Alpha-tocopherol transfer protein is important for the normal development of placental labyrinthine trophoblasts in mice

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Running title: α-tocopherol transfer protein during pregnancy

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Summary

α-tocopherol transfer protein (α-TTP), a cytosolic protein that specifically binds α-tocopherol, is known as a product of the causative gene in patients with ataxia associated with vitamin E deficiency. Targeted disruption of the α-TTP gene revealed that α-tocopherol concentration in the circulation was regulated by α-TTP expression levels. Male α-TTP-/− mice were fertile. However, placentas of pregnant α-TTP+/− females were severely impaired with marked reduction of labyrinthine trophoblasts, and the embryos died at midgestation, even when fertilized eggs of α-TTP+/− mice were transferred into α-TTP−/− recipients. The use of excess α-tocopherol or a synthetic antioxidant (BO-653) dietary supplement by α-TTP−/− females prevented placental failure and allowed full-term pregnancies. In α-TTP+/− animals, α-TTP gene expression was observed in the uterus, and its level transiently increased after implantation (4.5 days post-coitum). Our results suggest that oxidative stress in the labyrinth region of the placenta is protected by vitamin E during development, and that in addition to the hepatic α-TTP which governs plasma α-tocopherol level, the uterine α-TTP may also play an important role in supplying this vitamin.

Introduction

Vitamin E (α-tocopherol) is the most potent lipid-soluble antioxidant in biological membranes, where it contributes to membrane stability. Patients with ataxia and isolated vitamin E deficiency (AVED) have low or undetectable serum vitamin E concentrations exhibit neurological dysfunction and muscular weakness. It is now established that α-tocopherol transfer protein (α-TTP), a cytosolic liver protein known to specifically bind to α-tocopherol (1), is defective in AVED patients (2), indicating that α-TTP is a major determinant of plasma α-tocopherol level. Although α-tocopherol was initially identified as an anti-sterility factor to prevent
abortion (3), the mechanism of action and the molecule/s responsible for its anti-sterility effect remain unknown.

One of the reason for this is vitamin E was difficult to deplete from tissues and required elaborate manipulations to cause deficiency symptoms to occur in experimental animals. In this study, we established mice lacking α-TTP by targeted mutagenesis. This animal model for human AVED patients is suitable for examination of the complex pathophysiology of diseases associated with vitamin E deficiency and/or caused by oxidative stress. Here we examined the role of α-TTP in pregnancy and embryogenesis using our new animal model.

**Materials and Methods**

**Generation of α-TTP knockout mice.** An α-TTP targeting vector was constructed from an 8.8-kb α-TTP genome fragment encompassing exon 1. We inserted a fragment of PGK-neo cassette into the SmaI-SmaI site positioned 5’ and 3’ of exon 1, and flanked a 1.8-kb fragment of HSV-tk gene downstream of exon 2. AB2.2-Prime ES cells (Lexicon Genetics) or A3-1 ES (4) cells were transfected by electroporation with a linearized targeting vector. G418/gancyclovir-resistant clones were screened by PCR and then ES cells containing the disrupted allele were injected into C57BL/6J (CLEA, Japan) blastocysts as described previously (5). To obtain α-TTP-/- mutants, chimeras were mated with C57BL/6J females. α-TTP-/- mutant mice were produced from α-TTP+/- crosses. Genotypes were determined by PCR and confirmed by Southern blot analysis of DNA from tail tissue. The PCR primer pairs, ot198 (5'-AGCCCACACAAAAATGAAAAACGTCTCCAAG-3') and PGK-1 (5'-GCTAAAGCGCATGCTCCAGACTGCCTTG-3'), were used to detect α-TTP mutant allele. PCR primer pairs, ot198 and TTPN17 (5'-TCTCTGCAATGCCCGCTGCTGTCCCG-3'), were used to detect α-TTP wild allele. After an initial hot start at 94°C for 1 min, 35 cycles (94°C for 30 sec, 62°C for 1 min, and 72°C for 1 min and 20 sec) were run using Takara EX Taq (TaKaRa, Japan). The expected PCR products of wild and mutant allele were 990 and 950 bp, respectively. Genomic DNA from mutant mice were analyzed by Southern blotting using probe A including exon 1, and mouse α-TTP cDNA (ORF) probe, after digestion with EcoRI. The resultant two fragments, which had approximately the same number of nucleotides, were mixed and used for probe A. Mouse cDNA probe for α-TTP was prepared by RT-PCR with mouse liver
total RNA. In the next step, 15 µg of genomic DNA was electrophoresed on a 0.7% agarose gel and transferred onto a Hybond N+ membrane (Amerham, Arlington Heights, IL). The membranes were hybridized overnight at 42°C in a buffer containing 50% formamide, 5-strength SSPE, 0.5% SDS, 5-strength Denhardt’s solution, and 250 µg/ml denatured salmon sperm DNA with 32P-labeled probe. The membranes were washed for 30 min in double-strength SSC, 0.2% SDS and then in 0.5-strength SSC, 0.2% SDS at 65°C for 30 min. The 3.75 kb EcoRI fragment represents the wild type allele.

**Analysis of α-TTP expression by Northern blotting.** Total RNA was extracted from the liver of each adult mouse genotype, and from uterus, placentas and embryos of α-TTP+/+ mice using ISOGEN (Nippon Gene, Japan). Next, 10 µg of total RNA from liver, and 20 µg of total RNA from uterus, placenta and embryo were electrophoresed on 1% agarose gel, and transferred onto a Hybond N+ membrane. The membranes were hybridized and washed using the method described above for Southern blotting.

**Determination of Plasma α-tocopherol Concentrations.** Mice were fed a normal (36 mg α-tocopherol/kg diet) or α-tocopherol-supplemented diet (600 mg α-tocopherol/kg diet) after weaning. These diets were prepared from vitamin E deficient diet (Funabashi Farm, Chiba, Japan) supplemented with 5.0%(W/W) stripped corn oil (Tama Biochemical, Tokyo, Japan) and d-α tocopherol. D-α-tocopherol was kindly provided by Eisai Co. Ltd. (Tokyo, Japan). Blood samples were collected from overnight fasted animals and plasma was separated from whole blood by centrifugation. Plasma (50 µl) was diluted with 950 µl of phosphate buffered saline (PBS) and used for the following procedure. Diluted plasma was mixed with 1 ml of 6% pyrogallol in ethanol, and 2.0 µg of tocol was subsequently added as an internal standard and mixed vigorously. After incubation at 70°C for 2 min, 0.2 ml of 60% KOH was added and the mixture was incubated at 70°C for 30 min. In the next step, 5 ml of n-hexane and 2.5 ml of water were added and the mixture, mixed vigorously then centrifuged at room temperature. The hexane layer was saved while the hexane extracts were evaporated under nitrogen. The residue was redissolved in 100 ml of ethanol and subjected to high performance liquid
chromatography (HPLC) analysis and electrochemical detection. The HPLC system was an IRIKA P-530 (IRIKA, Kyoto) with an IRIKA RP-18 column (4x250 mm). The eluent was methanol/water/NaClO₄ at a ratio of 1000:2:7 (v:v:w) and a flow rate of 10 ml / min. Detection was performed with an IRIKA Amperometric E-520 detector. The retention time was 6.88 min for tocol, which was used as an internal standard, and 10.88 min for α-tocopherol, as described previously (6).

Embryo transfer. α-TTP⁺⁺ and α-TTP⁻⁻ embryos at 2-cell stage were transferred to the oviduct of α-TTP⁻⁻ or α-TTP⁺⁺ recipients on Day 0.5 of pseudopregnancy, respectively, as described previously (7). The recipients were sacrificed on 18.5 days post-coitum (dpc).

Viability of embryos in the uterus of α-TTP mutant mice. To determine the time of death, α-TTP⁺⁺ and α-TTP⁻⁻ mutant females were mated with C57BL/6J males, and then the pregnant females were sacrificed between at 9.5 and 14.5 dpc. Death of embryos was confirmed by lack of heartbeats.

Morphological appearance and histology. Embryos and placentas with and/or without the uterine horns were fixed with 10% neutral-buffered formalin for up to 24 hr. Embryos and uterine horn segments were subsequently processed into paraffin, sections, and deparaffinized for staining with hematoxylin/eosin before microscopic analysis.

α-tocopherol and synthetic antioxidant dietary supplementation. Mice were fed a commercial diet (CE-2, CLEA Japan, containing 45 mg/kg of d-α-tocopherol) after weaning. The α-TTP⁺⁺ and α-TTP⁻⁻ mutant females were mated with C57BL/6J males. At 0.5 dpc after mating, mice were fed α-tocopherol supplementation (CE-2 with supplementary d-α-tocopherol; 567 mg/kg), synthetic antioxidant (CE-2 with 0.65% BO-653) diet, or CE-2 as a control. Mothers were sacrificed at 18.5 dpc in order to examine the site of implantation and fetuses.
All experiments described in the present study were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Chugai Pharmaceutical, Shizuoka, Japan.

**Results**

**α-TTP Mutant mice.** In order to delete the initiation codon for α-TTP, a targeting vector was designed in which the entire exon 1 was replaced by a neomycin-resistance cassette (Fig. 1A). This targeting vector was introduced into embryonic stem (ES) cells by electroporation and then the ES cells were used to introduce vector into the mouse germline. We obtained three independent mutant mouse lines. Two lines (clone #L236 and L254) were derived from AB2.2-Prime ES cells and one line (clone #C229) was derived from A3-1 ES cells. The chimeras of these lines were bred with C57BL/6J to produce heterozygous mice for α-TTP. Mice of the L236 and C229 lines were bred and used for further analysis. When heterozygous mice were interbred, as expected for a recessive mutation, approximately one-quarter of the offspring were α-TTP-/- mutants (α-TTP+/+: α-TTP+/− : α-TTP−− = 63 : 105 : 74) (Fig. 1C). Both α-TTP+/− and α-TTP−− mice were normal in appearance and growth for at least 6 months. There were no significant differences among the genotypes in the plasma levels of VLDL, LDL, and HDL cholesterol as measured by HPLC (data not shown).

**Plasma α-tocopherol concentrations in α-TTP mutant mice.** When mice were fed a normal diet (36 mg α-tocopherol /kg diet), plasma α-tocopherol concentrations were about 400 µg/dl in α-TTP+/+ mice, half this level in α-TTP+/− mice, and undetectable in α-TTP−− mice (Fig. 2). These results indicate that α-TTP activity in the liver is a determinant of plasma α-tocopherol levels.

**Infertility of female α-TTP−− mice.** As shown in Table 1, α-TTP−− males were fertile. The α-TTP−− females became pregnant after mating, but none of the four or five tested delivered offspring (Table 1). Since α-TTP−− mutants were obtained from mating α-TTP+/− males and females in a Mendelian fashion, the α-TTP−− zygotes could develop to full-term. On the other hand, although fertilized eggs from α-TTP+/− mice could be...
successfully implanted into α-TTP−/− recipients, they failed to develop to full-term (Table 2). The number of live embryos (as determined by the presence of heartbeat) of α-TTP−/− mice markedly decreased between 11.5 and 14.5 dpc (Fig. 3).

The placentas and embryos of various maternal genotypes were not morphologically different at 9.5 dpc (data not shown). However, the embryos in the uteri of α-TTP−/− mutants showed developmental failure from 10.5 dpc, and the majority of these embryos showed neural tube malformations (Fig. 4E). In normal pregnancy, the labyrinth region of the placenta starts development from around 9-9.5 dpc, and then functions as a nutrient transport unit (8). Under normal embryogenesis, the allantoic vessels are seen by about 10 dpc where they penetrate the chorionic plate, and the ectoplacental plate is transformed into the labyrinthine part of the placenta (8). At this stage, the placenta could be divided into several well-defined layers such as the spongiotrophoblast layer and the labyrinth region. Histological examination showed a specific abnormality limited to the labyrinth region of the α-TTP−/− mutant at 10.5 dpc (Fig. 4C, F). In these mice, there was a marked reduction in the number of trophoblast cells, resulting in an abnormally small labyrinth. Furthermore, embryonic blood vessels were virtually absent in the trophoblast.

**Expression of α-TTP gene in the uterus.** In α-TTP+/− mice, expression of the α-TTP gene was observed in the uterus throughout pregnancy (Fig. 5), and the expression level of the α-TTP gene increased transiently after implantation on 4.5 dpc, and gradually decreased by parturition. Since α-TTP expression did not increase in pseudopregnant mice at 4.5 dpc (data not shown), implantation of embryos, or the development of embryos seems to have stimulated α-TTP gene expression. After about 4.5 dpc, the polar trophectoderm gives rise to extraembryonic ectoderm of the chorion, which later contributes to the trophoblast component of the labyrinth region, and the ectoplacental cone, which later produces the spongiotrophoblast layer (9). α-TTP was not expressed in the placenta at any time during development (Fig. 5). Although expression of the α-TTP gene in embryos was moderate, expression of this gene does not seem to be essential for embryonic development, since
α-TTP-/- eggs developed to full-term. These results suggest that α-TTP acts as a uterine factor and plays an important role in placental development.

**Rescue of embryos in uteri of α-TTP-/- mutants by diet containing excess amount of α-tocopherol or synthetic antioxidant.** To examine the effect of α-tocopherol dietary supplementation on the development of the placenta and embryos in uteri of α-TTP-/- mutants, diet was supplemented with α-tocopherol (567 mg/kg diet), either starting at 0.5 dpc after mating, or throughout the experiment. With this diet, plasma α-tocopherol levels in α-TTP-/- mice were maintained within the normal range, which were close to the levels in α-TTP+/− mice fed a normal diet (Fig. 2). This therapy, as well as supplementation of a synthetic antioxidant, BO-653 (10), had a pronounced effect on full-term development of embryos in the uteri of α-TTP-/- mutants (Table 3). The delivered pups showed normal growth and behavior and were fertile at adulthood.

**Discussion.**

Vitamin E was identified in the 1920s as a substance required for animals to have offspring (3). In this study, we generated α-TTP-/- mice with undetectable levels of plasma vitamin E even upon feeding with normal diet. Using these mice, we analyzed the infertility caused by vitamin E deficiency and found that the α-TTP-/- female mice have defective labyrinthine trophoblast formation during embryogenesis. The placental failure was effectively abrogated by α-tocopherol or synthetic antioxidant dietary supplement, indicating that vitamin E or other antioxidants are essential for the formation of labyrinthine trophoblasts. It is well known that the feto-placental system is prone to the attack of oxidants and that placental brush border membrane is most susceptible to peroxidation (11, 12). Oxygen free radicals are also involved in the induction of fetal anomalies. For example, excess oxygen radical activity has been reported to be associated with disturbed embryogenesis in diabetic pregnancy (13). Other studies have also shown a reduction in the severity of these diseases with administration of vitamin E during early pregnancy (14, 15). These findings, together with the present results, suggest that embryogenesis, especially the formation of the placental labyrinthine trophoblasts is more
susceptible to oxidative stress. Efficient functioning of the enzymic and non-enzymic reactive oxygen species scavengers ensures a normal intra-uterine fetal growth and development (12, 16). Mukherjea et al. demonstrated that α-tocopherol content in the placental membrane increases as gestation progressed (17, 18).

In this context, it is interesting to note that the expression of the α-TTP gene in the uterus of normal mice transiently increased around 4.5 dpc, possibly leading to an increase in α-tocopherol levels supplied to the embryo. On the other hand, it was also demonstrated that vitamin E crosses the placenta from the mother to the embryo, and interestingly, of the various forms of vitamin E transferred, the RRR-α-tocopherol, best ligand for α-TTP, crossed most efficiently (19). α-TTP expressed in the uterus may explain stereo-specific transport of tocopherols to the placenta, and upregulation of α-TTP expression may result in the increase in the transport of α-tocopherol to the placenta during embryogenesis. In addition to the hepatic α-TTP which governs plasma α-tocopherol level, the uterine α-TTP may also be the important factor for the feto-placental development. We established α-TTP disrupted mice as a model for vitamin E deficiency. This model should be a useful tool for the study of diseases caused by oxidation stress.

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Figure Legends

Fig. 1. Generation of α-TTP null mice. A, Mouse α-TTP locus, the targeting vector and the predicted structure of the α-TTP locus after homologous recombination. The neomycin cassette was inserted into Smal-Smal restriction site positioned 5’ and 3’ of exon 1. The PCR primer pairs, ot198 and PGK-1 and ot198 and TTPN17, were used to detect α-TTP mutant and wild allele, respectively. B, Southern blot analysis of EcoRI-digested genomic DNA. The probe A including exon 1, and mouse α-TTP cDNA probe did not hybridize to a 3.75kb fragment in homozygous mutant mouse genomes. C, Genotyping of offspring from heterozygous F1 intercrosses were analyzed by PCR. D, The expression of α-TTP was analyzed by Northern blot using total RNA from the liver. α-TTP+/+ and α-TTP+/- mice had undetectable and half levels of α-TTP mRNA in the liver compared with α-TTP++ mice, respectively. Blot was reprobed for cholesterol 7α-hydroxylase, which was used as loading control.

Fig. 2. Plasma α-tocopherol levels

Mice aged 4 and 11 weeks were maintained on normal and α-tocopherol supplemented diets. After overnight fasting, blood samples were collected from α-TTP+++, α-TTP++ and α-TTP+ mice for determination of α-tocopherol. Data are expressed as mean ± SD of six mice. Statistical analysis used Student’s t-test. *: Significantly different at P < 0.05.

Fig. 3 Viability of the embryos in the uterus of α-TTP mutant mice.

Over 70% of embryos died on 11.5 dpc. The proportion of live embryos in the uteri of α-TTP++ mice markedly decreased between 11.5 and 14.5 dpc. Data are expressed as mean number of embryos in three pregnancies.

Fig. 4. Morphological and histological appearance of embryos and placentas in uteri of homozygous mice.
A-C, Embryos and placentas in uteri of α-TTP+/+ mice.  D-F, Embryos and placentas in uteri of α-TTP−/− uterus.

A, B, D and E, Embryos at 10.5 dpc (×25).  Live embryos at 10.5 dpc in α-TTP−/− uteri were generally abnormal in appearance.  C and F, Placentas at 10.5 dpc (×40).  S: Spongiotrophoblast region,  L: Labyrinth region.  The development of labyrinth region in α-TTP−/− uterus was noted in less than 50% of those in α-TTP+/+.

Fig. 5. Northern blot analysis of α-TTP in uteri, placentas and embryos.

Total RNAs were isolated from uteri (on 0.5, 4.5, 8.5, 10.5, 15.5 and 20 dpc), placentas (on 8.5, 10.5 and 15.5 dpc) and embryos (on 8.5, 10.5 and 15.5 dpc) of wild type mice.  Blots were reprobed for β-actin, which was used as the loading control.
Table 1 Reproductive Rates of alpha-TTP mutant mice

| Genotype in parents | No. of mating | No. of implantation sites | No.(%) of resorptions | No.(%) of Live fetuses ** |
|---------------------|--------------|--------------------------|-----------------------|--------------------------|
| Female -/-          | Male -/-     | 5                        | 39                    | 39(100)                  | 0(0)                     |
| -/-                 | +/+          | 4                        | 35                    | 35(100)                  | 0(0)                     |
| +/+                 | -/-          | 6                        | 69                    | 6(9)                     | 63(91)                   |

*: Calculated from number of implantation site  
**: The mother were sacrificed on 18.5dpc  
The mice received a normal diet (CE-2; 45mg alpha-tocopherol /kg diet)

Table 2 Number of resorption sites and fetuses observed on 18.5 dpc after transfer of embryos into the oviduct of recipients mice

| Genotype of Embryos | No. of Embryos transferred | No.(%) of implantation sites | No.(%) of resorptions | No.(%) of Live fetuses ** |
|---------------------|-----------------------------|-----------------------------|-----------------------|--------------------------|
| +/+                 | -/-                         | 24                          | 21(88)                | 21(100)                  | 0(0)                     |
| -/-                 | +/+                         | 18                          | 10(56)                | 0(0)                     | 10(100)                  |

*: Calculated from number of embryos transferred  
**: Calculated from number of implantation site  
The mice received a normal diet (CE-2; 45mg alpha-tocopherol /kg diet)

Table 3 The effect of dietary supplementation of alpha-tocopherol for the gestation of alpha-TTP -/- mothers

| Genotype of female | Diet                | No. of litters | No. of implantation sites(mean) | No.(%) of resorptions | No.(%) of Live fetuses ** |
|--------------------|---------------------|----------------|-------------------------------|-----------------------|--------------------------|
| -/-                | CE-2#               | 4              | 35(8.8)                       | 35(100)               | 0(0)                     |
|                    | CE-2+alpha-toc##    | 4              | 34(8.5)                       | 0(0)                  | 34(100)                  |
|                    | CE-2+BO###          | 2              | 19(9.5)                       | 2(11)                 | 17(89)                   |
| +/+                | CE-2+alpha-toc##    | 2              | 15(7.5)                       | 0(0)                  | 14(93)                   |
|                    | CE-2+BO###          | 2              | 19(9.5)                       | 0(0)                  | 19(100)                  |

*: Calculated from number of implantation site  
**: The mother were sacrificed on 18.5 dpc  
#: 45mg alpha-tocopherol /kg diet  
##: 567mg alpha-tocopherol /kg diet  
###: 0.65% BO653 diet
**Fig. 1**

- **A**: Diagram of the targeting vector showing the Neo and TK genes flanked by wild and mutant alleles.
- **B**: Gel electrophoresis showing Alpha-TTP cDNA probe with bands for mutant and wild alleles.
- **C**: Gel electrophoresis showing bands for Alpha-TTP with mutant and wild alleles.
- **D**: Gel electrophoresis showing bands for cholesterol 7 alpha-hydroxylase with different genotypes.

**Fig. 2**

Bar chart showing plasma a-tocopherol concentrations (µg/dl) for normal and a-tocopherol supplementary groups at 4 weeks old and 11 weeks old.

**Fig. 3**

Line graph showing survival rate (%) over days post-coitum (dpc) for different genotypes: 
- Red line: +/-
- Black line: +/+
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