PERSPECTIVE

Physiological significance of Rag1 in retinal ganglion cell death

Although the transcription factor, nuclear factor-κB (NF-κB) is known to regulate cell death and survival, its precise role in cell death within the central nervous system (CNS) remains unknown. We previously reported that mice with a homozygous deficiency for NF-κBp50 spontaneously developed optic neuropathy. We examined the expression and activation of pro-apoptotic factor(s) that mediate optic neuropathy in *p50*$^{-/-}$ mice. Recombination activating gene 1 (Rag1) is known to regulate the recombination of immunoglobulin V(D)J. Experiments with genetically engineered mice revealed the involvement of Rag1 expression in the apoptosis of Brn3a-positive retinal ganglion cells (RGCs), and also showed the specific effects of a p50-deficiency on the activation of Rag1 gene transcription. Furthermore, a genetic analysis of murine neuronal stem-like cells clarified the biological significance of Rag1 in N-methyl-D-aspartate (NMDA)-induced neuronal apoptosis. The apoptotic regulating factors, Bax, and cleaved caspase 3, 8, and 9 were detected in HEK293 cells expressing the external molecule of Rag1, and a human histological examination revealed the expression of Rag1 in RGCs. A recent study indicated that Rag1 played a role in optic neuropathy as a pro-apoptotic candidate in *p50*$^{-/-}$ mice. This result may lead to new therapeutic targets in optic neuropathy.

The intracellular pathways related to cell survival regulate neuronal physiology during embryonic development as well as the pathogenesis of various neurodegenerative disorders. The NF-κB pathway was discovered in 1986 as a transcription modulator of the light chain of B lymphocyte immunoglobulins (Sha et al., 1995; Hoffmann et al., 2003). Subsequent studies identified NF-κB as a ubiquitously expressed dimeric transcription factor involved in numerous cellular processes, such as inflammation, differentiation, apoptosis, and oncogenesis. NF-κB is a dimer composed of members of the Rel family, which includes RelA(p65), RelB, and c-Rel (Hoffmann et al., 2003). The NF-κB family, which is primarily composed of p50/p65(RelA) heterodimers, has been detected in most animal cell types and is involved in cellular responses to stimuli such as stress and cytokines (Sha et al., 1995). NF-κB is sequestered in the cytoplasm of unstimulated cells by a class of inhibitors called IκBs. The degradation of IκB allows NF-κB to enter the nucleus, in which it specifically initiates the expression of target genes. Accordingly, the impaired regulation of NF-κB has been linked to various diseases, including cancer, inflammatory disorders, and autoimmune diseases, as well as deficiencies in the processes of synaptic plasticity and memory (Hoffmann et al., 2003). The NF-κB family also plays important roles in nervous system development and pathology by influencing neuronal apoptosis, neurite outgrowth, and synaptic plasticity (Baeuerle and Baltimore, 1988). However, the range of intercellular signals and transduction mechanisms that regulate NF-κB activity in neurons is broad and complex. Knockout mice have been extensively used to assess different gene components in the NF-κB pathway. For example, *p50*$^{-/-}$ mice exhibited the age-related degeneration of neuronal and non-neuronal cells, and the defective activation of NF-κB resulted in apoptosis in the striatal neurons of a Huntington disease model. Activated NF-κBp65 has been implicated in glutamate-induced neurotoxicity, NMDA-induced retinal neuronal cell death, retinal ischemia, and reperfusion injury in the CNS (Takahash et al., 2007). We previously reported that the number of retinal ganglion cells (RGCs) was significantly lower in p50-deficient (*p50*$^{-/-}$) mice than in its parental mice, *p50*$^{+/+}$ mice, suggesting that these animals exhibited features resembling those of human glaucoma (Takahash et al., 2007). However, the precise role of NF-κB in cell death within the CNS remains controversial. Therefore, we searched for a new target related to NF-κB pathways in neurons.

Verkoczy et al. (2005) reported that NF-κB was relevant in the B-cell receptor-mediated regulation of recombination activating gene (Rag) locus transcription. They suggested that immediately activated NF-κB pathways may facilitate quick antigen receptor-regulated changes in Rag expression, which is important for editing (Verkoczy et al., 2005). Rag genes encode two enzymes that play key roles in the adaptive immune system: both Rag1 and Rag2 mediate the recombination of V(D)J, a process that is essential for the maturation of B and T cells in the development and maturation of lymphocytes (Mombaerts et al., 1992). Rags have been detected not only in the immune systems of mammals and amphibians, but also in their nervous systems; Rag1 transcripts have been found in the murine CNS, particularly in areas of high neural density, such as the cerebellum and hippocampal formation (Chun et al., 1991; Fang et al., 2013). Rag1 may function in neurons to site-specifically recombine elements of the neuronal genome or prevent detrimental alternations in the genomes of long-lived cells. Although the role of the Rag1 locus in the CNS is currently unclear,

Rags are known to be regulated by NF-κB (Verkoczy et al., 2005). Based on the findings described above, we focused on Rag1 as a novel candidate target related to NF-κB pathways in neurons using *p50*$^{-/-}$ mice as a model of optic nerve neuropathy. Since no studies have been published on the expression of Rag1 in the visual system, we first confirmed the presence of Rag1, but not the Rag2 transcript in RGCs. Western blot analysis with antibody

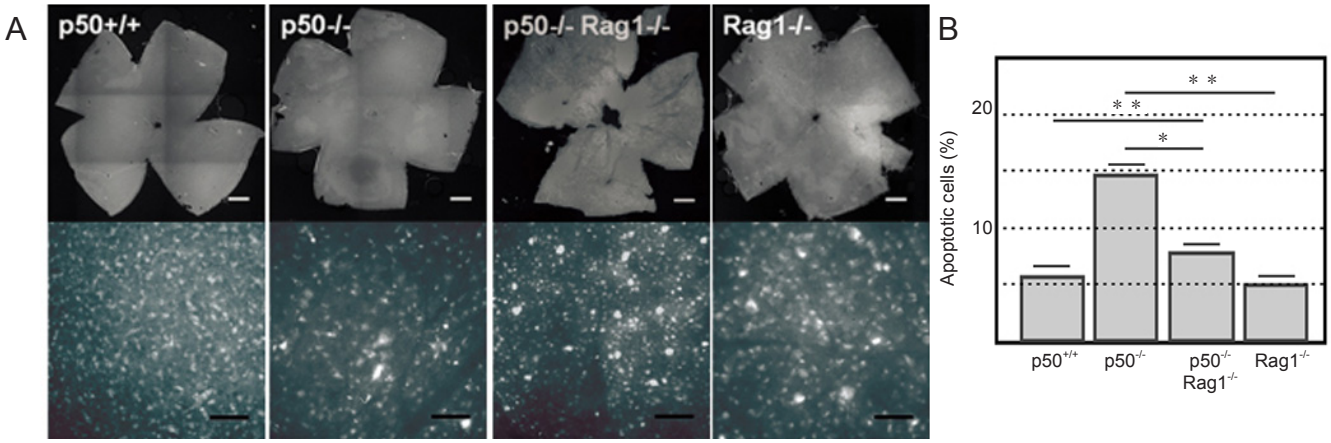

Figure 1 Effects of Rag1 knockdown in *p50*$^{-/-}$ mice.
(A) Fluorescence micrographs of flat-mounted retinal preparations 7 days after the injection of Fluoro-Gold into the superior colliculus of 6-month-old *p50*$^{+/+}$, *p50*$^{-/-}$, *p50*$^{-/-}$*Rag1*$^{-/-}$, and *Rag1*$^{-/-}$ mice. Upper panel: Scale bars: 500 μm; Lower panel: Scale bars: 50 μm. (B) The distribution of live, early apoptotic, late apoptotic, and dead cells was measured by flow cytometry using purified RGC samples from *p50*$^{+/+}$, *p50*$^{-/-}$, *p50*$^{-/-}$*Rag1*$^{-/-}$, and *Rag1*$^{-/-}$ mice. The quantification of the total apoptotic cell ratio, which was calculated as the total amount of late and early apoptotic cell ratios, revealed that this ratio was significantly higher in *p50*$^{-/-}$ mice than in *p50*$^{+/+}$, *p50*$^{-/-}$*Rag1*$^{-/-}$, and *Rag1*$^{-/-}$ mice (n = 8 per group). $*P < 0.005$; $**P < 0.001$. One-way ANOVA followed by Tukey's *post hoc* test.

against Rag1 showed the expression of Rag1 in retinal crude extracts derived from both *p50*$^{+/+}$ and *p50*$^{-/-}$ mice. The expression of Rag1 in the retina was significantly higher in *p50*$^{-/-}$ mice than in wild-type mice (Hirano et al., 2015). A positive regulator of the NF-κB family, such as RelA/c-Rel, may have mildly induced the expression of Rag1 in *p50*$^{-/-}$ mice. The absence of Rag1 in *p50*$^{-/-}$ mice resulted in a decrease in optic nerve neuropathy (**Figure 1**). In vertebrate embryonic development, the retina and optic nerve originate as outgrowths of the developing brain, and, thus, the retina is considered to be part of the CNS. Furthermore, three-dimensional cultures of mouse embryonic stem cell aggregates have demonstrated the autonomous formation of the optic cup, which develops into the outer and inner layers of the retina structure from brain balls (Eiraku et al., 2011). Glutamate is a major excitatory neurotransmitter in vertical pathways through the retina, in which RGCs first express the NMDA glutamate receptors that are typical in the brain (Takahash et al., 2007). Since genesis and neurotransmission in the brain and retina are closely related, it is plausible that Rag1, which has been detected in the hippocampus, is also expressed in the retina.

We assessed the precise role of Rag1 in the retina using experiments with *p50*$^{-/-}$ mice, which exhibit age-dependent decreases in RGCs. A lack of Rag1 in *p50*$^{-/-}$ mice diminished the loss of RGC, which was confirmed by several lines of evidence in the previous study (**Figure 1**). To validate the physiological function of Rag1 in NMDA-induced neuronal cell death, *in vitro* experiments were performed with the HEC293-Rag1 cell line, which stably expresses Rag1. Western blot experiments with whole cell lysates obtained from the HEC293-Rag1 cell line and its control cell line, HEC293-control, were subsequently performed in order to verify the physiological function of Rag1 in the apoptosis signal cascade. Expression of the apoptotic signaling factors Bax and cleaved caspase 3, 8 and 9 in the HEC293-Rag1 cell line was significantly higher than in the controls (Hirano et al., 2015). Rap75 and α-tubulin as internal controls in Rag1-overexpressing cell lysates were detected at similar levels to those in control lysates (Hirano et al., 2015). These results prompted us to speculate that Rag1 may play a role in the programmed cell death of RGCs, which was accelerated by NF-κB, in *p50*$^{-/-}$ mice. We found that Rag1 was also localized in the nucleus of RGCs in 15-month-old *p50*$^{-/-}$ mice in which the RGC number had already markedly decreased; therefore, we proposed that Rag1 may specifically influence apoptotic signaling in the nucleus (Hirano et al., 2015) (**Figure 2**).

Many questions still remain regarding the molecular mechanisms involved in Rag1 functions in the retina. However, Rag1 may play a role in RGCs that is entirely distinct from somatic recombination. The evidence for this lies in studies on the molecular structures of the recombinase enzymes themselves; although both Rag1 and Rag2 share several roles, *i.e.*, DNA cleavage and rearrangement of V(D)J recombination, only Rag1 contained the catalytic DNA-binding core of the recombinase (Chun et al., 1991; Mombaerts et al., 1992). These domains are known to be similar to the active site of several transposases and integrases. Kelch motifs, which mediate the interactions of Rag2 and Rag1, have been observed in numerous proteins (Hirano et al., 2015), and Rag1 may interact with an identified protein *via* a kelch motif in the retina. As in the mouse retina, we confirmed the localization of Rag1, but not Rag2, in RGCs in the human retina. A protein homology study revealed that the human Rag1

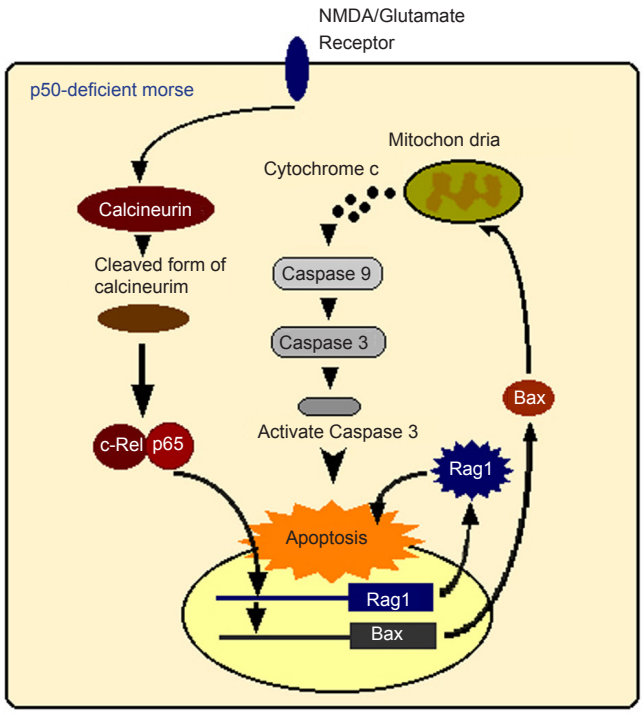

Figure 2 Signal cascade of cell death mediated by Rag1 in p50-deficient mouse.
A recent study demonstrated that the binding site of the hetero dimer p50-RelA(p65) could also be occupied by the homo dimer p50-p50, and may function as a repressor to regulate the role of p50-RelA(p65) as a transcription factor essential for neuronal responses. In p50-deficient neuronal cells, the c-Rel-RelA(p65) hetero dimer markedly induced Rag1 gene activation as a transcription factor. Rag1 may play a role in neuronal cell death signaling as a nuclear mediator. The cell death factors, Bax and cleaved caspase-3, and 9, were also clearly detected in Rag1-expressing cells.

molecule shared 90% homology with its mouse ortholog, and the catalytic domains, zinc-finger, recombinase, and RING-finger in the Rag1 molecule appeared to be conserved between species (Hirano et al., 2015). These results indicated that the physiological significance of Rag1 observed in mice may extend to the regulation of human RGC survival. We concluded that Rag1 may also be involved in the programmed cell death of RGCs in the human glaucomatous retina. Further studies on the role of Rag1 in RGCs are expected to contribute to the development of preventive and therapeutic treatments for human glaucoma.

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