PERSPECTIVE

Role of animal models in glaucoma research

Glaucoma is a neurodegenerative disease of the eye, and it presents with visual field defects accompanied by progressive degeneration of the optic nerve and retinal ganglion cells (RGCs). It is one of the major causes of blindness worldwide and affects 1 in 20 people over the age of 40. Glaucoma is caused by multiple factors, but it is usually associated with elevated intraocular pressure (IOP). Extensive studies have been carried out to discover therapeutic targets and to develop new drugs to treat ocular hypertension using experimental models of rodents (rats and mice) and non-human primates (e.g., cynomologus, rhesus, or marmoset monkeys), in which non-human primates provide unique insights into disease pathology that cannot be studied in rodents. Currently, almost all effective therapies in clinics aim to reduce IOP. However, not all glaucoma patients respond to this type of treatment and there is a subtype of glaucoma termed normal tension glaucoma (NTG), which is not accompanied by high IOP. Therefore, therapies targeting factors other than IOP are unmet medical need that could benefit cases when IOP reduction is not effective.

We have recently reported that different aspects of pathogenesis independent of IOP are demonstrated in two types of mouse glaucoma models (Sano et al., 2019) and that naturally occurring NTG-like neurodegeneration is observed in aged marmosets (Noro et al., 2019). In this perspective, we discuss various mouse models of glaucoma and the potential role of marmosets in glaucoma research.

Mouse models of glaucoma: There are a number of mouse models of glaucoma including high IOP models, NTG models, experimentally induced models and spontaneous models (Harada et al., 2019). The advantages of using experimentally-inducible models are that wild-type mice can be used and experimental conditions can be carefully controlled; for example the onset of disease. Experimentally-inducible models for high IOP glaucoma include laser treatment to damage the trabecular meshwork, microbead injection into the anterior chamber, cauterization of episcleral veins, and hypertonic saline injection into the episcleral veins; these methods aim to block the aqueous outflow leading to increased IOP. For NTG, examples include optic nerve injury, in which the optic nerve is crushed or transected, and intravitreal injection of *N*-methyl-D-aspartate; these methods cause acute RGC death independently of IOP. Although these experimental NTG models show RGC death, one may question if they are merely RGC death models because optic nerve injury does not necessarily cause glaucoma and there is no evidence to show increased *N*-methyl-D-aspartate receptor activation in RGCs of NTG patients.

The most widely characterized spontaneous model is DBA/2J mice, which present with a pigmentary form of glaucoma demonstrated with elevated IOP and optic nerve degeneration (Chang et al., 1999). Other spontaneous models with increased IOP include the pyrimidinergic receptor $P2Y_6$ knockout (KO) mice that present with age-dependent optic nerve and RGC degeneration accompanied by impaired visual function, due to excess production of aqueous humor from the ciliary body (Shinozaki et al., 2017); and Vav2/Vav3 KO mice demonstrating RGC loss and optic nerve head cupping with age, due to progressive iridocorneal angle closure (Fujikawa et al., 2010).

Spontaneous models for NTG include overexpression of the mutated genes associated with human NTG: optineurin E50K (Chi et al., 2010) and tank-binding protein 1 (Fingert et al., 2017). These models recapitulate a population of human glaucoma both genetically and phenotypically, but the late onset of disease (over 6–18 months of age) may not be very practical from an experimental point of view. In this respect, we have reported that mice deficient in glutamate transporters (GLAST or EAAC1) show NTG-like retinal degeneration from 3 or 5 weeks of age, respectively (Harada et al., 2007). Although the genetic association of GLAST or EAAC1 mutations with human glaucoma is yet to be established, the early onset of disease in these models is helpful for experiments on identifying therapeutic targets and interventions.

Multiple factors are involved in the pathogenesis of glaucoma, and although there are a number of mouse glaucoma models, each represents different pathological aspects. In humans, the lamina cribrosa (LC) is considered to be a putative site of optic nerve damage that causes characteristic pathology of glaucoma. In mice, this tissue is absent. Naturally, the features that cannot be reproduced in mice should be examined using other animal models. In the case of the LC, non-human primates may be ideal. Therefore, while taking advantage of the fast life cycle of mice to advance understanding in medicine, use of other animal models should be considered to unravel disease pathogenesis from a different perspective.

***N*-acetylcysteine (NAC) prevents retinal degeneration in EAAC1 KO mice, but not in GLAST KO mice:** Drug repositioning is an application of an existing drug to treat a different disease. One of the main advantages of drug repositioning is that it can save time and cost that is required to establish the safety of the drug. NAC is a *N*-acetyl derivative of cysteine that has historically been used as an antidote against paracetamol overdose, and more recently for various medical conditions including bronchopulmonary disorders, renal disorders and neurological and psychiatric disorders. It is liposoluble, so it can permeate across the cell membranes, and after entering the cells, it can be rapidly hydrolyzed and converted to cysteine. In neurons, the availability of cysteine is the rate-limiting substrate for the synthesis of glutathione (GSH), a powerful antioxidant, so supply of cysteine can increase GSH levels that may lead to neuroprotection. We have recently reported that daily NAC administration in EAAC1 and GLAST KO mice have differential effects on NTG-like retinal degeneration (Sano et al., 2019). We found that NAC administration protected RGCs in EAAC1 KO mice by increasing retinal GSH levels and reducing 4-HNE, an oxidative stress marker, but it failed to protect RGCs in GLAST KO mice. It was surprising to find such distinctive differences in the two models, because they both lack glutamate transporters, though different subtypes. EAAC1 is expressed in neurons and is involved in neuronal uptake of cysteine and glutamate. GLAST is mainly expressed in Müller glia in the retina and it plays a major role in removing excess glutamate, thereby protecting RGCs from glutamate neurotoxicity. Therefore, we speculated that in EAAC1 KO mice, RGCs die mainly because of the increased oxidative stress levels caused by the inability of RGCs to take up cysteine that is required for GSH synthesis. Supplementation of cysteine in neurons via NAC in EAAC1 KO mice restores the retinal GSH levels (Sano et al., 2019), and thus NAC exerts neuroprotective effects in this mouse model. On the other hand, we speculated that in GLAST KO mice, RGCs die mainly because of the increased glutamate neurotoxicity caused by the lack of glutamate removal from the extracellular space. Therefore, supplementation of cysteine via NAC could not prevent RGC death. Oxidative stress and glutamate neurotoxicity are both potentially involved in the pathogenesis of glaucoma. These findings demonstrated that EAAC1 and GLAST KO mice may represent different aspects of glaucoma pathogenesis and proved that they are both independently very useful models for glaucoma.

Aged marmosets present with naturally occurring NTG: The common marmoset (*Callithrix jacchus*), a small new world primate, is becoming increasingly attractive as an experimental animal model, particularly in neuroscience research. Like humans, the common marmoset is diurnal, and its brain and eyes are structurally well developed. The advantages of using the common marmoset over other non-human primates include (i) a high reproduction rate for a primate: their gestation period is about 5 months and multiple births are common; (ii) rapid postnatal development: they reach sexual maturation at 12 to 18 months of age; and (iii) ease of handling and breeding in laboratories. Common occurrence of multiple births is particularly useful for therapeutic studies as it enables direct comparison of the effects of treatment and placebo between littermates. In addition, their compact lifespan allows monitoring of aging or progressive disease effects in a relatively short period of time, suggesting it is a good model for aging research. Excitingly, generation of the transgenic marmoset was first reported in 2009 (Sasaki et al., 2009) and this technology provides a powerful tool for advances in medical research for various diseases.

We have recently reported that aged marmosets show glaucoma-like retinal and brain degeneration as well as the thinning of the LC (Noro et al., 2019). These marmosets had no genetic mutations in glaucoma-associated genes and no elevated IOP, suggesting that they show naturally occurring NTG (**Figure 1**). We used a number of *in vivo* imaging techniques including spectral-domain optical coherence tomography, multifocal electroretinogram and magnetic resonance imaging, for assessment of glaucomatous pathology in marmosets to follow up disease progression and to minimize animal sacrifice. We also demonstrated that increased oxidative stress and reduced brain-derived neurotrophic factor levels are observed in marmosets with glaucoma-like features. Brain-derived neurotrophic factor is a powerful neuroprotective agent especially for RGCs and its expression is reduced in glaucoma patient eyes (Gupta et al., 2014; Kimura et al., 2016). In addition, we found that the rate of incidence was 11%, which is similar to human glaucoma. However, with many aging research using non-human primates, if it takes decades before age-related conditions are apparent, one study could extend beyond a typical scientific career. Moreover, such long-term studies are extremely expensive, because

maintenance for non-human primates requires specialized facilities and staff. To this end, we are generating genetically modified marmosets with early onset of disease as a marmoset model of glaucoma. Our target gene is GLAST. Based on our mouse studies, we believe that the onset of retinal degeneration in GLAST KO marmosets will be within a few months rather than years, and thus, they will be a workable model that could contribute to advances in glaucoma therapy.

Conclusions and future perspectives: Glaucoma is an age-related disease, and recent drastic increase in life expectancy means that the number of glaucoma patients is also expected to rise. Animal models of glaucoma provide useful information on the pathogenesis and potential therapeutic targets for glaucoma, but we are still searching for a cure and the current therapies are limited to prevent or slow down the disease progression. Medicine is progressing and animal models are representing disease features closer to humans than before. As a result, novel therapeutic strategies in addition to reducing IOP are emerging. For example, delivery of a ciliary neurotrophic factor into the eye by implanting encapsulated human cells that are genetically modified to secrete therapeutic doses of ciliary neurotrophic factor is currently under clinical trials for glaucoma (ClinicalTrials.gov number, NCT01408472). Many studies with animal models pointed to the direction that neuroprotective effects of ciliary neurotrophic factor (and other agents) may be therapeutically useful for glaucoma, and success of this trial will prove that neuroprotection is effective in treatment of glaucoma. In summary, it is important to understand what each animal model offers, because there is no one model that represents the whole aspects of human glaucoma. Use of marmosets in glaucoma research may provide further insights into the molecular mechanisms involved in the onset and progression of glaucoma.

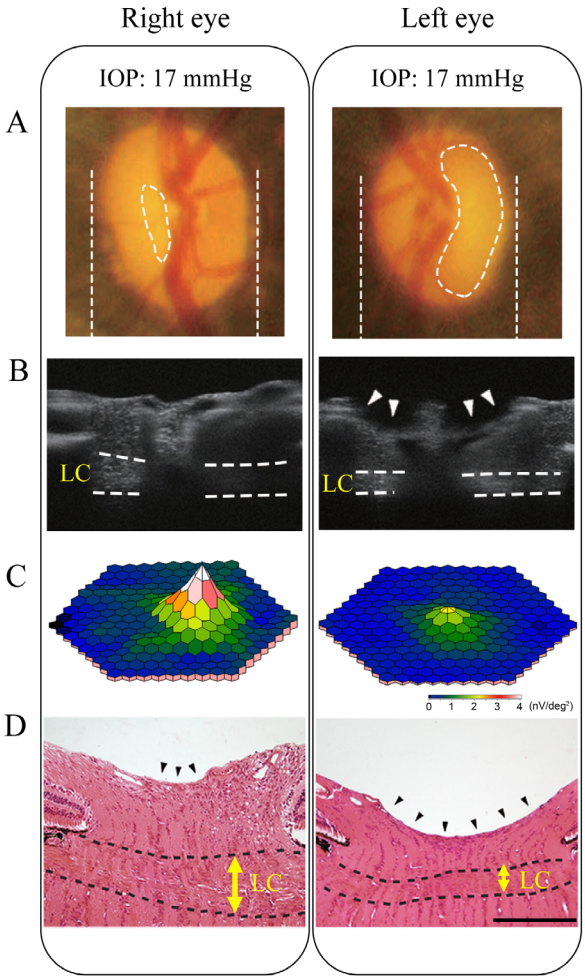

Figure 1 Eye examination of an aged marmoset with glaucoma-like degeneration (female, aged 12 years).
(A) Ocular fundus photographs. The edge of the cupping was traced from the three-dimensional images of the optic nerve head obtained by SD-OCT and the lines were superimposed on the fundus photograph. (B) *In vivo* imaging of the optic disc by vertical scan through the centre of the optic disc by SD-OCT. Arrowheads indicate the cupping of the optic disc and dotted lines indicate the LC. (C) Three-dimensional plots of the retinal responses as examined by multifocal electroretinogram. A higher score (white) indicates highly sensitive visual function. (D) Haematoxylin and eosin staining of the optic nerve head. Enhanced optic disc cupping (arrowheads) and thinning of the LC (dotted lines) are apparent in the left eye. Scale bar: 200 μm. Reproduced with some modification from Noro et al. (2019). IOP: Intraocular pressure; LC: lamina cribrosa; SD-OCT: spectral-domain optical coherence tomography.

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