学位論文内容の要旨
（Summary of Dissertation）

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学 位 論 文 題 名
（Dissertation Title）

A novel insight into the pathogenesis of refractory sight-threatening ocular diseases
（難治性眼疾患に対する新たな病態形成機序の解明）

【Background and Objectives】Age-related macular degeneration (AMD) and diabetic retinopathy (DR) are frequent retinal degenerative diseases, and responsible for the majority of cases of blindness. Both diseases are characterized by angiogenic and fibrotic formation at the retina or choroid. (1) Galectin-1, also known as *Lgals1*, has been demonstrated to contribute to angiogenesis through the modification of vascular endothelial growth factor (VEGF)-A signaling pathway. Previously, we showed the significant association of galectin-1 with the neovascular pathogenesis of DR. Here, we examined the involvement of galectin-1/*Lgals1* in ocular angiogenesis and fibrosis using the mouse model of laser-induced choroidal neovascularization (CNV). (2) Acrolein, a highly reactive unsaturated aldehyde, is known to facilitate glial cell migration, one of the pathological hallmarks in DR. Previously, we revealed that semicarbazide-sensitive amine oxidase (SSAO) plays a role in acrolein generation in retinal microvascular endothelial cells from patients with proliferative DR (PDR). In this study, we investigated the mechanism of acrolein generation in glial cells which lacks SSAO. (3) In addition, VEGF-A, a well-known angiogenic factor, contributes to the principal pathogenic events of DR. Transforming growth factor (TGF)-β, a multifunctional protein, regulates various pivotal biological responses and contributes to the pathogenesis of ocular diseases. However, concerning PDR, there have been no reports on the pro-angiogenic and fibrotic roles of TGF-β in Müller glial cells. We studied the molecular mechanism of TGF-β-induced VEGF-A production in Müller glial and the involvement of fibrovascular tissue formation in DR.

【Materials and Methods】(1) Laser photocoagulation was performed to induce CNV in *Lgals1* knockout and control mice. Seven days after laser treatment, the area of CNV and subretinal fibrosis were quantified. Gene expression levels of *Lgals1* and inflammatory mediators, as well as epithelial-mesenchymal transition (EMT) markers, were evaluated using real-time quantitative PCR (qPCR). Protein levels of phosphorylated SMAD family member (SMAD)2 and α-smooth muscle actin (SMA) were analyzed by immunoblot analysis. (2) Immunofluorescence staining was performed to show the localization of spermine oxidase (SMOX) in glial cells of fibrovascular tissues obtained from patients with PDR. Rat retinal Müller cell line 5 (TR-MUL5) cells were culture under either normoxic (20% O_2) or hypoxic (1% O_2) conditions. Expression levels of polyamine oxidation enzymes including SMOX were analyzed using real-time qPCR and enzyme-linked immunosorbent assay. Either hypoxia-inducible factor-1α (*Hif1*a) or *Hif2*a were knocked down by siRNA in TR-MUL5 cells. The transcriptional activity of SMOX in TR-MUL5 cells was evaluated using the luciferase assay. Levels of acrolein-conjugated protein, N^{ε}-(3-formyl-3,4-dehydropiperidino) lysine adduct (FDP-Lys), and hydrogen peroxide, were measured. (3) Human Müller glial cells were treated with various pro-fibrotic cytokines including TGF-β1 and -β2, to evaluate changes in gene expression using real-time qPCR. Immunoblot analyses were performed to detect the activation of TGF-β induced downstream signals.

Immunofluorescence analyses were conducted to evaluate the co-localization of VEGF-A and TGF-β1/2, TGF-β receptor (TβR), and myofibroblast markers in the fibrovascular tissues.

【Results】(1) Galectin-1/*Lgals1* was upregulated by CNV induction in the retinal pigment epithelium (RPE)-choroid complex, while deletion of *Lgals1* suppressed CNV together with VEGF receptor (VEGFR)2's downstream molecules. Loss of *Lgals1* also attenuated subretinal fibrosis and the expression of EMT markers including α-SMA and phosphorylated SMAD2. Supporting these *in vivo* findings, silencing of *LGALS1* in human RPE cells inhibited TGF-β1-induced EMT-related molecules and cell motilities. Conversely, overexpression of *Lgals1* enhanced CNV and subretinal fibrosis. AMD patient specimens demonstrated co-localization of galectin-1 with VEGFR2 in neovascular endothelial cells and with phosphorylated SMAD2 in RPE cells. (2) SMOX was localized in glial cells in fibrovascular tissues. Hypoxia induced SMOX production in TR-MUL5 cells, which were suppressed by silencing of *Hif1*a, but not *Hif2*a. Transcriptional activity of SMOX was regulated through HIF-1 binding to hypoxia response element sites in the promoter region of SMOX. Generation of hydrogen peroxide and FDP-Lys increased in TR-MUL5 cells under hypoxic conditions, which were abrogated by SMOX inhibitor. (3) Of various pro-fibrotic cytokines, TGF-β1/2 application to human Müller glial cells exclusively increased the mRNA expression of *VEGFA*, which was abolished by pretreatment with anti-TβR-neutralizing antibodies. Moreover, the administration of TGF-β1/2 upregulated *TGFB1* and *TGFB2* mRNA expression in an autocrine manner, which was suppressed by several signaling inhibitors. Supporting these findings, the administration of TGF-β1/2 increased the phosphorylation of these intracellular signaling. Immunofluorescence analyses showed co-localization of VEGF-A, TGF-β1/2, TβRI/II, and myofibroblast markers in glial cells in fibrovascular tissues from patients with PDR.

【Discussion】In the present study, for the first time, provide several findings on the molecular pathogenesis of AMD and DR. (1) Our previous study demonstrated that galectin-1 binds to VEGFR2 to mediate its downstream signal transduction in retinal microvascular endothelial cells, promoting angiogenesis in PDR. Importantly, the process of CNV-associated subretinal fibrosis has been reported mainly mediated by TGF-β1-SMAD2 signal transduction in RPE cells. Herein, we showed that galectin-1 promoted CNV formation and subretinal fibrosis, together with the underlying molecular mechanisms. (2) Dysregulation of SMOX alters intracellular polyamine levels and is associated with the progression of various human diseases (*i.e.*, diabetes). We found that *Hif1*a play a key role in regulated SMOX transcription in Müller glial cells under hypoxic condition. Hypoxic stimulation to Müller glial cells induced hydrogen peroxide and FDP-Lys production, both of which were suppressed by the potent SMOX inhibitor, suggesting that SMOX produces these two oxidative stress inducers in retinal Müller glial cells under hypoxic conditions. (3) Previous studies reported that the expression levels of TGF-β1 and -β2 in the vitreous fluid were higher in PDR eyes than in non-diabetic control eyes, and correlated with elevated vitreous VEGF-A levels. Here, we revealed that TGF-β-induced VEGF-A production and TGF-β autoinduction in Müller glial cell are via TβR together with SMAD-dependent pathway and SMAD-independent pathway, suggesting that the molecular pathogenesis of TGF-β in angiogenesis and fibrosis in Müller glial cells.

【Conclusion】Our current study highlights new insights on the molecular pathogenesis of AMD and DR, both of which are refractory sight-threatening diseases.