation. Thymus atrophy with a low % of CD8a+ cells and a high % of CD4+ MLN cells indicated a short life span of T and B lymphocytes in the rats. In the bone marrow of the two rats, not only pure red cell aplasia, but also BM aplasia was in progress. The damage to mature and immature erythroid cells preceded those of myeloid cells. Small-sized hepatocytes with small iron deposits were also considered to be in a state of pre-apoptosis. The complications of a homozygous gene mutation of liver-specific MTP became apparent along with their ageing.

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