The Role of c-*fos* in Cell Death and Regeneration of Retinal Ganglion Cells

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Purpose. To investigate the effect of c-*fos* on apoptotic cell death and regeneration of damaged retinal ganglion cells (RGCs) in tissue culture of retinal explants.

Methods. Retinas from transgenic mice carrying the exogenous c-*fos* gene under the control of the interferon (IFN)-α/β inducible Mx-promoter (Mx-c-*fos*), c-*fos*–deficient mice, and littermate control mice were dissected and cultured in a three-dimensional collagen gel culture system, followed by an analysis of TdT-dUTP terminal nick-end labeling (TUNEL) staining and measurement of neurites that emerged from explants.

Results. Compared with littermate control mice, Mx-c-*fos* transgenic animals showed a higher ratio of TUNEL positivity in the RGC layer from early in the culture period that correlated with the small number of regenerating neurites. In contrast, the c-*fos*–null mutated mice showed a still-lower ratio of TUNEL-positive cells. Nevertheless, the number of regenerating neurites was significantly lower in the initial phase, although the drastic increase in density of neurite regeneration was observed in the late period of culture.

Conclusions. These findings suggest that c-*fos* is involved in both apoptotic cell death and regeneration of damaged RGCs. Elucidation of the precise c-*fos*–mediated cascade involved in RGC apoptosis and regeneration is significant in realizing neuronal survival and regeneration. (*Invest Ophthalmol Vis Sci.* 2002;43:2442–2449)

The cellular immediate early gene c-*fos*, which encodes a nuclear phosphoprotein (c-Fos), is transiently induced in numerous cell types by many kinds of stimuli and conditions.[1] c-Fos is known to form a heterodimeric complex with Jun family proteins that works as the transcription factor activator protein (AP)-1.[1]

Expression of c-*fos* is strongly implicated in the activation program of cell death in various types of neuronal cells.[2,3] Recent studies have indicated that c-*fos* induction is required before the condensation of apoptotic chromatin and fragmentation of oligonucleosomal DNA can occur.[2,4–6] On the other hand, some reports indicate that c-*fos* is not essential for apoptosis in several tissues, including the central nervous system (CNS).[7,8] In addition, evidence shows that c-*fos* is positively involved in the process of neuronal survival and neurite extension.[9–12] Accordingly, the precise role of c-*fos* in cell death, survival, and regeneration of neuronal cells remains to be explained.

A variety of pathologic situations ranging from ischemic, traumatic insults to glaucoma are known to cause apoptotic cell death of RGCs.[13–15] If we can regulate activity of modulatory factors of apoptosis, diseases such as those mentioned here may be cured by the rescue of damaged RGCs, in that apoptosis may be the final common pathway of RGCs.[16,17] Furthermore, the induction of neurite regeneration is equally necessary for the functional recovery of vision after RGC damage.

Damage of RGCs immediately influences intracellular signaling pathways, which subsequently induce expression of genes involved in apoptotic cell death.[18–22] Among various kinds of factors related to the cellular emergency, such as injury and cell death, c-*fos* is a key factor in the cellular signaling system activated immediately after cell damage.[2–5]

Tissue culture of retinal explants has been a useful tool for the study of development, degeneration, and regeneration of retinal cells.[23,24] Culture of retinal explants in a three-dimensional collagen gel culture system is accompanied by the axotomy of RGCs, together with the disturbance in the cellular environment during its manipulation. In this study, we used this culture system, because the method has an advantage in that both cell death and neurite regeneration of RGCs can be simultaneously and quantitatively analyzed. The stable condition of retinal tissue fixed in collagen gel also enables us to observe both events through a longer period of culture, to demonstrate both the remarkable reduction of apoptosis and the retention of neurite regeneration capacity in RGCs in c-*fos*–deficient mice.

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Materials and Methods

Experimental Animals

C57BL/6CrSlc mice were purchased from Japan SLC Co., Ltd. (Hamamatsu, Japan). Mx-c-*fos*[25] provided by Ulrich Rüther (EMBL, Heidelberg, Germany) and c-*fos*–deficient mice[26] provided by Erwin F. Wagner (IMP, Vienna, Austria) were maintained by heterozygous mating in our animal facility. Mx-c-*fos* mice possess a constructed gene that causes them to express the protooncogene c-*fos* under the control of the IFN-α/β inducible Mx promoter.[25] In c-*fos*–deficient mice, the second exon and intron of the c-*fos* gene are replaced by a neomycin-resistant gene without the expression of functional c-Fos.[26] The c-$fos^{+/+}$ littermates were used as a control for both the heterozygous c-*fos*–deficient (c-$fos^{+/-}$) and homozygous c-*fos*–deficient (c-$fos^{-/-}$) mice. In case of Mx-c-*fos* mice, an intraperitoneal injection of 500 μg poly (I:C), a double-stranded polynucleotide (Amersham Pharmacia Biotech, Buckinghamshire, UK), dissolved in 0.1 M phosphate-buffered saline (PBS; pH 7.2) was performed 1 day before the cells were

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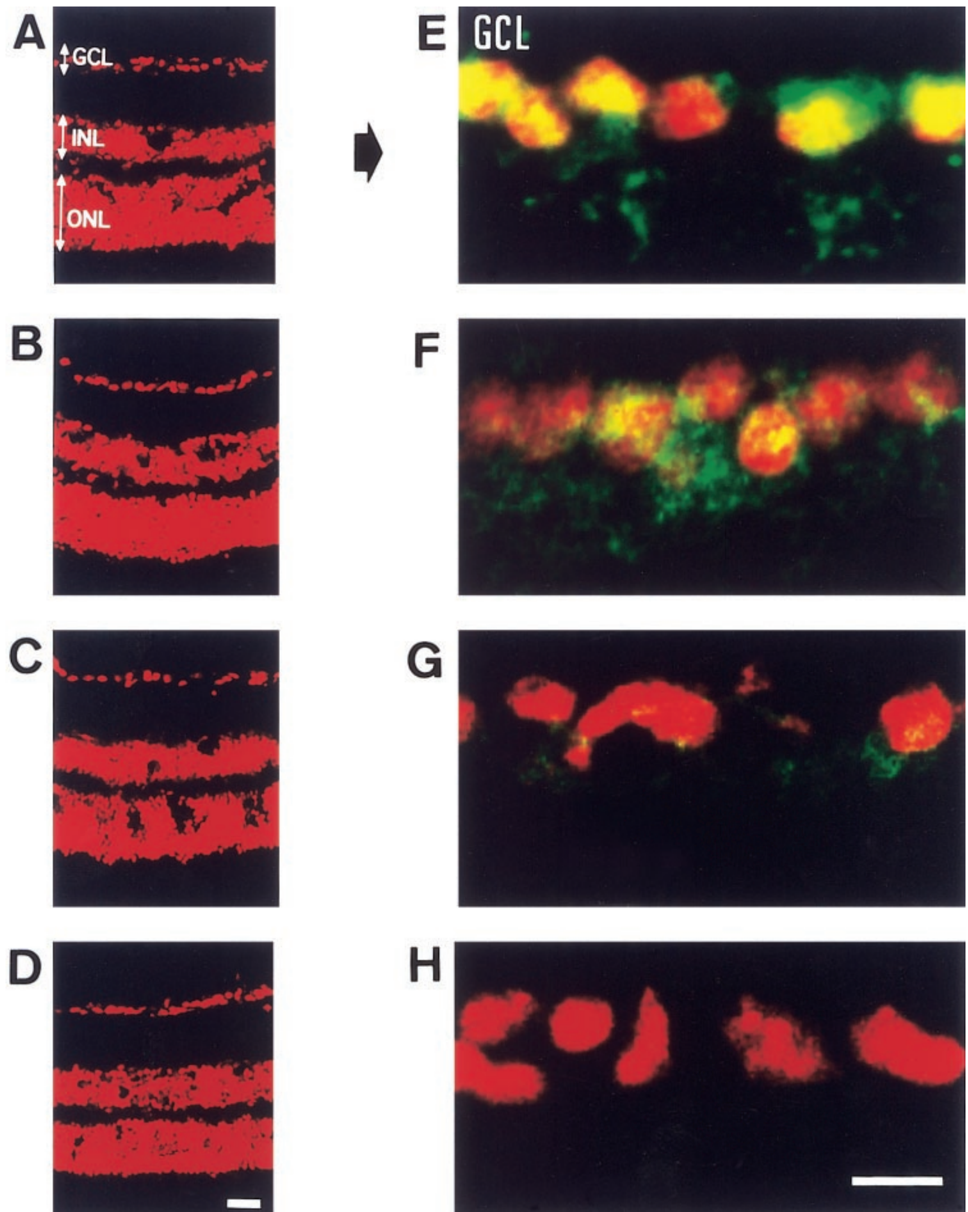

Figure 1. c-Fos immunohistochemistry of retinas. c-Fos expression (*green*) in retinas from Mx-c-*fos* (**A**, **E**), c-$fos^{+/+}$ (**B**, **F**), c-$fos^{+/-}$ (**C**, **G**), and c-$fos^{-/-}$ (**D**, **H**) mice was examined 4 hours after manipulation of the culture by immunohistochemistry. (**A–D**) PI staining for orientation of retinas (*red*). Compared with c-$fos^{+/+}$ (**F**), c-$fos^{+/-}$ (**G**) showed weaker immunoreactivity, whereas Mx-c-*fos* (**E**) displayed stronger c-Fos reactivity. No c-Fos reactivity was observed in c-$fos^{-/-}$ retina (**H**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: (**A–D**) 30 μm; (**E–H**) 10 μm.

cultured, to induce expression of the exogenous c-*fos* gene in vivo.[25] Injection of poly (I:C) into mice induces IFNα/β production very rapidly. Therefore, after intraperitoneal injection of poly (I:C) we can obtain a strong expression of c-Fos in Mx-c-*fos* mice.[25] Control littermates of Mx-c-*fos* mice injected with poly (I:C) (control-poly [I:C]) were used as the control for Mx-c-*fos* mice. For experiments, 8- to 12-week-old Mx-c-*fos*, c-$fos^{-/-}$, c-$fos^{+/-}$, c-$fos^{+/+}$, and control-poly (I:C) mice were used. All studies concerning animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Three-Dimensional Culture of Retinal Explants

All experiments (three sets of the experiment per group) were performed 6 hours after the onset of the light-exposure period. Mice were killed by ether anesthesia, and retinas were dissected under sterile conditions. With sharp blades, 500-μm square retinal explants were dissected 1.5 mm from the optic nerve head and were put into liquid collagen solution with the nerve fiber layer downward, as described previously.[23,24] The retinal explants were maintained at 37°C with 5% CO_2. The serum-free medium contained 2.7 mg/mL glucose, 5 μg/mL insulin, 16.1 μg/mL putrescine, 10% bovine serum albumin, 3.7 mg/mL $NaHCO_3$, 5.2 mg/mL Na_2SeO_3, and 3.6 mg/mL HEPES in minimum essential medium.

Immunohistochemistry

After fixation of retinal explants with 4% paraformaldehyde in 0.1 M PBS, cryostat sectioning was performed. After blocking with 5% goat serum and 3% bovine serum albumin in 0.1 M PBS, specimens were incubated at 4°C overnight, with rabbit anti-c-Fos (1:200; Calbiochem, Cambridge, MA), rabbit anti-Bax (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Bcl-2 (1:50; Calbiochem), rabbit anti-Bcl-XL (1:50; Calbiochem), or goat anti-p53 (1:200; Santa Cruz Biotechnology) antibodies. After washing, they were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, or anti-goat IgG for 1 hour at room temperature, followed by counterstaining using 20 μg/mL propidium iodide (PI; Molecular Probes, Eugene, OR) in aqueous solution. The specificity of antibodies was confirmed by omitting primary antibodies. Sections then underwent confocal microscopic observation (Radians 2000; Bio-Rad, Hertfordshire, UK). The degree of c-Fos, p53, Bax, and Bcl-XL immunopositivity in the ganglion cell layer

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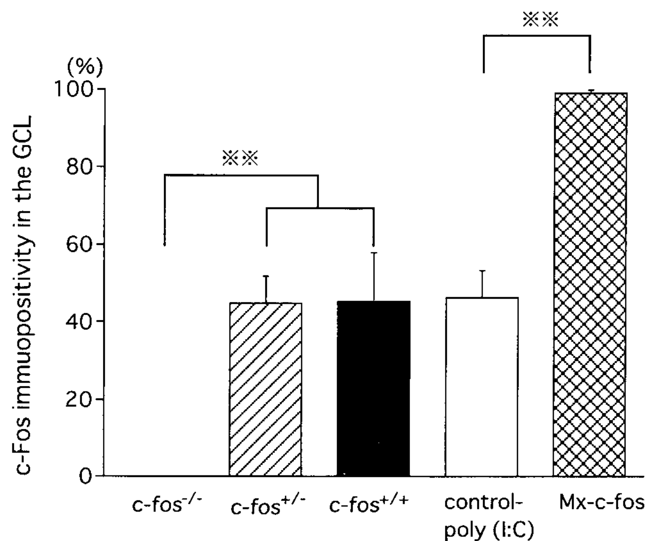

FIGURE 2. c-Fos immunopositivity in the GCL. The quantitative analysis of c-Fos immunopositivity in retinas from c-*fos*$^{+/+}$, c-*fos*$^{+/-}$, c-*fos*$^{-/-}$, control-poly (I:C), and Mx-c-*fos* were examined 4 hours after the culture. Data are expressed as the mean ± SD. $^{**}P < 0.01$, compared with c-*fos*$^{+/+}$/c-*fos*$^{+/-}$ or control-poly (I:C).

(GCL) was expressed through the ratios of immunopositive cells in relation to the total number of nuclei stained with PI. Statistical analysis was performed by the Mann-Whitney test (total sections examined were 18 from 6 explants per group). Total cells examined were 4904 in c-Fos, 3385 in p53 (not including c-*fos*$^{+/-}$), 3511 in Bax (not including c-*fos*$^{+/-}$), and 3594 in Bcl-XL (not including c-*fos*$^{+/-}$). For the immunostaining of Thy1.2 on regenerating neurites, retinal explants (day 14) embedded in the collagen gel were fixed with 4% paraformaldehyde in 0.1 M PBS and incubated with rat anti-Thy1.2 (1:100; Boehringer Mannheim Biochemica, Mannheim, Germany) for 4 days. After washing with PBS, they were incubated with FITC-conjugated anti-rat IgG for 1 day and observed under a fluorescence microscope.

In Situ Detection of Apoptosis by TUNEL Staining

Retinal explants were fixed as described, and 10-μm thick cryostat sections underwent TUNEL staining by an in situ apoptosis detection kit (Trevigen, Gaithersburg, MD), according to the instructions, and labeled nuclei were detected by streptavidin-FITC, as described before.[27] The detection of nonspecific positivity included omission of nick-end labeling reaction. Sections were counterstained with PI, as described here, and were inspected with use of the confocal microscope. The number of apoptotic cells in the GCL was expressed by the ratios of TUNEL-positive cells to the total number of nuclei stained with PI. Statistical analysis was performed by the Mann-Whitney test (total sections examined were 18 from 6 explants per group). Total cells examined were 4720 on day 0, 4480 on day 1, 4721 on day 2, and 4305 on day 14.

Measurement of Regenerating Neurites

Regenerating neurites were identified under a phase-contrast microscope, and the number of neurites per retinal explant was measured.[23,24] Branched neurites were counted as one. Statistical analysis was by the Mann-Whitney test. The number of explants examined in each group were 45 (c-*fos*$^{+/+}$), 28 (c-*fos*$^{+/-}$), 30 (c-*fos*$^{-/-}$), 27 (control-poly [I:C]), and 30 (Mx-c-*fos*).

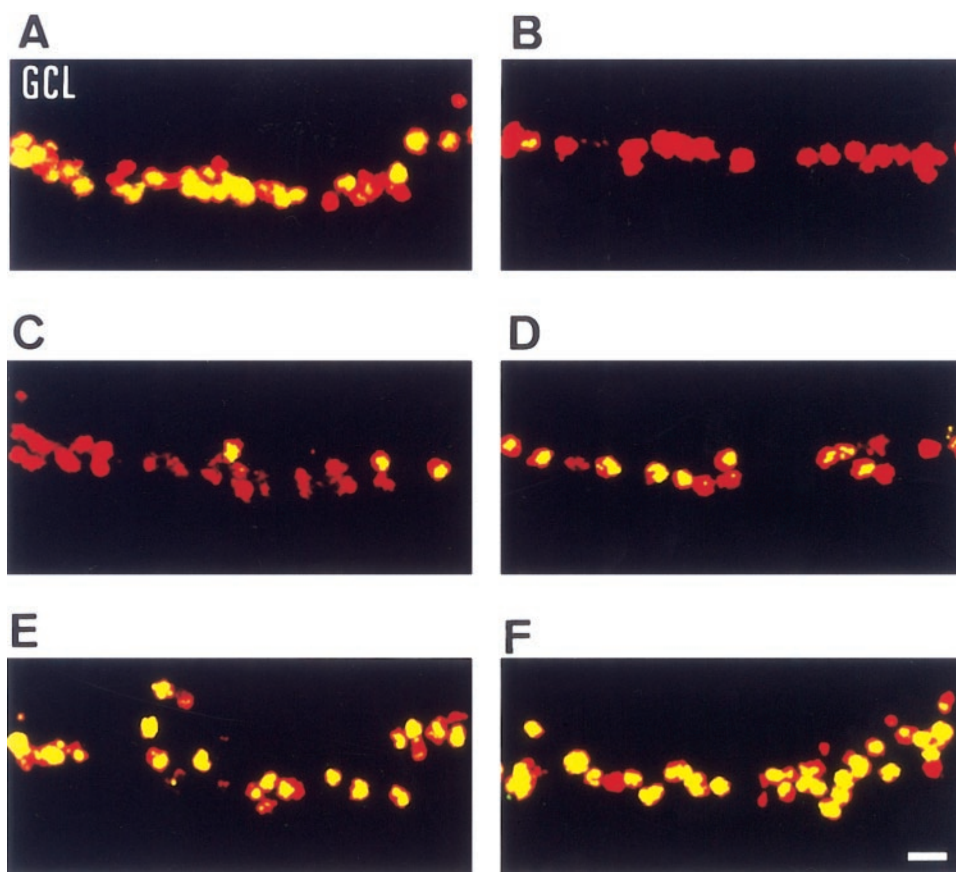

FIGURE 3. Apoptosis in the GCL of retinal explants. TUNEL staining was performed in the GCL from c-*fos*$^{+/+}$ (**A**), c-*fos*$^{-/-}$ (**B**), control-poly (I:C) (**C**, **E**), and Mx-c-*fos* (**D**, **F**). Positive cells were *yellow* because of superimposition of the *green* signal and *red* PI nuclear staining. Explants were from days 14 (**A**, **B**, **E**, **F**) and 2 (**C**, **D**). The ratio of TUNEL positivity in c-*fos*$^{-/-}$ retina (**B**) remained very low compared with that in c-*fos*$^{+/+}$ retina (**A**). In contrast, Mx-c-*fos* (**D**) showed a higher ratio of TUNEL positivity than did control-poly (I:C) (**C**) on day 2. Almost all the TUNEL-positive cells seemed to remain positive during the period of culture (**A**, **E**, **F**). GCL, ganglion cell layer. Scale bar, 10 μm.

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Results

Expression and Localization of c-Fos in RGCs

To observe the expression of c-Fos in RGCs, we performed c-Fos immunostaining and the quantitative analysis of c-Fos immunopositivity in the ganglion cell layer (GCL) 4 hours after the culture. For orientation of GCL, each retina was stained with PI (Figs. 1A–D). Mx-c-*fos*, control-poly (I:C), c-*fos*$^{+/+}$, c-*fos*$^{+/-}$, and c-*fos*$^{-/-}$ mice showed no basal expression of c-Fos protein in the GCL before the culture. In Mx-c-*fos* mice, however, intense c-Fos–positive RGCs were detected as early as 24 hours after the poly (I:C) injection in the GCL of intact retina—that is, just before beginning the culture (not shown). Strong c-Fos expression remained until 4 hours in culture (Figs. 1E, 2), whereas the immunoreactivity to c-Fos returned to the intact level by 24 hours in culture. In c-*fos*$^{+/+}$ (Fig. 1F) and control-poly (I:C) (not shown) retinas, c-Fos–immunopositive cells were detected in the GCL 4 hours after the culture (Fig. 2), and most of the reactivity disappeared by 24 hours in culture. c-*fos*$^{+/-}$ retina showed the same expression pattern described, with weaker intensity (Figs. 1G, 2). In c-*fos*$^{-/-}$ retina, no c-Fos expression was detected during the entire period (Figs. 1H, 2).

Detection and Quantitative Analysis of RGC Apoptosis

The TUNEL method was used to analyze RGC apoptosis in a serum-free environment. TUNEL staining of c-*fos*$^{+/+}$ retina showed very few positive cells on day 0, The ratio was 3.5% ± 1.9% (mean ± SD) to the total cell number in the GCL. On day 2, the ratio gradually increased to 29.5% ± 6.5%, reaching 88.6% ± 3.9% on day 14 (Figs. 3A, 4A). In contrast, the ratio of TUNEL positivity in c-*fos*$^{-/-}$ retina remained very low throughout the period of the culture. No positive cells were detected in the GCL on day 0. The ratio of TUNEL positivity was only 2.3% ± 1.9% on day 2 and 6.1% ± 2.8% on day 14. Both measurements were significantly different from those of c-*fos*$^{+/+}$ on the corresponding days ($P < 0.01$; Figs. 3B, 4A).

In contrast, Mx-c-*fos* mice showed a higher ratio of TUNEL positivity from the early period in culture. On day 1, the ratio of positivity was 63.6% ± 5.9%, which was significantly different ($P < 0.05$) from that of control-poly (I:C) (25.5% ± 13.6%). The ratio on day 2 was 82.6% ± 3.0%, which was also significantly different from that of the control-poly (I:C) (40.4% ± 8.7%; Figs. 3C–F, 4B). From these data, it can be concluded that the ratio of TUNEL positivity is correlated with the amount of c-Fos, and that c-Fos may play a key role in the process of RGC apoptosis.

GCL comprises not only RGCs but other kinds of cells, such as displaced amacrine cells. For example, 67.5% of cells in the GCL are RGCs in mice.[28] However, most of the apoptotic cells observed in the GCL were determined to be RGCs, because they were directly injured by the axotomy.

Expression of Apoptosis-Related Genes in RGCs

We subjected c-*fos*$^{+/+}$, c-*fos*$^{-/-}$, Mx-c-*fos*, and control-poly (I:C) retinas to immunohistochemistry for Bcl-XL, Bcl-2, Bax, and p53 to determine whether expression of these apoptosis-modulating factors was influenced by c-*fos* and performed a quantitative analysis of p53, Bax, and Bcl-XL immunopositivity in the GCL. Retinas were examined on day 2, because it had been shown by TUNEL staining that the ratio of TUNEL positivity showed the most distinct differences among c-*fos*$^{+/+}$, c-*fos*$^{-/-}$, Mx-c-*fos*, and control-poly (I:C) at that time point. In the case of p53 and Bax, Mx-c-*fos* demonstrated stronger reactivity than control-poly (I:C) did, and c-*fos*$^{-/-}$ showed the weakest reactivity of all (Figs. 5A–H, 6A, 6B). On Bcl-XL staining, the GCL of Mx-c-*fos* showed weaker reactivity than control-poly (I:C) did. In contrast, c-*fos*$^{-/-}$ showed more intense immunoreactivity than the others (Figs. 5I–L, 6C). In Bcl-2 staining, no distinct difference was observed among all groups (data not shown). Accordingly, expression of these factors is probably influenced by the amount of c-*fos* mRNA, and the amount of factors may have been related to the ratio of TUNEL positivity.

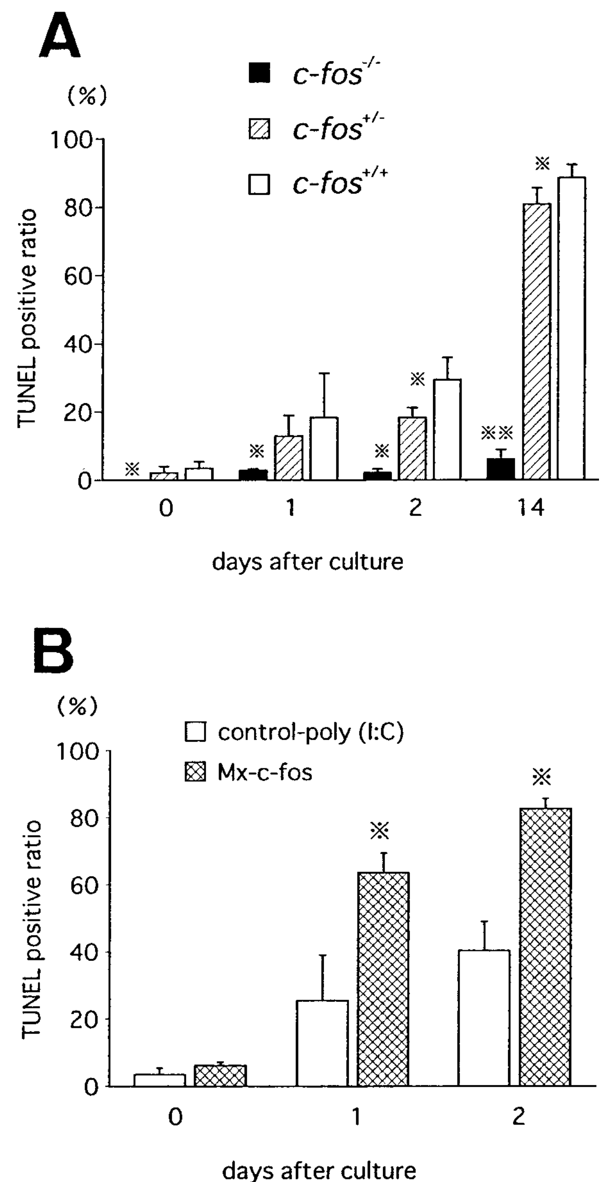

Figure 4. TUNEL positivity in the GCL of c-*fos*$^{+/+}$, c-*fos*$^{+/-}$, and c-*fos*$^{-/-}$ (**A**) and control-poly (I:C) and Mx-c-*fos* (**B**). Data are expressed as the mean ± SD. *$P < 0.05$; **$P < 0.01$, compared with c-*fos*$^{+/+}$ or control-poly (I:C).

Assessment of the Number of Regenerating Neurites

For the assessment of dependency of neurite extension on c-*fos*, the number of regenerating neurites in unit areas was counted and compared among c-*fos*$^{+/+}$, c-*fos*$^{+/-}$, and c-*fos*$^{-/-}$ and between Mx-c-*fos* and control-poly (I:C) on days 3, 6, 9, and 14. In c-*fos*$^{+/+}$, outgrowing neurites with growth cones were observed from retinal explants at 24 hours of culturing.

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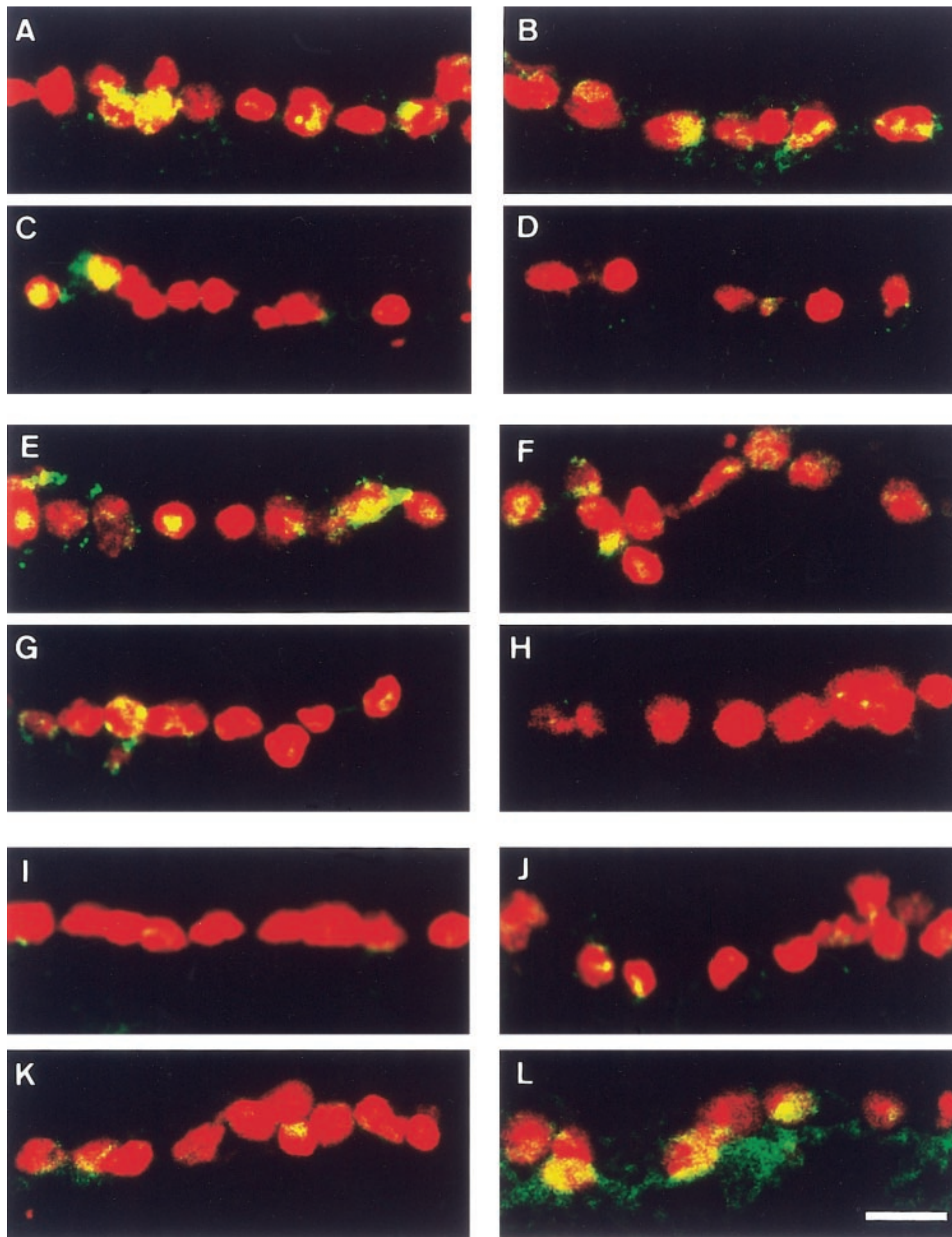

FIGURE 5. Expression of apoptosis-related gene products in the GCL. Immunohistochemistry of p53 (**A–D**), Bax (**E–H**), and Bcl-XL (**I–L**; *green*) in the GCL of Mx-c-*fos* (**A**, **E**, **I**), control-poly (I:C) (**B**, **F**, **J**), c-$fos^{+/+}$ (**C**, **G**, **K**), and c-$fos^{-/-}$ (**D**, **H**, **L**) on day 2. In p53 and Bax staining, Mx-c-*fos* showed stronger reactivity than did control-poly (I:C). In contrast, c-$fos^{-/-}$ showed the weakest reactivity of all. On Bcl-XL staining, Mx-c-*fos* demonstrated weaker reactivity than did control-poly (I:C). c-$Fos^{-/-}$ showed more intense immunoreactivity than the other groups. Nuclei of cells were stained with PI (*red*). Scale bar, 10 μm.

The neurites increased in number as time elapsed and elongated into collagen gel (Fig. 7A). By immunohistochemistry, almost all regenerating neurites were found to be Thy 1.2 positive, a specific marker for RGCs in the retina (data not shown).

In c-$fos^{+/+}$, neurites appeared by day 3 (13.9 ± 2.8 neurites/mm^2 explant), and the number of neurites increased as time elapsed (96.7 ± 17.2/mm^2 on day 6, 126.5 ± 22.3/mm^2 on day 9, and 136.0 ± 24.5/mm^2 on day 14; Figs. 7A, 8A). In c-$fos^{+/-}$, neurites were 13.2 ± 2.3/mm^2 on day 3, 101.8 ± 11.6/mm^2 on day 6, 141.9 ± 13.6/mm^2 on day 9, and 144.4 ± 37.5/mm^2 on day 14. No statistically significant difference was detected between c-$fos^{+/-}$ and c-$fos^{+/+}$ (Figs. 7A, 7B, 8A). In c-$fos^{-/-}$ retina, however, the number of neurites was significantly lower in the early period of culture than in c-$fos^{+/+}$ cultures. They were 0/mm^2 on day 3, 19.9 ± 8.9/mm^2 ($P <$ 0.01) on day 6, and 53.4 ± 19.0/mm^2 ($P < 0.05$) on day 9 (Fig. 8A). However, a substantial outgrowth of neurites was observed in the later period of culture. The density was 105.1 ± 33.4/mm^2 on day 14, which showed no significant difference from the c-$fos^{+/+}$ cultures (Figs. 7A, 7C, 8A).

In Mx-c-*fos*, the neurite number was markedly lower than during the early period and remained 17.7 ± 4.2/mm^2 on day 14—a highly significant difference when compared with that of control-poly (I:C) (115.6 ± 33.0/mm^2: $P < 0.01$; Figs. 7D, 7E, 8B).

DISCUSSION

To determine the functional importance of c-*fos* in damaged RGCs, we used tissue culture of retinal cells, which is a useful tool for the study of neuronal cell death and regeneration.[23,24] The stable condition of retinal tissue protected by collagen gel enabled us to observe neuronal cell death and regeneration through the long period of culture. Furthermore, the cell death cascade of damaged RGCs in retinal explants may share some mechanisms with RGCs in glaucoma, because RGC apoptosis of retinal explants may be related to the loss of trophic factors

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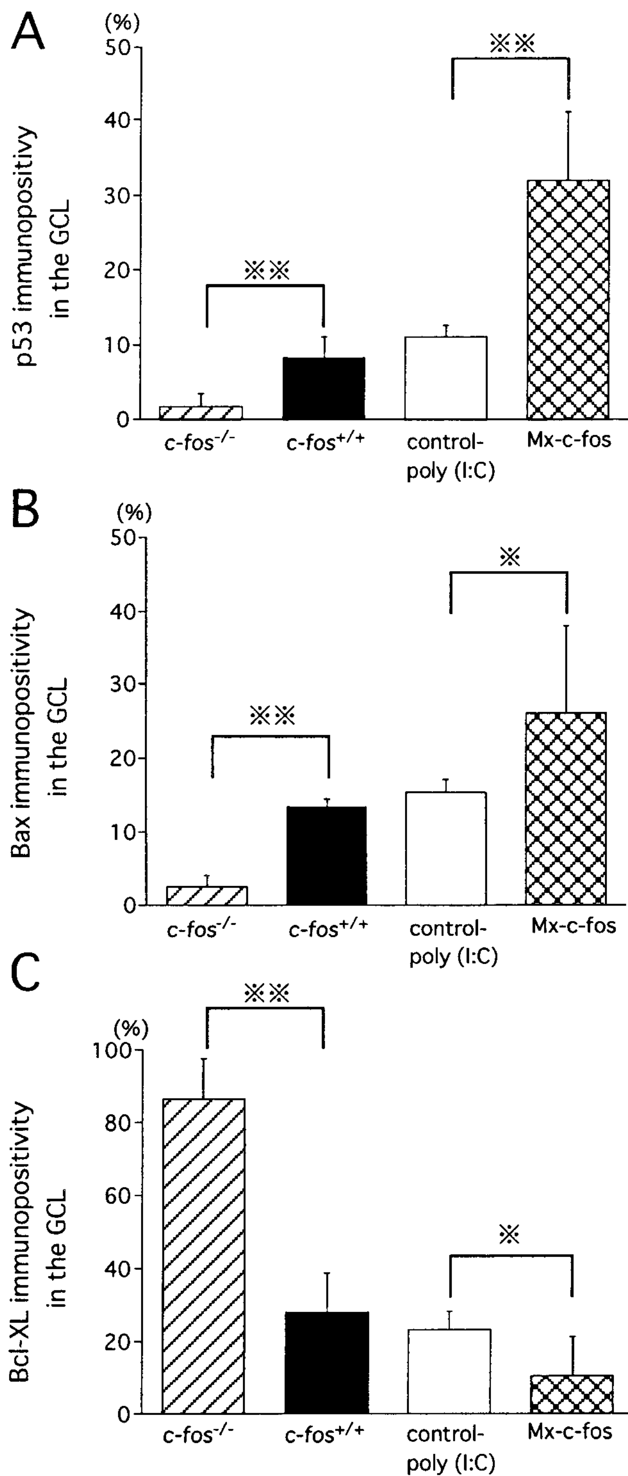

FIGURE 6. p53 (**A**), Bax (**B**), and Bcl-XL (**C**) immunopositivity in the GCL. The quantitative analysis of p53, Bax, and Bcl-XL expression in retinas from c-*fos*$^{+/+}$, c-*fos*$^{-/-}$, control-poly (I:C), and Mx-c-*fos* were examined on day 2. Data are expressed as the mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with c-*fos*$^{+/+}$ or control-poly (I:C).

caused by the disturbance of axonal transport from axon terminals to neuronal cell bodies.[14,29]

Although predominantly monocellular in nature, the GCL of the vertebrate eye consists not exclusively of RGCs, but of other kinds of cells as well, such as displaced amacrine cells. In mice retina, approximately 70% of the total number of cells was reported to be RGCs.[28] The exact number of the RGCs was not determined in our experiments, but considering that at least some cells other than RGCs were included in the count, the true ratios of apoptotic to total number of RGCs must have been slightly higher than those obtained. In contrast, most of the TUNEL-positive reactions in the GCL are likely to have come from RGCs, because the latter were directly injured by the axotomy.

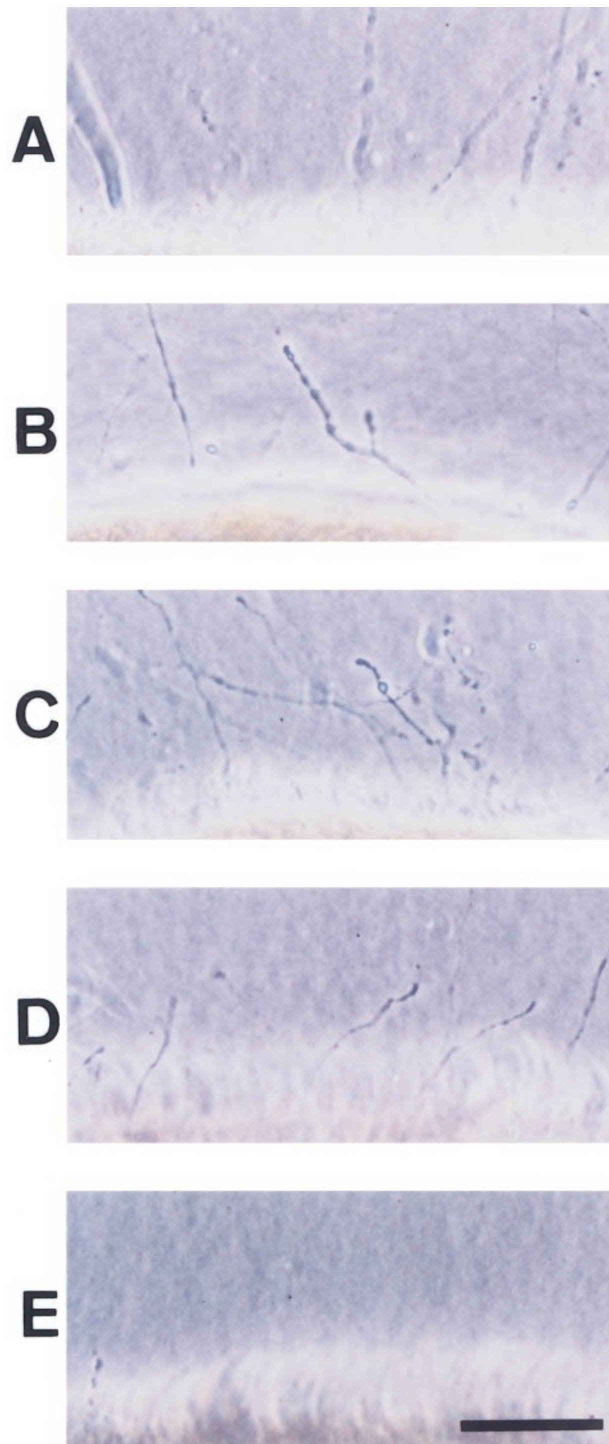

FIGURE 7. Neurite regeneration from retinal explants of c-*fos*$^{+/+}$ (**A**), c-*fos*$^{+/-}$ (**B**), c-*fos*$^{-/-}$ (**C**), control-poly (I:C) (**D**), and Mx-c-*fos* (**E**) after 14 days in culture. The number of regenerating neurites was counted under phase-contrast microscopy by moving focus point. Scale bar, 50 μm.

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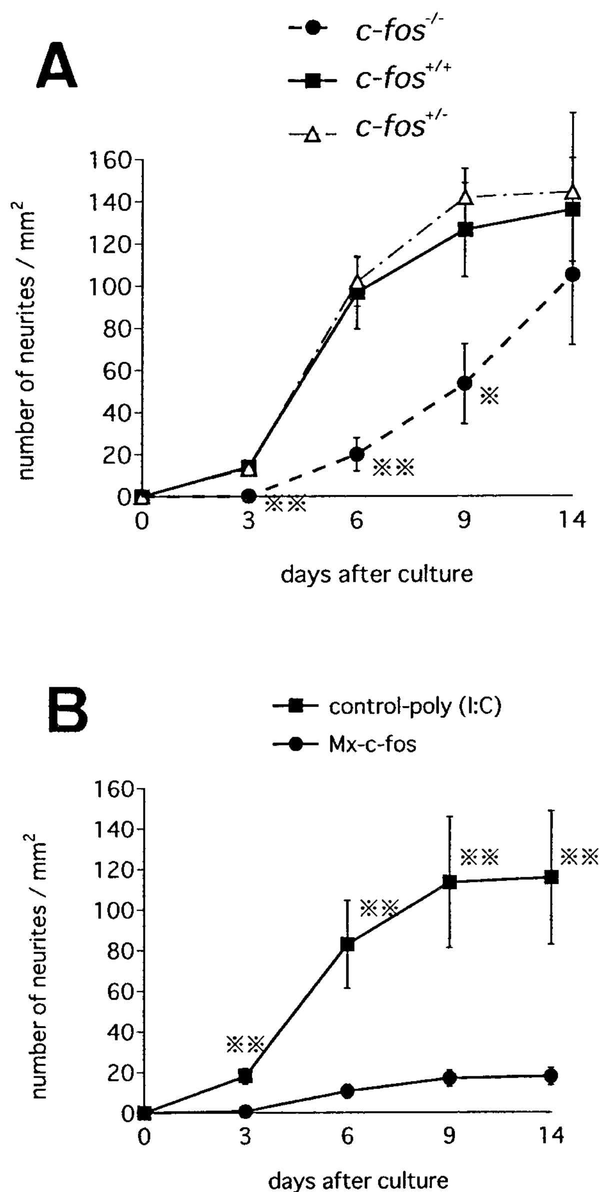

FIGURE 8. The number of regenerating neurites per retinal area of c-*fos*$^{+/+}$, c-*fos*$^{+/-}$, and c-*fos*$^{-/-}$ (**A**) and control-poly (I:C) and Mx-c-*fos* (**B**). There were significantly fewer neurites in c-*fos*$^{-/-}$ retina than in c-*fos*$^{+/+}$ retina, during the early period of culture. However, a substantial outgrowth of neurites was observed in the later period of culture. In Mx-c-*fos*, the neurite number was markedly lower compared with the control-poly (I:C). Data are expressed as the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with c-*fos*$^{+/+}$ or control-poly (I:C).

Unlike in vivo injury models, most dying cells and neurites seemed to remain until the later period of the culture. The reasons may be the collagen gel culture system that makes it difficult for the cells to be phagocytosed in the absence of blood flow and a series of immunologic reactions occurring in this in vitro environment.[30,31] Furthermore, we could compare control littermates with Mx-c-*fos* and c-*fos*–deficient mice, because these mice showed grossly normal retinal development.[3,32] We also examined intact retinas from Mx-c-*fos* and c-*fos*–deficient mice, and no significant differences of cell density in the GCL were detected between Mx-c-*fos* or c-*fos*–deficient retina and control littermate retina (data not shown).

Yoshida et al.[33] and Otori et al.[34] indicate that focal retinal injury or ischemic injury induces transient c-*fos* expression in RGCs, although the precise role of c-*fos* is unclear. In this study, transient c-*fos* expression was also seen in the GCL of the control retina. From our findings in TUNEL experiments, the following results were obtained: In the retina of c-*fos*-null mutant mice, most of the axotomized RGCs escaped apoptosis; and, in contrast, in the retina of overexpressed c-*fos*, many RGCs were involved in apoptotic cell death from the early phase of culture. It is notable that in Mx-c-*fos* retina overexpression of c-*fos* was transiently induced for a short period before and after the manipulation, and it returned to the intact level by 24 hours of culture. This finding indicates that the remarkable increase in the number of apoptotic RGCs caused by axotomy may occur in a short and restricted period immediately after the culture. In other words, whether the cell death cascade is activated could be determined by the amount of c-Fos in RGCs in a short period immediately after the axotomy.

In neurons of the CNS, one of the major cell-death–related gene families is *bcl-2*, which includes *bcl-2*, *bcl-x$_L$*, and *bax*. Bcl-2 and Bcl-XL, which have anti-apoptotic activity, are known to be functional antagonists of Bax. Bcl-2 and Bcl-XL can complex with Bax to form a heterodimer and neutralize its pro-apoptotic function.[17,35] In addition, these cell-death genes are thought to be mediated by p53, which is activated by cellular damage. Once activated after damage, p53 regulates a number of the target genes as a transcription factor. After the stimulation of apoptosis, p53 increases the expression of *bax*, or decreases the expression of *bcl-2*, leading to a change in the concentration of these gene products in the cell.[17,36] Many reports have suggested that transcriptional regulation of apoptosis-related genes, such as p53, *bcl-x$_L$*, and *bax*, is involved in the process of apoptotic cell death in the retinal neuron.[18,20–22,37] Our results show that *bcl-x$_L$* downregulation and p53 and *bax* upregulation may be related to RGC apoptosis of cultured retinas and that the expression of these factors may be involved in part of the c-*fos*–mediated pathway. It is not clear, however, whether c-*fos* directly regulates the expression of these genes in the apoptotic processes of damaged RGCs. Further work is needed to elucidate the precise mechanism of the c-*fos*–mediated cell death cascade in RGCs.

From the assessment of neurite regeneration in Mx-c-*fos* retina, it can be concluded that the significantly low level of neurite regeneration in Mx-c-*fos* is the cause of its higher ratio of TUNEL-positive RGCs, because cells in the process of apoptosis are unable to extend neurites. Furthermore, the mean number of neurites of c-*fos*$^{-/-}$ was significantly lower than that of c-*fos*$^{+/+}$ in the early period of culture, suggesting that c-*fos* plays an important role in neurite regeneration, particularly in the earlier period after RGC damage. However, because RGCs survived more in c-*fos*$^{-/-}$ retina, it was expected that more regenerating neurites arose from survived RGCs. In good accordance with this speculation, the data showing a substantial increase in density of neurite regeneration in the later period of culture.

The rate of neurite regeneration in c-*fos*–deficient retina shows that c-*fos* may play a role in accelerating neurite regeneration in the early period after RGC damage, because many surviving RGCs could not regenerate neurites promptly. In c-*fos*–deficient retina, however, the majority of axotomized RGCs successfully regenerated neurites in the late period of culture, perhaps because they escaped apoptosis. These results indicate that c-*fos* may be involved in two contrasting phenomena (i.e., cell death and regeneration) in damaged RGCs. However, we cannot exclude the possibility that RGCs in c-*fos*$^{-/-}$ mice are not apoptotic but are functionally abnormal.

From the findings mentioned, it may be possible to rescue the acutely damaged RGCs from cell death by the artificial control of c-*fos* expression in the retina in a short period just after the damage. To control expression of c-*fos*, several meth-

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ods may be used, such as introduction of antisense oligonucleotides[38] and administration of fentanyl.[39] If these methods are applied just after damage, they can be useful in saving RGCs from cell death and in inducing regeneration of RGC neurites. In addition, another approach to prevention of RGC apoptosis was demonstrated by findings in this study. c-Fos is a component of transcription factor AP-1. The expression of c-*fos* in damaged RGCs can be expected to upregulate or downregulate several apoptosis-related factors that are in the c-*fos*–mediated cascade. We found that p53, *bax*, and $bcl\text{-}x_L$ are candidates for such factors. Therefore, controlling expression such factors as apoptosis-related genes regulated in the c-*fos*–mediated cascade may be a suitable strategy to ensure neuronal survival and regeneration.

In conclusion, c-*fos* is essential for apoptosis in damaged RGCs, and the initial expression of c-*fos* induces apoptosis in damaged RGCs in a dose-dependent manner. c-*Fos* also may be involved in the mechanism of neurite regeneration in damaged RGCs. Understanding the effect of c-*fos* on damaged RGCs may be necessary in developing strategies to promote successful neuronal survival and regeneration.

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