Optic Nerve Compression and Retinal Degeneration in Tcirg1 Mutant Mice Lacking the Vacuolar-Type H⁺-ATPase a3 Subunit

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Abstract

Background: Vacuolar-type proton transporting ATPase (V-ATPase) is involved in the proper development of visual function. Mutations in the Tcirg1 (also known as Atp6V0a3) locus, which encodes the a3 subunit of V-ATPase, cause severe autosomal recessive osteopetrosis (ARO) in humans. ARO is often associated with impaired vision most likely because of nerve compression at the optic canal. We examined the ocular phenotype of mice deficient in Tcirg1 function.

Methodology/Principal Findings: X-ray microtomography showed narrowed foramina in the skull, suggesting that optic nerve compression occurred in the a3-deficient (Tcirg1⁻/⁻) mice. The retina of the mutant mice had normal architecture, but the number of apoptotic cells was increased at 2–3 wks after birth. In the ocular system, the a3 subunit accumulated in the choriocapillaris meshwork in uveal tissues. Two other subunit isoforms a1 and a2 accumulated in the retinal photoreceptor layer. We found that the a4 subunit, whose expression has previously been shown to be restricted to several transporting epithelia, enriched in pigmented epithelial cells of the retina and ciliary bodies. The expression of a4 in the uveal tissue was below the level of detection in wild-type mice, but it was increased in the mutant choriocapillary meshwork, suggesting that compensation may have occurred among the a subunit isoforms in the mutant tissues.

Conclusions: Our findings suggest that a similar etiology of visual impairment is involved in both humans and mice; thus, a3-deficient mice may provide a suitable model for clinical and diagnostic purposes in cases of ARO.

Introduction

Vacuolar-type proton transporting ATPase (V-ATPase) is a multi-subunit complex formed from a membrane peripheral V₁ sector and a membrane-spanning Vo sector. The V₁ sector has catalytic sites for ATP hydrolysis, whereas the Vo sector is responsible for proton translocation [1]. Mammals express multiple subunit isoforms of the V-ATPase components in a tissue-specific manner [2]. The a subunit is a hydrophobic protein (approximately 100 kDa) and forms the Vo sector with proteolipids c and e subunits. The mammalian genome contains 4 genes for the a subunits, namely, a₁–a₄ [3,4]. The a₁, a₂, and a₃ subunits are present in various tissues at different levels. In contrast to the ubiquitous expression of the a₁, a₂, and a₃ subunits, the expression of the a₄ subunit is restricted to several types of ion-transporting epithelial cells [4,5].

The a₃ subunit of V-ATPase constitutes a transmembrane segment of the proton pump in late endosomes and lysosomes and functions in the luminal acidification of these organelles [3,6]. Genetic defects in this subunit are responsible for a severe form of autosomal recessive osteopetrosis (ARO) in humans [7]. ARO is a life-threatening condition that causes increased bone density, which leads to decreased bone strength, resulting in multiple fractures and inflammation in bone tissues. V-ATPase with the a₃ subunit is highly expressed in the cell surface of bone-resorbing osteoclasts and is responsible for acid secretion into the extracellular space between the osteoclasts and bone surface. Its deficiency causes ARO because of defective bone remodeling. This is also true for mice carrying mutations in the Tcirg1 (also known as Atp6V0a3) locus, which encodes the a₃ subunit [8,9]. Reflected on the expression of the a₃ subunit in various cells and tissues, Atp6i and oc mutations in Tcirg1 cause a wide range of phenotypes, and the mice rarely survive for more than 1 month after birth [10]. The mutant animals exhibit malfunctions in systemic calcium homeostasis and develop rickets [11]. The a₃ subunit is also required for the normal secretion of insulin and the bacteria-killing function of macrophages [12,13].

Ocular complications are often associated with ARO in humans. This phenotype is thought to be a result of nerve compression at the optic canal because of the malfunction of bone resorption by osteoclasts [14]. The oc/oc mutant mice, which carry a mutation in the Tcirg1, are defective in the optomotor response, suggesting impairment of their vision. This defect can be corrected by bone marrow cell transplantation soon after birth, which supports the hypothesis that the dysfunction of osteoclasts in the

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Figure 1. Ocular histology of wild-type and Tcirg1−/− mouse. Appearance of eyes of wild-type (A) and homozygous for Tcirg1−/− (B) at 3-wks after birth. The enucleated eye of a 3-wks Tcirg1−/− was practically same in size compared with that of the wild-type littermate (C). Technovit embedded eyes were sectioned and stained with toluidine-blue (D–K). Structure of retina (F, G), ciliary body (H, I), and optic nerve (J, K) were indistinguishable in the wild-type (F, H, J) and Tcirg1−/− (G, I, K) eyes. L, lens; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; C, choroid; NPE, non pigmented epithelium; PE, pigmented epithelium; ON, optic nerve. Bars, 1 mm (D and E) or 100 μm (F–K).

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Results

Retina architecture in Tcirg1−/− mice

The oc mutation is a spontaneous mutation in the Tcirg1 locus, which encodes the a3 subunit of V-ATPase [8]. Previous studies have shown that the oc/oc mice have near-normal electroretinograms [21]; however, they are defective in the optomotor response [15]. The Tcirg1−/− mutant mice carrying a deletion mutation frequently show eyes stained with stigma (Fig. 1A, B). This symptom appeared in animals of about 3 weeks of age, either in 1 or in both eyes. We examined the histology of the ocular tissues from wild-type and Tcirg1−/− mice and found no changes in the size of eye balls (Fig. 1C) and no obvious structural alterations in either the anterior or posterior parts of the ocular tissues (Fig. 1D–K), as was previously reported for oc/oc mutant mice [21]. The retinal layers were organized normally in the Tcirg1−/− mutant eyes (Fig. 1G). Retinal pigment epithelium (RPE) cells were present and there were no obvious structural changes in the uveal tissues rich in choroidal melanocytes. The ciliary bodies were also normal, with pigmentation in the pigmented epithelial cell layer of the Tcirg1−/− mutants (Fig. 1H, I), showing that melanin pigmentation occurs even in the absence of the V-ATPase a3 subunit [22]. The appearance of the optic nerve was similar in both genotypes (Fig. 1J, K).

Recently, we showed that the a3 subunit is required for the digestive function of macrophages [13]. Therefore, we considered the possibility that the loss of a3 might cause defects in vascular remodeling after birth, a developmental process that involves macrophage-like hyalocytes [23]. However, histological examination showed normal regression of the retinal and vitreous capillaries in the Tcirg1−/− mutants (Fig. 2). Thus, the morphology of the eyes of both mutant and wild-type mice 3 weeks after birth was the same (Fig. 1D, E).

Optic nerve compression in Tcirg1−/− mice

The V-ATPase with a3 subunit is highly accumulated in the bone resorbing osteoclasts, and its deficiency causes severe osteopetrosis because of defective bone remodeling [24]. Impaired visual function in ARO patients is thought to be the result of nerve compression at the foramina [14]. We then examined the anatomy of the skulls of wild-type and Tcirg1−/− mutant mice by x-ray microtomography to determine whether this nerve compression also occurs in mice (Fig. 3 and Videos S1 and S2). In the mutant mice, the optic canals were narrower than in the wild-type or heterozygous mice throughout their postnatal development, suggesting impairment of foramina formation. The optic canals in the wild-type and heterozygous animals became wider after birth, however, those in the mutant animals were constricted, likely reflecting the defective bone resorption (Fig. 3B). The compression of the nerve often causes tissue degeneration. We examined the apoptosis in the retina during the postnatal development for 3-wks (Fig. 4). In wild-type animals, considerable cell death in retina took place one week after birth, then the cell death became occasional at later stages [25]. In the mutant retina, there remained numerous TUNEL-positive nuclei in the inner nuclear layer (INL) 3-wks after birth (Fig. 4). In the wild-type, the TUNEL-positive cells became less frequent in the ganglion cell layer (GCL) and in the outer nuclear layer (ONL) during the postnatal development, however, in the mutant retina, cell death increased in the ONL and GCL (Fig. 4G). GCL is the sites of nuclei of retinal ganglion cells whose axons leave the orbit through optic canal, the late onset cell death at this location suggested the presence of neuronal compression and axonal damage in the Tcirg1−/− mutant mice. Similar to our observation, it has been reported that optic nerve injury causes apoptosis in the retinal ganglion cells [26]. These results support the hypothesis that bony compression is causative for impaired vision in Tcirg1−/− mutant mice. In the mutant mice, the border between the INL and OPL (outer plexiform layer) appeared less smooth at 3-
Figure 3. Narrowed optic foramen in Tcirg1<sup>−/−</sup> mouse. Micro-computed tomography scan sections of skull of wild-type and Tcirg1<sup>−/−</sup> mice at 1-wk (P7), 2-wks (P14) and 3-wks (P21) after birth (A). 3-D models were constructed and horizontal sections (caudal up, rostral down) generated by ImageJ software. Video S1 (wild-type) and S2 (Tcirg1<sup>−/−</sup>) show the stack of the horizontal sections of 2-wks pups. (A) Images showing the widest opening at optic foramina indicated by white triangles. (B) Comparison of the distance across the optic foramina between wild-type and Tcirg1<sup>−/−</sup> or V-ATPase in Ocular Tissue.
weeks of age (Fig. 4E and F), whereas separation of the layers occurred normally during the postnatal development (Figs. 2 and 4).

**Distribution of the V-ATPase α subunits in ocular tissues**

Our observation was compatible with the canonical view that bony compression at foramina causes neural degeneration in retrograde fashion, then brings loss of vision. However, it is also possible that V-ATPase is essential for the maintenance of retinal function directly. We examined the expression patterns of the α3 subunit in the ocular system, as well as the expression patterns of the other α subunits, because the presence of each V-ATPase α subunit in this tissue has not been well defined.

We stained Technovit section of the eye with antibodies for each α subunit. The specificity and reactivity of the antibodies has been well established in previous studies [12,22,27,28,29]. In the photoreceptor layer of the retina, the α1 and α2 subunits were detected, but the expression of the α3 subunit was below the level that could be detected by immunofluorescence histology (Fig. 5). The α3 subunit was highly expressed in the choriocapillary meshwork between the RPE cells and sclera, but it was not found in the RPE cells (Fig. 5A–C). Weak but significant staining was also seen in ciliary bodies (Fig. 6 C–D). In the anterior part of the eye, the α3 subunit was found in the capillary-rich tissue between the muscle and nonpigmented epithelial (NPE) and pigmented epithelial (PE) layers (Fig. 6A, C, D).

![Image of Tcilg1 -/-](image_url)

**Figure 4. Degeneration of retinal layer in wild-type and Tcilg1 -/- mouse.** Apoptotic cells were detected by TUNEL assay (green) on cryosections of wild-type (A, C, E) and Tcilg1 -/- mutant (B, D, F) tissues at 1-wk (P7), 2-wks (P14), and 3-wks (P21) after birth. The nuclei are shown with TOPRO-3 staining (blue). Three images of regions (1×10^5 μm^2) at retina were obtained from one section, and three sections were used for quantification in one eye. At least three eyes were examined in the experiments. Student’s t-test (two-tails) was used for evaluating statistical significance. Bars, 50 μm.

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The ocular ciliary epithelium is known to express the \( B_1 \) subunit of V-ATPase on the cell surface [16]. The \( B_1 \) subunit is expressed specifically in epithelial cells. The presence of \( B_1 \) suggests that the V-ATPase in ciliary epithelium is composed of a combination of specific subunit isoforms, because V-ATPases resides on the plasma membrane of renal epithelial cells assemble preferentially with the \( a_4 \) and \( B_1 \) subunits in the Vo and V1 sectors, respectively [30]. Indeed, we found that the \( a_4 \) subunit was present in the PE cells (Fig. 6B, E) and that this expression pattern was similar to that of the \( B_1 \) subunit (Fig. 5K, L). The signals of \( a_1 \) and \( a_2 \) are also shown by arrows. Distribution of kidney-specific type \( B_1 \) subunit (K, L) and ubiquitously expressed \( B_2 \) subunit (M, N), constituting the V1 catalytic sector of V-ATPase were visualized as well. The nuclei are shown with TOPRO-3 staining (blue). Bar, 50 \( \mu \)m.

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The ciliary NPE is considered to be an anterior extension of the photoreceptor layer, whereas the ciliary PE represents a continuation of the RPE of the retina. Consistent with this tissue architecture, the \( a_4 \) subunit, which was shown to be expressed in the PE of the ciliary epithelium, was highly expressed in RPE cells (Fig. 5D–F). This distribution is similar to that of the \( B_1 \) subunit (Fig. 5K, L).

In the ciliary body, CD31, a marker protein for the capillary endothelium, was colocalized with the \( a_3 \) subunit. A part of the \( a_3 \) signal overlapped with CD31 in the choriocapillary meshwork underlying the retina (Fig. 7D–F). These results indicated that the \( a_3 \) subunit was highly expressed in the capillary tissues. In addition to the endothelial cells, stronger signals were observed in the CD31-negative cells in the chorioid (Fig. 7A, D). The signal in the chorioid disappeared upon the loss of the \( a_3 \) subunit in \( Tg^{G1-} \).
mice (Fig. 8B). Interestingly, in the Tieg1−/− mutant tissues, the α4 subunit of V-ATPase was present in the choriocapillary meshwork, while the expression of the α4 subunit was virtually absent in the wild-type cells (Fig. 8C–F). Immunohistochemistry analyses on RPE/choroid layer confirmed that the amount of α4 subunit increased approximately 1.5-folds in the Tieg1−/− mice (Fig. 8G).

These results suggested that the function of α3 in choroid could be compensated by the α4 isoform.

**Discussion**

V-ATPase with the α3 isoform functions as a proton-secreting system in the plasma membrane of osteoclasts [6]. The loss of this protein results in a characteristic phenotype of osteopetrosis in both humans and mice, demonstrating its essential role in extracellular acidification and bone resorption. Osteopetrosis is often associated with impaired vision. Defective formation of the foramina, where the optic nerve passes, is presumed to be the cause of the impaired vision. In this study, we showed that mice lacking the α3 subunit have a narrowed optic canal. Although the retina of the mutant mice had normal architecture, the number of apoptotic cells was increased. These observations are consistent with the view that retrograde retinal degeneration occurs due to optic nerve compression.

We showed that the α3 subunit is expressed in extraretinal tissues, i.e., uveal choriocapillary meshwork. The capillary system in these tissues is known to be fenestrated; thus, small molecules and solutes can pass through without cellular activity but large molecules must be transported actively by transcytosis involving the endocytic and recycling pathways [31]. V-ATPase with the α3 subunit is a component of endosomes and lysosomes; thus, its activity may be important in these exchange processes. The expression pattern of the α3 subunit raises a possible scenario that V-ATPase with α3 may play a role in exchanging material across the endothelial cells, and loss of this function may result in increased cell death in the INL and ONL, where the nuclei of interneurons and photoreceptors reside, respectively, and may affect the OPL, the layer containing synaptic contacts between the interneuron and photoreceptor cells. Although the expression levels of α3 subunit are low in the retina, minor amounts of V-ATPase with α3 subunit is likely to be associated to the endosomes and lysosomes, thus it is also possible that dysfunction of these endocytic compartments may lead increased cell death in retinal tissue.

One form of osteopetrosis results from the loss of the anion-conducting channel, Clc-7. The loss of the clcn7 gene is accompanied by severe impairment of visual function and atrophy in the neural retina, due to the loss of lysosome function in the neural retina and RPE [21,32]. Mutations in the Tieg1 gene, which encodes the V-ATPase α3 subunit, account for 50% of human ARO cases. Combined with mutations in Clcn7 (chloride channel), genetic defects in these genes are responsible for two-thirds of cases of ARO [33]. Although defective vision is associated with both mutations, the results of our study and those of previous studies suggest that the underlying mechanisms are different, because V-ATPase with the α3 subunit is less abundant in RPE cells or in other part of retina, whereas the Clc-7 protein is highly expressed in neural tissues [21]. The loss of the V-ATPase α3 subunit results in early mortality (∼3 weeks after birth) compared to the clcn7 mutants that survive for 2–3 months; therefore, the α3 mutant mice may not survive long enough to develop retinal degeneration. Hematopoietic stem cell transplantation (HSCT) is one of the first choices for the treatment of ARO and lessens the severity of V-ATPase α3 mutation both in humans and mice [15,33]. However, HSCT may not be an effective treatment for ARO patients with Clc-7 dysfunction, who often also have neurological problems due to defective lysosomal function in neuronal cells [33,34]. This implies that precise genetic diagnosis is necessary to determine the benefit of HSCT in patients with ARO.

In this study, we found that the α4 subunit was highly expressed in PE and RPE cells. Despite the relatively high expression of the α4 subunit in retinal tissues, there have been no reported cases of visual impairments due to the loss of α4 function in humans. This may be because the other α subunit(s) compensate the lack of α4. There have been no reports of mutant mice lacking α4 function. Similar to the loss of α4 function, mutations in the ATP6V0A2
Figure 8. Ectopic expression of a4 subunit in uveal choriocapillary meshwork of Tcirg1−/− mouse. Technovit sections of wild-type (A, C, E) and Tcirg1−/− eye were stained with anti-a3 antibodies (A, B) or anti-a4 antibodies (C–F) and then viewed under laser microscope. TOPRO-3 (blue) were used for counter stain of nuclei. Antibodies (C–F), and then viewed under laser microscope. TOPRO-3 (blue) were used for counter stain of nuclei.

Materials and Methods

Antibodies, reagents, and animals
Specific antibodies against each isoform of the mouse V-ATPase subunit a have been described previously [27,30]. Anti-B1 and B2 antibodies have been described previously [30]. Fluorescent dye– conjugated secondary antibodies were obtained from Jackson Immunoresearch. C57Bl/6 and ICR mice were purchased from SLC Japan. The modification of the Tcirg1 locus and creation of mutant mice (Tcirg1−/−) have been described previously [13]. All experiments involving animals were conducted in accordance with the institutional guidelines of the Institutional Animal Care and Use Committees of the Institute for Scientific and Industrial Research (ISIR), Osaka University and the Committee of Doshisha Women’s College (DWC). The animal experiments were approved by the Committees (Dosan19-01-0 at ISIR, Osaka Univ. and Y09-018 at DWC).

Immunohistochemistry and immunoblot analysis

Tissues were dissected and fixed overnight at 4°C with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2), which was freshly made from paraformaldehyde (Sigma-Aldrich). The fixed tissues were embedded in Technovit 7100 resin after dehydration through an ethanol series and cut (3 μm thickness) by means of a rotary microtome. The sections were incubated for 1 h at 4°C in a PBS-based blocking buffer containing 0.2% gelatin, 0.2% saponin, 1% bovine serum albumin, and 1% normal goat serum. Subsequently, the sections were incubated overnight at 4°C with antibodies diluted in the blocking buffer. After being washed with the blocking buffer, the sections were incubated with the appropriate secondary antibody for 1 h at room temperature and extensively washed with PBS.

For in situ TUNEL assays, the fixed tissues were embedded in OCT compound (Sakura Finetech Japan), and retinal cryosections (5 μm thickness) were obtained by using a cryomicrotome. The sections were processed for TUNEL with fluorescein-dUTP (Roche) and counterstained with TOPRO-3 (Invitrogen). The slides were mounted in VectorShield mounting medium and examined under a confocal microscope (Zeiss LSM-510).

The RPE/choroids was isolated under stereomicroscope and lysed in extraction buffer containing 50 mM Tris-Cl (pH 7.4), 1% SDS plus Complete proteinase inhibitors (Roche) and 1 mM phenylmethylsulfonyl fluoride by sonication for 20 sec. The protein concentration of the lysate was determined by the BCA colorimetric assay (Pierce). The lysates (20 μg protein) were run through 5–20% SDS-polyacrylamide gels, transferred onto PVDF membrane and probed with the primary antibodies and horseradish peroxidase conjugated secondary antibodies (Jackson Immunoresearch). The blots were developed by ECL system (GE Healthcare) and images were obtained and quantified in LAS-1000 lumino-image analyzer (Fuji Film). The β-actin was detected with a mouse anti-β-actin antibody (Abcam) and used as the internal marker.

locus, which encodes the a2 subunit, do not seem to cause visual impairment, whereas the loss of the a2 subunit results in an abnormal assembly of the extracellular matrix and skin (cutis laxa), probably because of defective posttranslational glycosylation [35]. Because the retina expresses several a subunits in combination, functional subunits may compensate for the loss of a single subunit; thus, loss of either a2 or a4 alone may not be sufficient for the development of a defective visual phenotype. We have shown the upregulation of the a4 subunit in the retinal layer of a3 knockout mice, while the a4 subunit was below the detectable level in the wild-type animals. Along with this current finding, the Tcirg1−/− mice accumulates the a2 subunit in the islets of Langerhans [12]. These results suggest the presence of mechanism compensating a loss of particular subunit isoform with the other isoforms, adding further layers of complication in genotype/phenotype relationships. This hypothesis will be addressed by creating compound mutants with mutations in each of the a subunits.
Computed tomography and modeling

Mice were anesthetized with ketamine and xylazine, and their bone anatomy was scanned using a microCT scanner (Latheta LCT-100, Aloka) with 0.06 mm ×0.06 mm ×0.06 mm resolution. The 3-D models were generated by NIH ImageJ software on a Macintosh computer. The maximum opening of foramina was measured on coronal sections on the ImageJ software by a person who did not share genotype information of the specimen. Statistic significance was evaluated with Student’s t-test on Microsoft Excel program.

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Supporting Information

Video S1 Foramina of wild-type mouse. Found at: doi:10.1371/journal.pone.0012086.s001 (0.38 MB AVI)

Video S2 Foramina of mutant mouse. Found at: doi:10.1371/journal.pone.0012086.s002 (0.35 MB AVI)

Author Contributions

Conceived and designed the experiments: GHSW YW. Performed the experiments: NK HT GHSW. Analyzed the data: NK HT GHSW YW. Wrote the paper: NK HT GHSW YW.