Autophagy in Neurodegeneration and Neuroinflammation in Glaucoma

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INTRODUCTION

This pilot project aimed for improved molecular understanding of autophagy regulation in glaucoma.

We hypothesize based on accumulating evidence that the molecular regulation and outcomes of autophagy during glaucomatous neurodegeneration are cell type-specific. Cellular stress, including mitochondrial energy depletion, leads to activation of the autophagy pathway as detected in human glaucoma and animal models. Although autophagy serves as a physiological adaptation mechanism to improve cell survival, experimental studies of glaucoma support that dysfunction of this metabolic homeostasis mechanism in retinal ganglion cells (RGCs) may also lead to early synapse loss, axon transport deficits, axon loss, and soma death (through a cross-talk with apoptosis signaling). On the other hand, dysregulation of autophagy in glial cells may stimulate various signaling pathways that are linked to glia-driven innate and adaptive immune responses with neurodegenerative potential (such as cytokine signaling, toll-like receptor signaling, inflammasome activation, and autoantigen presentation).

To explore cell type-specific molecular regulation and temporal outcomes of autophagy, we model glaucoma in transgenic mouse lines with RGC-targeted or glia-targeted specific deletions and systematically analyze transgenic animals and wild-type controls for cell type-specific outcomes of experimental glaucoma at different sites of injury at different time points.

DESIGN & METHODS

Our ongoing work pursues two opposing experimental paradigms of decreased autophagic flux (by deletion of Rab7 that promotes the fusion of autophagosomes with lysosomes and late endosomes), or compromised autophagy regulation (by deletion of mTOR that is an upstream negative regulator). Related transgenic lines are generated by breeding the specific f/f lines into Thy1-cre/ERT2 or GFAP-cre/ERT2 (or Cx3cr1-cre/ERT2) for RGC- or astroglia (or microglia)-targeted conditional deletion, respectively.

Additional lines, similarly generated by the cre/lox, include GFAP/IκKβ to also study the role of NF-κB (a transcriptional activator of inflammatory mediators and neuroinflammation in glaucoma) in molecular regulation of autophagy in astroglia. This is because our previous studies of glaucomatous human donor eyes and animal models of glaucoma pointed to a role for NF-κB in cell type-specific regulation of autophagy, as well as its potential as an immunomodulatory and neuroprotective treatment target for glaucoma.

To model glaucoma, intraocular pressure (IOP) elevation is induced by microbead/viscoelastic injections into the anterior chamber of transgenic mice and age/sex-matched wild-type background controls (C57BL/6J) with/without cre. The IOP is measured by a TonoLab, and the IOP-time integral is calculated for each mouse eye for group matching (besides neuron counts within each group).

At different time points of ocular hypertension, we characterize transgenic effects on the glial inflammatory activation phenotype (through the analysis of glial morphology by GFAP/Iba immunolabeling, and analysis of cytokine/chemokine profiles by multiplexed bioassays in retina, optic nerve head, and intraorbital optic nerve samples). We also analyze neuron survival and function (by counting RGC somas/axons and recording PERG responses).

Next, we isolate RGCs and astroglia by immunomagnetic cell selection and analyze the isolated cell type-specific protein samples for a diverse set of molecular outcomes, such as expression of markers related to autophagy (LC3-II, Atg5, Atg7, and p62) or apoptosis (Atg6, and proteolytic caspase activity/cleavage), expression of molecules linked to glial inflammation signaling (such as molecules involved in cytokine signaling, toll-like receptor signaling, or inflammasome activation), and transcriptional activity of NF-κB. Besides the preselected set of molecules, cell type-specific protein samples are analyzed by isotope labeling-based quantitative mass spectrometry (LC-MS/MS) to also gain high-throughput molecular information about transgenic effects on other components of cell survival or cell death pathways, and glia-driven inflammation.

RESULTS

For setting a baseline for our proposed molecular analysis in transgenic mouse models, we first analyzed the cellular outcomes of ocular hypertension in mice and characterized the cell type-specific molecular responses to experimental mouse glaucoma. While we are progressing towards the generation and cell type-specific analysis of Rab7 and mTOR lines, our initial studies have focused on the outcomes of astroglial IκKβ deletion.

Similar to C57BL/6J, microbead/viscoelastic injections in transgenic mice resulted in moderate IOP elevation (23.2±3.6 mmHg). The increased IOP could be maintained for a longer period (up to 12 weeks) if a second injection was given at week 4. After 12 weeks of ocular hypertension, a prominent decrease was detectable in the number of remaining RGCs and axons (~50% loss), and the PERG amplitude (~30% reduction) in microbead/viscoelastic-injected ocular hypertensive eyes relative to saline-injected normotensive controls. However, despite a similar increase in IOP, comparison of neuron counts and PERG responses between transgenic and wild-type groups (matched for the IOP-time integral) indicated an over 40% less neuron loss in astroglial IκKβ-deleted ocular hypertensive mice compared to wild-type ocular hypertensive controls. Besides increased neuron survival and function, astroglia-targeted inhibition of NF-κB by IκKβ deletion suppressed neuroinflammation in ocular hypertensive retina and optic nerve head. This was evident by decreased glial activation and ~four-fold lower titers of pro-inflammatory cytokines (including TNF-α and IFN-γ).

We next isolated mouse retina and optic nerve head astroglia for protein analysis. Our previous studies verified the isolated cell type-specific samples by retrograde labeling of RGCs, analysis of RGC and glia morphology, and analysis of specific cell markers by RT-PCR and immunoblotting. Our updated isolation technique allowed even increased protein yields, and immunoblotting of the isolated proteins for specific cell markers indicated more than 95% purity. **Figure 1** shows two-color immunoblots of astroglia samples (isolated from mouse retina or optic nerve head) using antibodies to an astroglia marker, GFAP (red), or neuron markers, NeuN or NFP (green).

Analysis of astroglia-specific protein samples by isotope labeling-based quantitative LC-MS/MS identified thousands of proteins (based on >3 unique peptide matches and a false discovery rate of <1%), including those with over two-fold up-regulated or down-regulated expression with ocular hypertension ($p<0.05$). Functional groups of the astroglia proteins up-regulated with ocular hypertension included cellular assembly and organization, cellular function and maintenance, cell survival, growth and proliferation, molecular transport, protein synthesis, cell adhesion, cell-cell interaction, cell signaling, and stress response. The Ingenuity Pathways Analysis of proteomics datasets linked the ocular hypertension-induced molecular alterations to various canonical pathways associated with inflammatory activation, including cytokine signaling, toll-like receptor signaling, and NF-κB activation pathways. We also detected a number of proteins linked to autophagy activation in retina and optic nerve head astroglia after 6 and 12 weeks of ocular hypertension. We presented these findings at ARVO 2017 meeting.

More recently, we analyzed astroglia proteins isolated from GFAP/IκKβ mice and detected prominent transgenic effects on ocular hypertension-induced inflammation signaling. Interestingly, our high-throughput datasets from GFAP/IκKβ mice indicated a significant down-regulation of astroglial mTOR. We also detected opposing variation of mTOR and BECN (beclin) expression, supporting the cross-talk between these two important proteins. In addition, WIPI (WD repeat domain phosphoinositide-interacting protein), WDFY (WD repeat and FYVE domain containing protein), LC3 (microtubule-associated protein 1 light chain 3), and SQSTM (sequestosome), which are required for autophagy progression and autophagosome formation, exhibited up-regulation in GFAP/IκKβ. **Figure 2** presents significantly ($p<0.05$) up-regulated (red) or down-regulated (green) components of the autophagy activation pathway in transgenic versus wild-type ocular hypertensive animals.

Figure 1

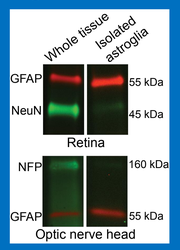
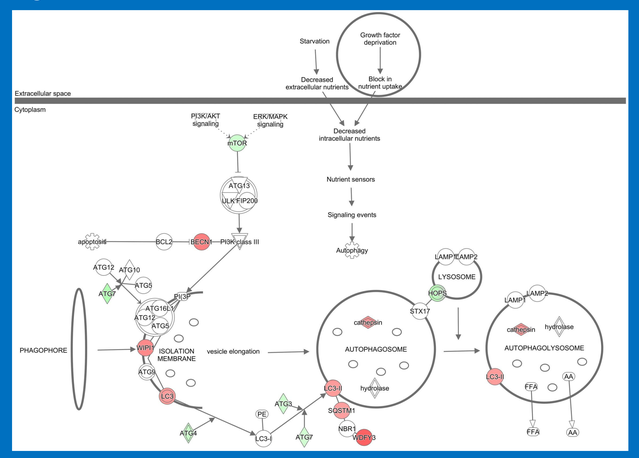


Figure 2



CONCLUSIONS

These findings further support the role for astroglial NF-κB in cell type-specific regulation of autophagy in mouse glaucoma. Predominant activation of NF-κB in glaucomatous astroglia might repress autophagy through the activation of autophagy inhibitor, mTOR (also supported by our previous observations). This likely reflects an autoregulatory feedback loop to control cell survival versus cell death and inflammatory outcomes. However, NF-κB may also activate autophagy through ubiquitin-binding protein p62 (SQSTM). Our ongoing studies explore these molecules (and many more) in cell type-specific molecular regulation of autophagy at different sites of injury at different time points of injury by modeling glaucoma in transgenic mice.

Given the complexity of glaucomatous neurodegeneration that involves multiple sites of injury, diverse cell types, time-dependent responses, and cell type-specific pathways, a new treatment strategy targeting multiple sites, multiple cell types, and multiple pathways at the same time is likely to be the most effective. Since autophagy can impact both RGC survival/axon integrity and glia-driven neuroinflammation in glaucoma, it seems to be a promising treatment target with multi-target potential. The new information should therefore have translational implications in development of immunomodulatory and neuroprotective treatments for glaucoma.

NEXT STEPS

To speed the scientific understanding of molecular processes towards new treatment possibilities for glaucoma patients, cell type-specific analysis is essential. We continue to systematically analyze the temporal course of cell type-specific responses to cell type-targeted transgenic deletion of specific molecules in retina and optic nerve (head) of mouse eyes with or without induced ocular hypertension.

Ongoing studies are expected to further characterize cell type-specific, site-specific, and temporal outcomes of glaucoma towards the development of new treatment strategies for immunomodulation and neuroprotection in glaucoma.

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