Amino-Terminal Phosphorylation of c-Jun Regulates Apoptosis in the Retinal Ganglion Cells by Optic Nerve Transection

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PURPOSE. To examine the involvement of c-Jun and c-Jun N-terminal phosphorylation (JNP) in apoptosis of retinal ganglion cells (RGCs) after the optic nerve (ON) transection.

METHODS. The expression and phosphorylation of c-Jun protein and apoptosis in RGCs were examined after ON transection in wild-type mice and mice in which both phosphoacceptor serines of Jun have mutated to alanines (c-Jun[AA] mice). The fluorescent tracer 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was applied to the superior colliculi (SC) and the right ON was severed after 7 days. After two more weeks, the average number of RGCs per field was calculated.

RESULTS. JNP and TUNEL-labeled apoptotic nuclei were detected in the ganglion cell layer (GCL) of the retina of the wild-type mice in response to ON transection. The numbers of TUNEL-positive nuclei in the c-Jun[AA] mice was reduced in comparison to those in wild-type mice. Retrograde labeling showed that the number of the RGCs in the retinas on the injured side of the c-Jun[AA] mice was significantly higher than in wild-type mice 14 days after the lesion.

CONCLUSIONS. These results suggest that there is a partial but significant contribution of JNP to the induction of apoptosis in RGCs by ON transection. (Invest Ophthalmol Vis Sci. 2002;43:1631–1635)

Retinal ganglion cells (RGCs) die by apoptosis after optic nerve (ON) transection.1 RGC death after axotomy can be blocked by cycloheximide, suggesting that the protein's synthesis is required.2 There are gene-specific factors that regulate the transcription of target genes by binding to specific recognition elements, which usually are located in the upstream promoter region of the gene.3 These factors usually increase the rate of transcription of the gene and therefore increase the formation of mRNA and protein. We have reported the activation of transcription factor in the retina, cornea, and lens.4–19

The heterodimeric transcription factor AP-1 has been implicated in diverse biological processes, such as cell proliferation, cell differentiation, and apoptosis.20 and c-Jun is an important component of this transcription factor. A way of regulating of c-Jun's and thereby AP-1's activity is by c-Jun N-terminal phosphorylation (JNP) at serines 63 and 73 through the Jun N-terminal kinases (JNKs).21,22

c-Jun and JNP seem to be involved in inducing apoptosis in neurons, because overexpression of dominant negative alleles of c-jun blocks apoptosis in cultured neurons,23 and the presence of JNP is associated with neuronal cell death.24,25 Recently, we reported that mice having both phosphoacceptor serines mutated to alanines (c-Jun[AA] mice) are resistant to epileptic seizures and neuronal apoptosis induced by the excitatory amino acid kainate.26 In this study, involvement of c-Jun phosphorylation in RGC apoptosis after ON transection was examined in c-Jun[AA] mice.

MATERIALS AND METHODS

Experimental Animals

c-Jun[AA] mice were generated as described previously.27 The animals are anesthetized with a ketamine cocktail containing ketamine (3.75 mL [100 mg/mL]), acepromazine (0.3 mL [10 mg/mL]), rompun-xylazine (1.9 mL [20 mg/mL]), and sterile saline (23 mL) with the dosage of 0.4 mL/100-g mouse. After partial craniotomy, access to the ON was gained by suctioning the overlying cerebral cortex, and the right ON was completely severed between the optic foramen and the chiasm.28

The animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

Mice were anesthetized with the ketamine cocktail and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) containing 0.5% picric acid at room temperature. After enucleation, the eyes were postfixed overnight in the same fixative containing 20% sucrose. On the next day, 12-μm-thick frozen sections were cut on a cryostat and mounted onto gelatin and poly-L-lysine-coated slides. To assure comparability of results between various animals, equatorial sections were used containing the ON head as standard anatomic reference.29

Immunocytochemistry and Apoptosis Assays

The slides were dried for 1 hour, rinsed in PBS twice, and incubated with phosphaSer63-specific c-Jun antibody (rabbit polyclonal; New England Biolabs, Beverly, MA) or anti-c-Jun antibody (rabbit polyclonal, H79; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 12 hours. Binding of the primary antisera was localized using an avidin-biotin immunoperoxidase kit (Vector Laboratories, Burlingame, CA). After three 5-minute washes in TNT (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20), sections were incubated with 1:50 dilution of rhodamine tyramide in 1× amplification diluent (DuPont NEN Life Science Products, Boston, MA). Apoptotic cells were detected with a kit (In Situ Cell Death Detection Kit, Fluorescein, TUNEL; Roche Molecular Biochemicals, Indianapolis, IN). For statistical analysis of

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c-Jun(AA) mice. TUNEL-labeled RGCs were detected more sparsely than in the wild-type mice (k, l). Most c-Jun- and phospho-c-Jun-labeled nuclei (b, c, f, g) in the wild-type retina were large and round without apoptotic-appearing nuclei. In contrast, TUNEL-labeled nuclei showed various signs of apoptosis, such as nuclear shrinkage, condensation of the chromatin, and nuclear fragmentation (j, k). Magnification, ×400.

RESULTS

Assessment of RGC Survival

To identify RGCs, we labeled these cells before any experimental manipulation with the fluorescent tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR), using previously described techniques that involve the application of the tracer to the superior colliculi (SC).30 In brief, the DiI suspension was prepared by mixing 3 mg DiI in 1 mL saline containing 1% to 3% Triton X-100 (DuPont NEN). Sonication and repeated agitation produced a mixture of dissolved DiI and small DiI crystals in suspension. The mice were anesthetized, each midbrain was exposed, and, after removing the pia overlaying the SC, a small pledget of gelatin sponge (Spongostan Film, Ferronsan, Denmark) soaked in DiI suspension was laid over the SC to mark the RGCs by retrograde axonal transport. Seven days after the DiI application, the right ON was completely severed, as described earlier.

Retrograde Labeling of RGCs

To identify RGCs, we labeled these cells before any experimental manipulation with the fluorescent tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR), using previously described techniques that involve the application of the tracer to the superior colliculi (SC).30 In brief, the DiI suspension was prepared by mixing 3 mg DiI in 1 mL saline containing 1% to 3% Triton X-100 (DuPont NEN). Sonication and repeated agitation produced a mixture of dissolved DiI and small DiI crystals in suspension. The mice were anesthetized, each midbrain was exposed, and, after removing the pia overlaying the SC, a small pledget of gelatin sponge (Spongostan Film, Ferronsan, Denmark) soaked in DiI suspension was laid over the SC to mark the RGCs by retrograde axonal transport. Seven days after the DiI application, the right ON was completely severed, as described earlier.

Assessment of RGC Survival

Two weeks after the ON dissection, the mice were given a lethal dose of ketamine cocktail. The eyes were enucleated, and the retinas were detached and prepared as flattened wholemounts in 4% PFA in 0.1 M PBS solution. At approximately the same distance (0.3 mm) from the optic disc, six fields of identical size (0.077 mm²) were randomly chosen, and labeled cells were counted under a fluorescence microscope (magnification, ×800) by observers blinded to the identity of the mice, and the average number of RGCs/mm² was calculated.31 The location of the fields was specified to avoid variations in RGC density as a function of distance from the optic disc.31 For statistical analysis, six animals were used for each wild-type and c-Jun(AA) mice. The results were evaluated with the Aspin-Welch t-test.

RESULTS

c-Jun, JNP, and TUNEL labeling were absent in retinas of untreated contralateral eyes (3 not shown) and 7 days after ON transection (Figs. 1a, 1e, 1i), when many c-Jun- and JNP-positive nuclei were detected in the GCL of treated wild-type eyes (Figs. 1b, 1c, 1f, 1g). In more than 20 surgically altered retinas of c-Jun(AA) mice, no JNP immunopositive cells were detected (Fig. 1h). Most c-Jun and phospho-c-Jun labeled nuclei in the wild-type retinas were large and round, showing no sign of apoptotic nuclei (Figs. 1b, 1c, 1f, 1g). TUNEL-labeled RGCs were also detected 3 and 7 days after ON transection in the wild-type mice (Figs. 1j, 1k). These TUNEL-labeled nuclei revealed various signs of apoptosis, such as nuclear shrinkage and condensation of the chromatin (Figs. 1j, 1k). In c-Jun(AA) mice, TUNEL-labeled RGCs were detected more sparsely compared with the wild-type mice (Fig. 1l).

The numbers of c-Jun- and JNP-positive nuclei were high 3 and 7 days after the lesion and declined after 14 days in wild-type mice. The numbers of c-Jun-positive nuclei in the c-Jun(AA) mice was reduced to 41% in comparison to wild-type mice (Fig. 2a). The numbers of TUNEL-positive nuclei in the c-Jun(AA) mice was reduced to 25% in comparison to wild-type mice (Fig. 2b).

Retrograde labeling of RGCs with DiI was performed 1 week before ON transection to determine whether the c-Jun(AA) mice were more resistant to secondary neuronal death after axotomy (Fig 3). Fourteen days after the lesion, the number of RGCs per square millimeter in the retinas on the uninjured side of the c-Jun(AA) mice (n = 6) was not significantly different from that in wild-type mice (n = 6; Fig 4). In contrast, the number of RGCs per square millimeter in the retinas on the injured side of the c-Jun(AA) mice (n = 6) was significantly higher (P < 0.001) than in wild-type mice (n = 6).

DISCUSSION

The present study investigates the expression and phosphorylation of c-Jun protein and signs of RGC apoptosis after ON transection. JNP- and TUNEL-labeled apoptotic nuclei was detected in the GCL of mice retina in response to ON transection. Most c-Jun- and JNP-labeled nuclei (Figs. 1b, 1c, 1f, 1g) in the wild-type retina were large and round and appeared to have no apoptotic nuclei. These results suggest that JNP and apoptotic changes in the RGCs are temporally different. JNP may be involved in the initiation of cell death of RGCs, as reported in 3T3 fibroblasts in response to the alkylating agent.32

The number of TUNEL-positive nuclei in the c-Jun(AA) mice was reduced in comparison to those in wild-type mice; however, this was only a hint that mutant mice may exhibit less apoptosis. In addition, more cells could become necrotic and die without TUNEL positivity. Therefore, it remained to be determined whether the AA mutation has a survival-promoting
Retrograde labeling of RGCs with DiI is a well-established procedure and was feasible in mice once the axotomy itself had been performed. The number of remaining cells in mutant and wild-type mice was then compared, to substantiate the claim that the c-Jun(AA) mice are more resistant to secondary neuronal death after axotomy. Fourteen days after the lesion, the number of RGCs in the retinas on the injured side of the c-Jun(AA) mice was significantly higher than in wild-type mice, suggesting that the c-Jun(AA) mice are more resistant to secondary neuronal death after axotomy. It was also suggested that JNP was not involved in the cell genesis of RGCs, because the number of the RGCs in the retinas on the uninjured side of the c-Jun(AA) mice was not significantly different from that of wild-type mice.

Transcription of Jun is autoregulated by the c-Jun protein. The numbers of c-Jun–positive nuclei in the c-Jun(AA) mice was reduced in comparison to that in wild-type mice, suggesting that JNP is required for efficient Jun expression.

The mechanism that links the JNP and apoptosis has not been determined. FasL is suggested to be one important target gene regulated by JNP during apoptosis, in both in vitro and in vivo systems. Experimental and human glaucoma lead to the interruption of retrograde axonal transport in ganglion cell axons and to apoptotic cell death in RGCs. Phosphorylation of c-Jun, in conjunction with the gene that is regulated by c-Jun, may provide a clue for further understanding of apoptotic cell death after glaucoma or ON injury.

**FIGURE 2.** (a) The number of c-Jun– and phospho-c-Jun–immunoreactive cells after ON transection. The number of nuclei was high 3 and 7 days after the lesion and declined after 14 days in wild-type mice. The number of c-Jun–positive nuclei was reduced and no phospho-c-Jun was detected in c-Jun(AA) mice. (b) The numbers of TUNEL-positive cells after ON transection. The number of TUNEL-positive nuclei in the c-Jun(AA) mice was lower than in wild-type mice. Data reflect average numbers of labeled RGCs per retinal section and are derived from seven independent experiments. Error bars, SD. *P < 0.01; **P < 0.01.


