Effects of 0.4% ripasudil hydrochloride hydrate on morphological changes in rabbit eyes

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Abstract
- We evaluated the cellular structure changes after continuous use of ripasudil hydrochloride hydrate in rabbit eyes which might affect its own efficacy and adverse effects. Two pigmented Dutch rabbits and 1 Japanese white rabbit were instilled with 0.4% ripasudil hydrochloride hydrate to the left eye twice daily. The right eye was observed as the control. Both eyes of all 3 rabbits were then enucleated for histopathologic examination by light and electron microscope at 1mo in 1 of the pigmented Dutch rabbits, 3mo in the other pigmented Dutch rabbit, and in the Japanese white rabbit after instillation. Microscopic observations showed increase intercellular space widening in trabecular meshwork, ciliary body, and iris stoma, increase pigmented granule number and size in iris epithelial cells, and decrease actin filament in iris muscle fiber cells. Consequently, ripasudil hydrochloride hydrate decreases the intraocular pressure by improving the conventional outflow and may also facilitate the unconventional outflow via intercellular space widening without serious side effects.
- KEYWORDS: ripasudil hydrochloride hydrate; actin filament; side effect; rabbit

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INTRODUCTION

Ripasudil (K-115) is a Rho kinase (ROCK) inhibitor that is commercially available to treat open angle glaucoma in Japan\textsuperscript{1-2}. ROCKs are serine/threonine kinase that regulates smooth muscle contraction. In mammals, ROCKs exist in 2 isoforms (ROCK1 and ROCK2) which being expressed in many tissues, including human trabeculocytes and ciliary muscle cells\textsuperscript{3}. ROCK inhibitors inhibit myosin light chain phosphatase resulting in actomyosin-based cellular relaxation in many types of cells including trabeculocytes, ciliary muscle cells, and vascular endothelium\textsuperscript{4}. The levels of mRNAs for ROCK and ROCK substrates are higher in trabeculocytes compared to ciliary muscle cells. Trabeculocytes are also more sensitive to ROCK inhibitors than ciliary muscle cells\textsuperscript{5}. The exact molecular mechanisms of the conventional aqueous outflow improvement are not well understood, but it has been hypothesized that cellular relaxation and intercellular adhesion relaxation cause widening empty spaces in the juxtaacanalicular region and increased vacuoles in endothelial cells improve outflow volume\textsuperscript{5}. Previous animal model studies also found that ROCK inhibitors may improve blood flow to the optic nerve\textsuperscript{6}, increase ganglion cell survival\textsuperscript{7}, and reduce bleb scarring in glaucoma surgery\textsuperscript{8}.

Ripasudil has a good ocular penetration property, first described by Isobe et al\textsuperscript{9} using radioactive distribution assay. Ripasudil showed high intraocular penetration through the transcorneal route within 15min after instillation with very low transcleral and systemic absorption. The radioactive distribution assay showed the drug concentration in the anterior chamber and periocular soft tissue in 15min, but only a little was detected in the posterior retina, choroid, and extraocular tissue around the optic nerve without contralateral drug absorption. Due to the drug distribution property, which mainly affects the anterior chamber structure, myosin light chain containing cell changes in the anterior segment including trabeculocytes, vascular endothelium, epithelium, fibroblasts, and smooth muscle in these regions might have been affected by this medication. Ripasudil can be used to treat open angle glaucoma as primary or adjuvant therapy\textsuperscript{10-11}.

However, to our knowledge, no previous investigators reported ultrastructural changes in an in vivo study. A morphologic study may not only define the exact molecular mechanisms

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of ripasudil, it may also postulate future side effects of this medication. The purpose of this study is to evaluate the cellular structure changes after continuous use of ripasudil in rabbit eyes, and to investigate its efficacy and adverse effects.

MATERIALS AND METHODS

Ethical Approval All experiments were conducted under proper anesthesia. We considered ethical issues and paid careful attention to minimizing pain. All experiments were also performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the approval of the animal experiment Ethics Committee of Kitasato University (No.2015-176).

Animals The experiments were performed on 6 eyes of 3 rabbits (2 male Dutch rabbits and 1 male Japanese white rabbit). They were purchased at 8 weeks of age and raised in a temperature- and humidity-controlled environment (25°C and 60%, respectively) until 40 weeks old, weight 2.0, 1.9, and 3.0 kg respectively. Ripasudil 0.4% was instilled twice a day to the left eye of all 3 rabbits, and the right eye was the control. After 1mo of continuous 0.4% ripasudil instillation to the left eye of all 3 rabbits, the first pigmented Dutch rabbit was euthanized with an overdose intravenous injection of pentobarbital sodium. The 2 remaining rabbits were instilled continuously with ripasudil for 3mo. After 3mo of the experiment, the remaining 2 rabbits were euthanized in the same manner. The eyeballs were immediately enucleated for the histological examinations.

Light and Electron Microscopy The enucleated eyes were fixed overnight in 2.5% glutaraldehyde +0.1 mol/L phosphate-buffered saline (PBS). The enucleated eyeballs were grossly examined before horizontally incised near equator. Vitreous body and lens were removed. Corneal ring was excised circumferentially 1 mm from limbus. Retina was grossly examined which no gross morphological abnormality was detected. Wedge shape specimen of the limbal region including trabecular meshwork, ciliary body and iris was taken from each quadrant. The pieces were rinsed three times with 0.1 mol/L PBS, immersed in 1% OsO₄, dehydrated and embedded in Quetol 812 resin. The resin was then polymerized and cured at 60°C for 48h. Each block of tissue specimen was cut into 0.5-µm-thick sections, stained with toluidine blue and examined under a light microscope (BX50; Olympus, Tokyo, Japan). Tissue was also cut into ultrathin sections at a thickness of 80 nm, double-stained with lead citrate and uranyl acetate, and examined using an electron microscope (JEM-1230; JEOL, Tokyo, Japan).

RESULTS

Microscopy of Trabecular Meshwork Comparing the control eyes (Figure 1A, 1D), after 1-mo instillation, the trabecular meshwork showed obvious increased intercellular spaces between the trabeculocytes (Figure 1B, 1E). The trabeculocytes became more thinned and rod-shaped at 3mo (Figure 1C, 1F). The scleral vascular plexus near the trabecular meshwork and the efferent vessel, which drained into the episcleral plexus increased slightly in size after the 1- and 3-month instillations. However, the pigmentary changes in the trabecular meshwork were unremarkable at any timepoints.

Microscopy of Ciliary Body and Iris Comparing the control eyes (Figure 2A, 2D), the interstitial space of the ciliary body was widened at 1 and 3mo (Figure 2B, 2E, and 2C, 2F, respectively). Comparing the control eyes (Figure 3A, 3D), the iris stromal tissue also showed gradually increasing intercellular spaces between the cells in 1 and 3mo (Figure 3B, 3E, and 3C, 3F, respectively). The histiocytes, fibroblasts,
and interstitial cells showed no significant changes by the light microscope. No abnormal inflammatory cells were detected in the iris stromal tissue. Melanocytes in the stroma were not observed in the microscopic examination. The clumping of melanin containing cells, especially at 3mo was detected in iris pigmented epithelial cells. Moreover, the pigmented granules in the iris pigment epithelial cells were increased in size and amount. Comparing the control eyes (Figure 3G, 3J), the sphincter and dilator muscles in the iris showed an increase in intercellular spaces, and muscle fiber bundles were also thin (Figure 3H, 3K, 3L). The actin filaments were diminished without affecting the other organelle in the sphincter muscle fibers. Z-bands (dense bodies) were also observed (Figure 3M).

DISCUSSION

In the microscopic findings of the trabecular meshwork, the space between cells also widened after prolonged exposure to ripasudil over the time period of the present study. Moreover, thinning of the iris muscle fiber and loss of actin filament were detected. After 3mo of ripasudil instillation, subclinical histological changes were detected without the functional disturbance.

In rabbit eyes, the anterior chamber has no true trabecula, and the vascular anatomy of the outflow pathways differs from that of primates[12]. Rabbits do not have a true Schlemm’s canal or collecting system as do primates. Many small intrascleral vessels (the trabecular plexus) may play an important role in the aqueous drainage system in rabbits. This vascular plexus becomes the densest adjacent to the trabecular meshwork, and drains the aqueous humor through the perilimbal blood vessels. In all mammals except primates, an iris pillar which originates from the anterior surface of the iris to the posterior surface of the corneoscleral junction, forms the completely enclosed angle called the space of Fontana which further develops to Schlemm’s canal as a result of the huge development of the ciliary muscle. The actin cytoskeleton modifying medication is known to inhibit the myosin light chain phosphorylation processes causing changes in cell shape, cell-cell adhesion, and cell-matrix adhesion[13]. The vascular plexus in the sclera also increases in vascular diameter with increased vacuolization in the vascular endothelium and the trabecular epithelium. These results confirm the cellular structure changes in the trabecular meshwork, which promotes the conventional outflow rate. Not only increasing the aqueous flow through the trabecular system, ripasudil also improves the permeability of the chamber angle trabecular plexus and the iris and ciliary body vasculature. The outflow facility increased 2 and 2.2 times from baseline as a result of the Isobe et al[9] and Honjo et al[14] experiments which were confirmed previously by the two-level constant-pressure perfusion method.

The unconventional outflow is comprised of uveoscleral and uveovortex outflows. The uveoscleral outflow drains the aqueous humor through the iris root and the anterior surface of the ciliary body then reaches the suprachoroidal space that drains into the episcleral vein. The uveovortex outflow drains the fluid into the iris vessel and the vortex veins. Because of the lower hydrostatic pressure in the suprachoroidal space than that in the anterior chamber, it causes the driving force of the aqueous to the uveoscleral outflow. These unconventional outflows depend on the net osmotic resorption of aqueous humor into the uveal venous circulation, extracellular matrix spaces, and episcleral venous pressure[15]. The results of our

Figure 2 Ciliary body  A, D: Control; B, E: 1mo after instillation; C, F: 3mo after instillation. The interstitial space is widening after ripasudil instillation.
A study showed obviously widening of the space between cells in the trabecular meshwork cells, ciliary body, and iris stoma. The relaxation of the vascular endothelium might improve vascular permeability and blood flow to the target organ\cite{16}.

As previously described, the unconventional outflow was determined by the fluid flow through the space between cells, drain into the vessel in the iris stroma and the suprachoroidal space. Continuous ripasudil instillation which resulted in myosin light chain-based cellular relaxation, space between cell-cell and cell-matrix become widened, might cause an increase in the unconventional outflow. The uveoscleral outflow was measured with a perfusion technique using fluorescein isothiocyanate-dextran in studies by Isobe et al\cite{9} and Honjo et al\cite{14} in which they reported non-significant increases from the baseline of 30% and 15%, respectively. Because these studies were performed after one instillation.

**Figure 3 Iris** A, D, G, J: Control; B, E, H, K: 1mo after instillation; C, F, I, L: 3mo after instillation. After ripasudil instillation, the stroma (B, E, C, F) and muscles (H, K, I, L) show increased intercellular spaces between the cells. The muscle fiber bundles are thin (K, L). Actin filaments are diminished (black dot circles). Z-bands (dense bodies) are observed (black arrows, M).
of a ROCK inhibitor, the ultrastructural change, which may prove to increase the unconventional outflow in the present study, occurred after continuous use of the ripasudil for at least 1mo. The unconventional outflow measuring method would be required to confirm this result after 1mo instillation of ripasudil.

The main cause of failure of glaucoma filtration surgery is postoperative subconjunctival scarring in the filtration bleb. There is an evident base indicating that human Tenon’s fibroblasts from subconjunctival space play a key role in the scarring process via proliferation, migration, and contraction processes. The transdifferentiation of fibroblasts into myofibroblasts is a crucial step in wound healing and scar formation[17]. Myofibroblasts are responsible for fibrosis via increased extracellular matrix synthesis, granulation tissue formation, and wound contraction[18]. A ROCK inhibitor, as an actin cytoskeleton inhibitor, functions as a potent antiscarring agent by inhibiting the transdifferentiation of Tenon’s fibroblasts into myofibroblasts, improving cellular migration, and inhibiting cell contraction during the wound healing process[18]. The results of the present in vivo study revealed that the prolonged exposure to ripasudil did not affect the stromal fibroblast structure. Even though the previous studies confirmed the antiscarring effect of ripasudil, no structural confirmation was made from this study.

The other ultrastructural changes found in the present study might indicate the potentially subclinical adverse effects in the future after long-term clinical use of ripasudil, such as an increase in the size of melanin granules in the iris pigment epithelium, iris stromal cells, and the anterior layer of melanocytes. Latanoprost-induced iris darkening (LIID), the most common ocular side effect of latanoprost, was initially speculated to arise from either a proliferation of melanocytes in the iris stroma or an increase in melanin granule numbers within stromal melanocytes. The morphological findings in LIID patients’ peripheral iris tissue obtained after trabeculectomy, showed significant change in the sizes of the intracellular melanin granules without change in the number of the granules or cellular proliferations. This effect was largely found in the anterior border rather than in the deep stroma[19]. As seen in the present study, continuous use of ripasudil might cause iris darkening in the future.

Regarding the experimental procedures, some limitations need to be acknowledged. First, there is 2 eyes of only 1 animal sample in the group of 1 and 3mo, respectively. Second, we did not measure intraocular pressure during experiment. Thus, the relationship between morphological changes and intraocular pressure still unknown. In the future, these evaluations should be included to confirm this limitation. However, this limitation does not affect our conclusions, because the histopathological differences in the trabecular meshwork, the ciliary body, and the iris were obvious. Inoue et al[20] showed significantly intraocular pressure lowering effect of ripasudil after 1 and 3mo after treatment which corresponding to morphological change found in this study.

In conclusion, ripasudil, as a potent ROCK inhibitor, inhibits myosin light chain containing cells such as epithelium, endothelium, fibroblasts, and smooth muscle cells in the anterior segment resulting in cellular relaxation, and increases intercellular space and cellular permeability.

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