Activation of caspase-3 in axotomized rat retinal ganglion cells in vivo

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Abstract Recently, we have shown that inhibition of caspase-3-like caspases is the most effective treatment strategy to protect adult rat retinal ganglion cells from secondary death following optic nerve transection. In the present study, we localized active caspase-3 in axotomized retinal ganglion cells in vivo and demonstrated a co-localization of the active p20 fragment and TUNEL-staining in some of these cells. In line with this, we detected an enhanced cleavage and activity of caspase-3 protein in retinal tissue after lesion, while caspase-3 mRNA expression remained unchanged. These data suggest caspase-3 as an important mediator of secondary retinal ganglion cell death following axotomy in vivo.

Key words: Retinal ganglion cell; Axotomy; Apoptosis; Caspase activation; Caspase-3

1. Introduction

Following optic nerve (ON) transection, the vast majority of retinal ganglion cells (RGCs) dies by apoptosis [1,2], with a maximum of cell death occurring between days 5 and 7 post-lesion [3]. Various potential neuroprotective agents such as brain-derived neurotrophic factor, NT-4 and glial cell line-derived neurotrophic factor have already been successfully tested in this experimental paradigm [4–7]. However, none of them was able to rescue more than 50–60% of RGCs at day 14 after ON transection. In recent years, a family of cysteine proteases, named caspases [8], has been characterized. It could be demonstrated in vitro and in vivo that these proteases are important mediators of apoptotic cell death during development and in disease states [9–12]. Recently, we have shown that inhibition of caspases rescues axotomized RGCs from secondary neuronal death in vivo, with specific inhibition of caspase-3-like caspases being the most effective [13]. We therefore hypothesized that caspase-3 is the major mediator of RGC death after ON transection. In the present study, we investigated retinal caspase-3 activation in more detail employing reverse transcription (RT-) PCR, Western blot analysis, caspase activity assays and immunohistochemistry.

2. Material and methods

2.1. Surgery and drug administration

Adult Sprague-Dawley rats (200–250 g, Charles River Wiga, Sulzfeld, Germany) were used in this study. Transection of the ON approximately 2 mm from the posterior eye pole without damaging the retinal blood supply was performed as described before [13]. In a subset of animals, the specific and irreversible inhibitor of caspase-3-like caspases benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethylketone (z-DEVD-cmk: Bachem, Germany) dissolved in 2% dimethylsulfoxide (DMSO, Sigma/Germany) was injected intravitreously at a concentration of 4000 ng and a volume of 2 μl on days 0 and 4 following ON transection by means of a small glass electrode according to a protocol described in more detail elsewhere [13].

2.2. Caspase-3 fluorogenic activity assay

Animals received an overdose of chloral hydrate and the eyes were removed either 6 h, 2 days, 3 days post-lesion or on day 4 post-lesion approximately 6 h following the second caspase inhibitor injection (see above). Untreated contralateral retinae served as controls. Retinae were homogenized, lysed (150 mM NaCl, 50 mM Tris pH 8.0, 2 mM EDTA, 1% Triton, containing 0.1 mM PMSF, and 2 μg/ml pepstatin, leupeptin and aprotinin) for 10 min at 37°C and cell debris pelleted at $14\,000 \times g$ for 15 min. The protein concentration of the supernatant was determined using the BCA reagent (Pierce, Rockford, IL, USA). The caspase-3 fluorogenic activity assay was performed with 30 μl of fresh protein lysates (control ($n = 9$), axotomy day 4 ($n = 6$), axotomy day 4 treated with z-DEVD-cmk on days 0 and 4 ($n = 4$)) which were incubated with 100 μM Ac-Asp-Glu-Val-Asp-AMC (DEVD-AMC), a fluorogenic substrate of caspase-3. Fluorescence was determined as optical density (OD) units, every 15 min for 2 h using 360 nm excitation and 460 nm emission wavelengths (Cyto-Flour 2350) and calculated as the increase in OD per μg protein over time. Statistics were performed applying one-way ANOVA followed by a Duncan test.

2.3. Western blot analysis for active caspase-3

Proteins of axotomized day 4 retinal tissue and contralateral controls processed as described above were separated on SDS-PAGE (50 μg 12% versus 20 μg 15%), transferred to a nitrocellulose or PVDF membrane and blocked with 5% skim milk in PBS-T (0.1% Tween 20). Proteins were detected by incubating with rabbit anti-caspase-3 (1:1500 CM-1 [14], 3 h at room temperature (RT)) followed by goat anti-rabbit HRP (1:2000, 1 h at RT), washing for three times in PBS-T and applying the ECL-Plus reagent (Amersham, Arlington Heights, IL, USA) following the supplier's instructions.

2.4. RT-PCR for caspase-3

For RT-PCR experiments, after dissection, retinae were immediately snap-frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (Gibco Life Technologies GmbH, Germany) following the manufacturer's protocol. RT-PCR was performed according to standard protocols. For cDNA synthesis, 2.5 μg total RNA was reverse-transcribed with Superscript II (Gibco BRL) in a volume of 50 μl, applying the manufacturer's instructions. The reaction was primed using random primers (300 ng). For a subsequent PCR reaction, 100 ng of ethanol-precipitated cDNA was used as template. The primer sequences and cycling conditions for semi-quantitative PCR were the following: 94°C for 3 min, annealing for 30 s and extension for 30 s at 72°C. Caspase-3 primer, forward: TACCCTGAAATGGGCTTGTGT, reverse: GTTAACACGAGTGAGGATGTG, annealing temperature 50°C, 28 cycles, and G3PDH primer, forward: GTGATGCTGGTGCTGA, reverse: GCTAAGCAGTTGGTGG, annealing temperature 50°C, 23 cycles.

2.5. Active caspase-3 immunohistochemistry and TUNEL co-localization

Immunolabelling was performed on 4% PFA-fixed, 16 μm cryostat

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sections of control retinae and retinae from day 6 post-lesion [7]. Sections were pre-incubated (10% NGS+0.3% Triton in PBS: 1 h RT), followed by CM-1 antibody [14,15] (1:250–1:1000, 4°C overnight) and anti-rabbit IgG-Cy3 (1:125: 1 h RT). Omission of the first antibody served as negative control in any immunohistochemical procedure. Double-labelling of TUNEL/caspase-3 was performed according to a protocol published elsewhere [15] with a modified TUNEL procedure [3]. Labelling was visualized by fluorescence and confocal microscopy.

All experiments were confirmed at least three times with tissue from different animals.

3. Results

To study axotomy-induced changes of caspase-3 mRNA expression, we used total retinal tissue for RT-PCR at 6, 12 and 24 h, as well as on days 3 and 6 following ON transection. As indicated in Fig. 1a, caspase-3 mRNA remained unchanged at all time-points investigated suggesting that caspase-3 might only be regulated on translational or post-translational levels in lesioned retinal tissue.

Measurement of the caspase-3 activity indeed revealed that ON transection results in a nearly 2-fold increase in cleavage of the fluorogenic caspase-3 substrate DEVD-AMC on day 4 post-lesion, which was statistically significant ($P < 0.04$) when compared to controls (Fig. 2). Compared to all other investigated time-points (data not shown), the differences were most pronounced on day 4 post-lesion indicating that caspase-3 gets activated until day 4 following ON transection. In addition, we were able to show that caspase-3 activity can be effectively reduced to 72% of controls by injecting the specific and irreversible inhibitor of caspase-3-like caspases into the vitreous body of the lesioned eye (Fig. 2, $P = 0.0525$).

To examine whether the increased caspase-3 activity is due to an increased cleavage of the 32 kDa proform of caspase-3 protein into the active fragments, we performed Western blot analysis applying the CM-1 antibody which has a preference

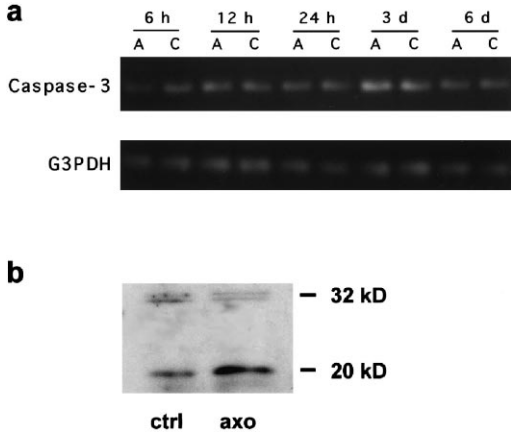

Fig. 1. Time-course of caspase-3 mRNA expression following ON transection and Western blot analysis for active caspase-3. (a) Retinal caspase-3 mRNA expression at 6, 12 and 24 h as well as 3 and 6 days after axotomy (A) compared to control retinae (C). At none of the investigated time-points, any changes in caspase-3 mRNA levels were detectable. Below, corresponding expression of G3PDH mRNA as housekeeping gene. (b) Western blot for caspase-3 applying the CM-1 antibody with a preference for the active p20 fragment. Whereas the amount of the 32 kDa zymogen proform slightly decreased after axotomy (axo), there is an upregulation of the active 20 kDa fragment of caspase-3. Note that there is a constitutional p20 expression in unlesioned control retinae (ctrl).

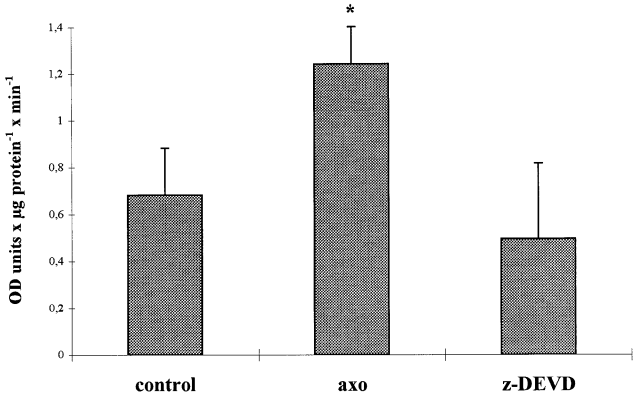

Fig. 2. Retinal activity of caspase-3 following ON transection and z-DEVD treatment. Data are given as mean OD units ± S.E.M. per min and µg protein as a measurement of DEVD-AMC cleavage by active caspase-3. Compared to control retinae, the activity of caspase-3 on day 4 following ON transection is significantly increased. Intraocular injection of 4000 ng z-DEVD-cmk on days 0 and 4 post-lesion results in a massive reduction of caspase-3 activity supporting the neuroprotective effect of z-DEVD as a specific inhibition of caspase-3 in axotomized RGCs. *, statistically significant when compared to control ($P < 0.04$).

for the active p20 fragment of caspase-3. In good agreement with our results from the activity assay, we detected increased levels of active caspase-3 in retinal tissue on day 4 following ON transection when compared to unlesioned contralateral controls (Fig. 1b). Interestingly, Western blot analysis confirmed a basal caspase-3 activity in control retinae.

Employing the CM-1 antibody for immunohistochemistry, we could localize the increased caspase-3 activity in axotomized RGCs on day 4 (data not shown) and day 6 post-lesion (Fig. 3). RGCs displaying strong fluorescence after axotomy as presented in Fig. 3b were never detected in control tissue (Fig. 3a). In contrast, we observed active caspase-3 in the photoreceptor cell layer of both control and axotomized retinae (Fig. 3). Double-labelling with TUNEL-staining revealed that on post-lesional day 6, some RGCs were positive for active caspase-3 and TUNEL simultaneously whereas others were only reactive for either one (Fig. 3c and d). By using confocal microscopy (Fig. 4), we observed an exclusively cytosolic localization of active caspase-3 whereas TUNEL-staining was most pronounced in the nucleus.

4. Discussion

In the present study, we provide evidence that caspase-3 activation following ON transection in vivo is located in axotomized RGCs which are supposed to die shortly thereafter. First, we examined whether ON lesion regulates retinal caspase-3 mRNA expression. Since we failed to detect such a regulation, we hypothesize that ON transection results in a translational or post-translational regulation of caspase-3 activity in axotomized RGCs. This is in contrast to results obtained after traumatic brain injury in vivo, where marked changes in caspase-3 mRNA expression occur in the entire affected region resulting in a rapid and simultaneous activation of caspases and subsequent neuronal death within hours [16]. The lack of mRNA regulation in our lesion paradigm may be explained by the fact that RGC death following axotomy occurs over an extended period of 3–4 days [3] and is

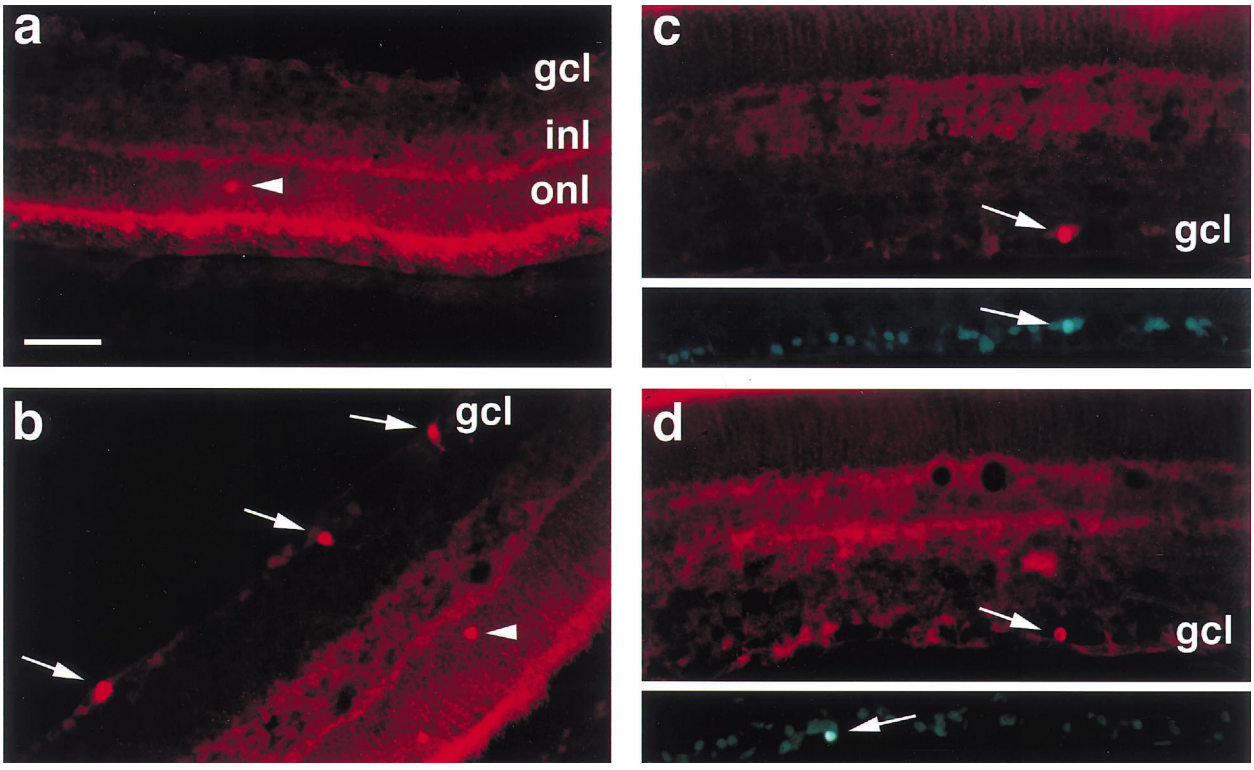

Fig. 3. Immunohistochemistry for active caspase-3 and double-labelling for TUNEL. Immunolabelling was performed applying the CM-1 antibody which recognizes processed caspase-3 (p20 fragment) preferentially to unprocessed zymogen. For double-labelling experiments, immunohistochemistry for active caspase-3 was followed by TUNEL-staining. (a) Micrograph of a control section. Note the lack of immuno-positive cells in the ganglion cell layer (gcl). An arrowhead points to a cell in the outer nuclear layer (onl) positive for active caspase-3. inl: inner nuclear layer. Scale bar: 50 μm. (b) Micrograph of a section 6 days following ON transection stained with the CM-1 antibody. Note the strong fluorescence in single RGCs (arrows) which could never be observed in controls indicating an activation of caspase-3 in axotomized RGCs. Immuno-positive cells in the onl as observed in controls are marked with arrowheads. (c) Micrograph of a section 6 days following ON transection double-labelled for active caspase-3 and TUNEL. Arrows point to a cell in the gcl positive for active caspase-3. Below the identical gcl is shown stained for TUNEL at the same magnification. Note that the cell stained for active caspase-3 is TUNEL-positive as well (arrow). (d) Representative micrograph 6 days following ON transection double-labelled for active caspase-3 and TUNEL (below). Arrows point to RGCs positive for either active caspase-3 or TUNEL without double-staining.

executed only in a limited subset of axotomized RGCs at the same time. Another explanation might be that the RGC population represents only about 0.57% of the entire retinal tissue [17]. Thus, modest changes in caspase-3 mRNA expression in single cells might escape detection by RT-PCR of total retinal tissue.

Secondly, we investigated the activity of caspase-3 protein by specific substrate cleavage in retinal tissue from controls and after axotomy. In line with our neuroprotection study which suggested that caspase activation after axotomy is a rather early event occurring between days 0 and 4 post-lesion [13], we detected a significantly increased activity of caspase-3

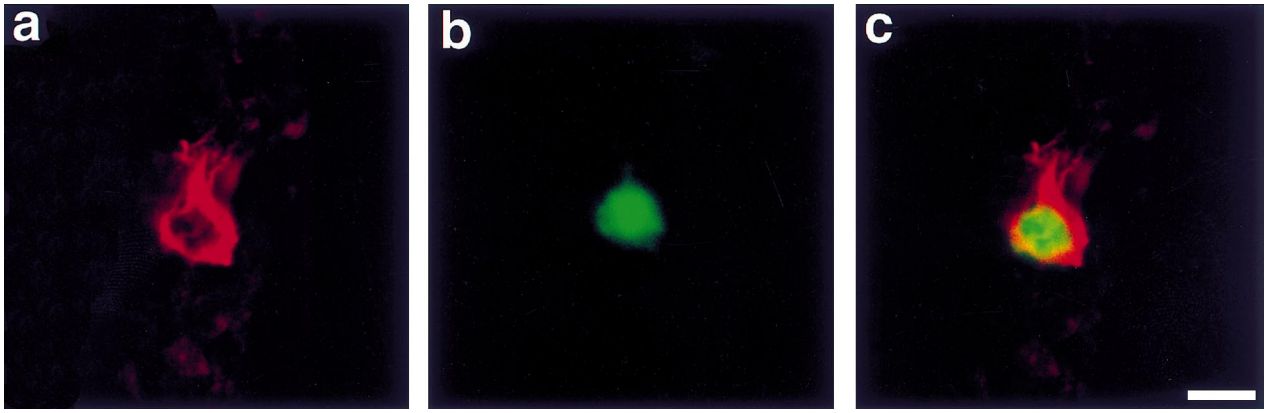

Fig. 4. Confocal microscopy of an axotomized RGC, double-stained for active caspase-3 and TUNEL. (a) Immunolabelling for active caspase-3. Note that the staining is located in the cytoplasm, completely sparing the nucleus. (b) TUNEL-staining of the cell shown in a. Note that the staining is mainly but not exclusively present in the nucleus, indicating that the cell represents an advanced stage of cell death where the nuclear membrane is already damaged. (c) Superimposition of b and c. Scale bar: 5 μm.

on day 4 post-lesion. Treatment with an intraocularly injected inhibitor resulted in a marked decrease in caspase-3 activity suggesting that the neuroprotective action of z-DEVD-cmk [13] is based on specific caspase inhibition.

Next, we performed Western blot analysis employing the CM-1 antibody [14,15] that preferentially detects the active p20 fragment of caspase-3. In line with observations from ischemia studies in vivo [15] and confirming our results of the activity assay, we observed an increased level of the active p20 fragment of caspase-3 on day 4 after axotomy. This supports our hypothesis that caspase-3 is regulated on the post-translational level following ON transection. The baseline levels of active caspase-3 in control retinae are in good agreement with our observations from affinity labelling experiments [13]. Moreover, by immunohistochemistry, we found few photoreceptor cells reactive for active caspase-3 in the outer nuclear layer of control and axotomized retinae as well as labelled photoreceptor inner segments appearing to be above background (Fig. 3), which could account for the basal caspase activity in controls. Recent studies support this observation showing that light exposure especially in albino rats results in the damage of photoreceptor cells [18,19] involving apoptotic processes [20]. Since the complete number of caspase-3 substrates has not yet been identified, caspase-3 could alternatively play a physiological role in cell metabolism.

Finally, we studied the cellular localization of caspase-3 activation employing the CM-1 antibody for immunohistochemistry. As expected, caspase-3 activation following ON transection occurred selectively in RGCs which are the only cell type directly affected by the lesion. As indicated by an increased caspase-3 activity on day 4 post-lesion, we detected immuno-positive cells at this time-point as well as on day 6 following axotomy. Since it is known that the number of TUNEL-positive RGCs reaches its maximum between days 5 and 7 following axotomy [3], we hypothesized that caspase-3 activation precedes TUNEL-staining in the same cell. To test this, we performed double-labelling experiments on post-lesional day 6 in which we found a co-localization of TUNEL-staining/caspase-3 reactivity in a few cells whereas others were only stained for one or the other. Confocal microscopy revealed the expected cytosolic localization of active caspase-3 that has been described after ischemia as well [15]. In contrast, TUNEL-staining displayed a typical nuclear distribution [3] with a weak fluorescent signal in the cytosol suggesting a damaged nuclear membrane which one would expect in advanced stages of apoptosis.

Taken together, these data indicate that secondary death of the RGC population following ON transection is not a synchronized event that occurs simultaneously in all cells, but rather a process lasting several days. Thus, at an advanced stage of cell death, single cells shortly show activation of caspase-3 followed by a time-window in which caspase-3 and TUNEL-staining overlap until caspase-3 labelling diminishes and the cell dies apoptotically. Thus, caspase-3 is suggested as one of the final and major executioners of RGC death following axotomy in vivo.

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