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Axonal degeneration, regeneration and ganglion cell death in a rodent model of anterior ischemic optic neuropathy (rAION)

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Abstract

Using laser-induced photoactivation of intravenously administered rose Bengal in rats, we generated an ischemic infarction of the intrascleral portion of the optic nerve (ON) comparable to that which occurs in humans to investigate optic nerve axon degenerative events following optic nerve infarct and the potential for axon re-growth. Animals were euthanized at different times post infarct. Axon degeneration was evaluated with SMI312 immunolabeling, and GAP-43 immunostaining was used to identify axon regeneration. Terminal dUTP nick end labeling (TUNEL) was used to evaluate retinal ganglion cell (RGC) death. There was significant axon structural disruption at the anterior intrascleral portion of the ON by 3d post-infarct, extending to the posterior ON by 7d post-stroke. Destruction of normal axon structure and massive loss of axon fibers occurred by 2 weeks. GAP-43 immunoreactivity occurred in the anterior ON by 7d post-infarct, lasting 3–4 weeks, without extension past the primary ischemic lesion. TUNEL-positive cells in the RGC layer appeared by 7d post-insult. These results indicate that following induction of ischemic optic neuropathy, significant axon damage occurs by 3d post-infarct, with later neuronal death. Post-stroke adult rat retinal ganglion cells attempt to regenerate their axons, but this effort is restricted to the unmyelinated region of the anterior ON. These responses are important in understanding pathologic process that underlies human non-arteritic anterior ischemic optic neuropathy (NAION) and may guide both the appropriate treatment of NAION and the window of opportunity for such treatment.

Keywords

optic nerve; stroke; axonal degeneration; GAP-43 immunostaining; retinal ganglion cell; TUNEL; microglia

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1. Introduction

Non-arteritic anterior ischemic optic neuropathy (NAION) is the most common cause of sudden optic nerve (ON)-related visual loss in persons over the age of 55. NAION is a pure central nervous system (CNS) white-matter infarct of the retinal ganglion cell (RGC) axons connecting the retina and higher CNS structures. The pathogenesis of this disease is unclear. It is hypothesized to be due in some cases to nocturnal hypoperfusion of the ON head (Hayreh et al., 1999) and in others to autoregulatory disruption of the blood supply to the ON head (Arnold, 2003). Because NAION represents a pure CNS axonal infarct, NAION-associated histological changes are believed to be representative of other CNS axonal infarcts. Documented CNS white-matter infarct changes include Wallerian degeneration (Thomalla et al., 2005), changes in axon structure (Wakita et al., 2002), and oligodendrocyte and RGC dysfunction and death (Goldenberg-Cohen et al., 2005). Post-stroke inflammatory changes also have been recognized as an important component of long-term dysfunction after infarction (Castellanos et al., 2002; Gee et al., 2007).

Because NAION is a non-fatal condition, few clinical specimens are available (Knox et al., 2000). In particular, there is a dearth of early clinical material (Tesser et al., 2003). Until recently, the lack of an easily available, reproducible animal model of NAION made it difficult to study the in vivo pathological processes resulting from this disease and from other isolated CNS axonal infarcts. We recently developed rodent models of AION (rAION) (Bernstein et al., 2003; Goldenberg-Cohen et al., 2005). These models are generated using laser-induced photoactivation of intravenously administered rose Bengal within the optic nerve capillaries, resulting in ON infarction within the intrascleral and immediately retroscleral portions of the ON, followed by RGC death (Bernstein et al., 2003; Goldenberg-Cohen et al., 2005; Zhang et al., 2009). The histopathologic changes in the ON and retina in these models are similar to those in the few reported human NAION specimens (Knox et al., 2000; Tesser et al., 2003).

Despite the axon loss known to be associated with NAION and other white-matter infarcts, the in vivo axon structural alterations occurring after NAION are less well understood. We therefore evaluated directly the structural axonal changes associated with rAION, as well as the early retinal cellular responses associated with ischemic axonal degeneration, in the hope that the findings would improve our development of clinically effective treatments of white-matter infarcts in general and of NAION in particular.

2. Materials and Methods

2.1. Animal model

All animal experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four adult Sprague-Dawley outbred rats (weight 120-150g; Harlan Corp; Indianapolis, IN) were kept under a 12-hr light-dark cycle and allowed free access to food and water. Optic nerve ischemia was produced in deeply anesthetized animals (ketamine/xylazine, 80mg/5mg/kg) as described previously (Bernstein et al., 2003). Briefly, 1 ml/kg intravenous rose Bengal (RB) (2.5 mM) was injected via tail vein and photoactivated in the vessels of the nerve using a 532 nm wavelength laser light with a 500 μ m spot size for 12 seconds. This induction results in >50% RGC loss by 21 days post-induction (Bernstein et al., 2007b). The right eye of each animal received laser treatment; the left eye was used for a normal control. At 1 and 3 days post-induction, the retinas from both the treated and untreated eyes were evaluated clinically through pupils dilated with 1% tropicamide. Animals were euthanized 1, 3, 5, 7, 14 days and 1 month post-induction using CO₂ inhalation. Four rats were used in each time point.

2.2. Specimen preparation

Eyes were removed immediately after the animals were euthanized and then fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PF-PBS) for 1 hr. The anterior segments were then removed. The remaining posterior segments were post-fixed in the same fixative for an additional 12 hr, transferred to 20% sucrose for cryoprotection, and then embedded in Tissue-Tek O.C.T. (Sakura, Tokyo) within chilled methyl-butane in dry ice. The blocks were kept in -80°C until sectioning. The posterior segments, including about 3 mm of the ON stump, were serially sectioned longitudinally at $10\mu\text{m}$ thickness.

2.3. Immunohistochemistry

Sections were air-dried at room temperature for 10 min and successively incubated with 5% blocking serum in PBS for 30 min, followed by a primary antibody overnight at 4°C , and then a secondary fluorescent antibody conjugated with either Cy-2 (green, donkey anti-rabbit; 1:300) or Cy-3 (red, donkey anti-mouse; 1:100) (Jackson ImmunoRes, West Grove, PA; 1:100) for 1 hr at room temperature. The primary antibodies used were mouse monoclonal SMI312 (Sternberg Monoclonals, Baltimore, MD; 1:500), ED1 (Serotec, Raleigh, NC; 1:400), rabbit polyclonal Iba1 (Wako Chemicals USA, Inc., Richmond, VA; 1:800), and GAP (growth-associated protein)-43 (Abcam Inc., Cambridge, MA). SMI312 antibody labels neurofilaments of the ON axons. ED1 antibody reacts against recently blood-borne (hematogenous) or extrinsic macrophages (Milligan et al., 1991). Iba1 antibody recognizes an ionized calcium-binding receptor membrane protein specifically expressed in inflammatory cells, including macrophages and microglia (Ito et al., 2001). A rabbit polyclonal anti-laminin antibody (kind gift of Dr. M.A. Rodrigues) was used at a dilution of 1:1000. For double-labeling, sections were incubated with the two primary antibodies from different species overnight and then two secondary fluorescent antibodies conjugated with either Cy-2 or Cy-3. TUNEL labeling was performed with a kit from Roche Diagnostics (Indianapolis, IN) following the company's protocol. Finally, slides were incubated with Hoechst nuclear stain (Molecular Probe, Eugene, OR; 1:2000) for 30 sec and mounted with Gel Mount Aqueous Mounting Medium (Sigma, St Louis, MO). Sections were visualized with a fluorescent digital microscope (Zeiss Axioscop) as well as a laser confocal microscope (Zeiss LSM510).

3. Results

3.1. Post-ON infarct axon neurofilament changes

We have shown previously (Zhang et al, 2009) that 3d after ON stroke, there is a marked infiltration of ED1(+)/Iba1(+) cells associated with axon structural disruption in the region of the ischemic infarct of the intrascleral portion and adjacent pre- and post-scleral portion of the optic nerve. In the current study, we found that more pronounced axon damage is present by 7d (Fig.1A), with loss of SMI312 immunoreactivity extending posteriorly along the entire nerve, as well as intraocularly into the retinal nerve fiber layer (NFL). The loss of SMI312 immunoreactivity is more pronounced at 14d after the stroke (Fig. 1B), indicating the speed of Wallerian degeneration.

3.2. TUNEL labeling and microglial response in the retina

Few, if any, TUNEL-positive cells are detectable in the outer nuclear layer (ONL), inner nuclear layer (INL) or RGC layer in normal control retinæ of naïve adult rats (Fig. 2A). Immediately following rAION induction, almost no TUNEL positivity is seen in any of the retinal layers (1 day; Fig. 2B and 3 days; 2C); however, large numbers of TUNEL-positive cells are seen in the RGC layer alone at 7d post-ON stroke (Fig.2D).

TUNEL semi-quantification at 7d post-induction was performed, using counts of cross-sections from nine slides generated from eyes from three different animals (three slides per eye) with ON stroke. Three high-power fields were counted per slide. TUNEL-positive cells were divided by the total number of cells (nuclei) within the RGC layer, excluding spindle-shaped endothelial cells. In the three eyes with rNAION, there was an average of 42.5% TUNEL-positive cells. Interestingly, in all three eyes, we observed that TUNEL-positive cells were present on one side of the retina but not on the other side on the same section (not shown), indicating a regional distribution of RGC death, consistent with a previously published study (Bernstein et al., 2007a).

As noted above, ED1 antibody recognizes a protein expressed by newly generated monocytes/macrophages (Milligan et al., 1991), whereas Iba1 antibody recognizes the ionized calcium channel adapter protein-1 and thus can be used as a marker for identifying all immune cells, including extrinsic macrophages and microglia (Ito et al., 2001). In normal (control) retinas, ED1(+) cells are rarely seen (Fig. 3A), but scattered Iba1(+) cells are demonstrable in the RGC layer, inner plexiform layer (IPL) and INL (arrows, Fig. 4A). As late as 7d post-infarct, no significant changes occur in the number of retinal ED1(+) cells (Fig. 3C), but there is a significant increase of Iba1(+) cells demonstrable in all inner retinal layers by at 7d post-ischemia (Fig. 3B). These ED1(+)/Iba1(+) cells probably represent microglial cells. Thus, in the retina following ON infarct, rAION induces RGC-specific cell death by apoptosis, with retinal activation of intrinsic microglia, rather than by recruitment of systemic macrophages.

3.3. ON-GAP-43 immunolabeling post-infarct

In control rat ON and retina, GAP-43 immunoreactivity is not present (Fig. 4). Post-ON infarct, GAP-43 immunoreactivity remains minimal until 7d post-stroke, at which time a GAP-43 immuno-positive signal is demonstrable either on one side of the ON or across the entire nerve, depending on the severity of the infarct.

GAP-43 immunostaining of a section through the retina and ON (Fig. 4C), reveals that GAP-43 immunoreactivity localizes exclusively to the NFL and anterior ON region, up to the intrascleral portion of the ON, but is not present posterior to the intrascleral region (Fig. 4C, asterisk). This site likely represents the boundary of the primary ischemic lesion, as seen in previous studies (Zhang et al., 2009). In addition, there is significant axon loss after 7d post-insult as indicated by SMI312 axon staining, and GAP-43 immunoreactivity is also demonstrable within the retinal NFL. A GAP-43(+) signal is still present in the ON and retina at 14 days post-infarct (Fig. 4D, and inset-high magnification, arrows) but has largely disappeared by 1 month post-infarct (Fig. 4E).

Following rAION induction, there can be variability of infarct severity among animals, as well as potential differences in the GAP43 regenerative response. As immunohistochemical GAP43 quantification is difficult in a complex tissue such as the retina-optic nerve complex, we induced rAION to an estimated ultimate loss of 50% RGCs in four additional animals and immunostained for GAP43 and SMI 312 expression 14d post-induction (Fig. 5).

GAP43 expression is undetectable in control retina and optic nerve, whereas axon neurofilaments fill the optic nerve (Fig. 5 A-C). Fourteen days post-induction, there is strong regional GAP43 expression, with minimal GAP43 expression detectable beyond the intrascleral portion of the ON (Fig. 5D&5G). SMI 312 immunostaining also reveals regional neurofilament loss (Fig. 5E&5H), with areas of residual intact neurofilaments. There are areas of GAP43 expression in areas of SMI312 activity (Fig. 5D-5I; arrows), but in some focal areas, SMI312-positive neurofilaments are negative for GAP43 labeling (Fig. 5E&5H, arrowheads).

3.4. ON scarring post-infarct

Because GAP43-positive RGC axons are only seen at a limited distance beyond the intrascleral portion of the ON (see Fig. 4C&4D), we interpreted this as a failure of axon regeneration. As glial scarring can inhibit axon regrowth (Shen et al., 2009), we immunostained for the presence of the extracellular protein laminin within the glial scar (Grimpe and Silver, 2002). Strong laminin staining was detected at 14d post-infarct in the region of the original ischemic lesion (Fig. 6B, arrows), suggesting extensive vascularized glial scar formation, whereas there were only low levels of laminin expression in control ONs (Fig. 6A; control).

4. Discussion

The results of our study reveal that in 7d-14d post-infarct rat ONs, the affected nerves show progressive disruption of normal axon structure only in the region of acute ischemia. Although in the current study, RGCs begin to die around 7d post-stroke, the retinal regions containing RGCs associated with the infarct do not demonstrate extrinsic macrophage migration. Instead, there is activation and increase of microglia, as demonstrated by the ED1(-)/Iba1(+) cells seen within the retina. These microglial cells presumably are there to remove degenerating RGCs and associated membrane debris. This suggests that without additional manipulation, extrinsic macrophage invasion only occurs in the region of the primary infarct. In this setting, the role of extrinsic macrophages is complex and may be multifactorial, as invading macrophages have been shown to be neuroprotective (Yin et al., 2006), but these macrophages may also secrete factors that block axon regrowth (Shen et al., 2010).

The SMI312-neurofilament antibody marker shows that, starting from 3d post-stroke, there is significant disruption of axons in affected ON fiber bundles (Zhang et al., 2009). Interestingly, SMI312 immunostaining posterior to the ischemic infarct is relatively intact at this time, but progresses more rapidly along the nerves at 7d and 14d post-stroke. These results suggest that, similar to other models of Wallerian degeneration, axon degeneration following focal axonal stroke progresses distally along the nerve.

RGC-neuron death results from ON ischemic axon dysfunction and degeneration. In our current model, the TUNEL-positive RGC death is similar to that seen in ON transection models, in which RGCs begin dying via apoptosis 5-7 days after injury, followed by loss of approximately 90% of all RGCs by 2 weeks (Villegas-Perez et al., 1988, 1993). In our model of axon ischemia, however, death-associated RGC changes are demonstrable by 7d post-ischemia, but the time course of RGC death is more prolonged. Indeed, at 2-3 weeks post-infarct, about 50% ganglion cells are still present (Goldenberg-Cohen et al., 2005; Slater et al., 2007). In our study, 42.5% of cells were TUNEL-positive at 7d post-rAION induction, whereas Slater et al. (2008) suggested that the peak of rAION-induced early apoptosis as measured by Annexin V staining occurred several days later (10 days). This difference may represent individual variation or different methodology used. Nevertheless, both studies show delayed apoptosis following the ON infarct. This delay in the initiation of RGC death in rAION suggests a potentially prolonged "treatment window" in our model; i.e., initiation of treatment within 7-10d post-infarct may have the potential to rescue and preserve RGCs. If we assume that similar phenomena occur in human NAION, then such a treatment window may exist in that condition as well.

In the current study, there was little change in the number of ED1(+) cells in the retina of rAION-induced eyes at the times the eyes were examined; however, IBA1(+) microglia did indeed increase in activity as determined by changes in morphology within the inner retina at 7d post-ON infarct. This suggests that unlike the primary infarct region in the ON, the

reactive inflammatory retinal changes occur from cells intrinsic to the retina at the time of insult. This response is similar to that seen in retinas of eyes that undergo ON axotomy (Zhang et al., 2004) and indicates that following ON stroke, there is maintenance of an intact blood-retinal barrier.

GAP-43 protein is a major component of growth-cone membranes in developing rat brains (Skene et al., 1986). It helps regulate the growth state of axon terminals and is associated with regeneration of injured neurons (Skene, 1989; Benowitz and Routtenberg, 1997). In our model, GAP-43 immunoreactivity is present in the proximal anterior ON head axons 7d post-ischemia. Increased expression is observed at the proximal side of the infarct and extends through the retinal NFL. This may represent an attempt by injured RGCs to repair and/or regenerate their axons.

Following ON axotomy, GAP-43 immunoreactivity significantly increases from 6-20d post-insult (Doster et al., 1991). This likely represents a regenerative process similar to that in our rodent ON ischemia model. GAP-43 expression in retinal flat mount specimens post-total axotomy reveals that GAP-43 protein expression is demonstrable in axons extending from the ON to the periphery of the retina; a distance of about 3-4 mm from the optic disc (Doster et al., 1991). In these reports, GAP-43 immunostaining was demonstrable throughout the entire retina and in the ON proximal to the lesion. The observation that GAP-43 is asymmetrically expressed on one side of the ON in some of our specimens of rNAION suggests that the ischemic infarct lesion in the rat does not involve the whole ON but rather is segmental or regional in distribution. This is confirmed by the focal nature of ON damage as assessed in cross-sections and described in earlier reports using this model (Bernstein et al., 2003).

Similar to results described in studies using an ON crush model (Ohlsson et al., 2004), in rNAION, GAP-43 immuno-positive axons are demonstrable only proximal to the injury site from day 7 and are not present in the distal portion of the injured ON. This suggests that in both crush and ischemic models, axon regeneration does not extend past the site of injury. The presence of increased laminin expression in the region of the infarct suggests that this is due, at least in part, to a reaction to post-insult development of glial scar tissue. It is also possible, however, that following an isolated CNS ischemic axon insult in an immunologically intact animal, a host of mechanisms, such as release of degenerated myelin products and a high degree of selective regulation of the immunologic response in different CNS regions, may combine to limit axon regeneration and long-term repair. Manipulation of these responses within a defined time period after an ischemic insult to the optic nerve may therefore be a useful approach in reducing permanent nerve damage and improving nerve function in ischemic axonopathy.

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Fig. 1. SMI312 immunoreactivity 7 and 14d post-ON stroke

A. 7 days post-infarct. SMI immunostaining reveals distal axonal destruction extending into the posterior ON, with sparing of peripheral axonal fibers (arrows). There is also reduced intensity of SMI312 immunostaining in the RGC layer of the retina. **B. 14 days post-infarct.** Severe ON axon loss is seen both in the intrascleral portion and in the more posterior ON (arrows) compared with 7d (compare figures A and B) after ON ischemia. Significantly reduced SMI312 immunoreactivity is also seen in the peripapillary retinal nerve fiber layer. Scale bar=50 μ m

Fig. 2. TUNEL labeling in rat retina at different times post-ON infarct (RGCL: retinal ganglion cell layer) **A.** TUNEL stain of naive control retina, and at: **B.** 1 day; **C.** 3 days; **D.** 7 days post-ON stroke. No TUNEL(+) cells are observable in the retina prior to 1 week post-infarct. One week post-ON infarct, abundant numbers of TUNEL(+) cells are demonstrable in the RGCL (arrows). Scale bar=30 μ m

Fig. 3. Iba1 (green) and ED1 (red) double immunolabeling in the retina of control, 3 days, and 7 days post ON-infarct eyes

A. Control retina. Iba1(+) cells are demonstrable in the RGCL, IPL (inner plexiform layer) and INL (Fig. A). Rare ED1(+) cells are also occasionally detectable in the normal retina (not shown). **B.** Retina 7d post-ON infarct. Many more Iba1+ cells with intense staining and shorter cellular processes are demonstrable in the RGCL and IPL (arrows). **C.** Isolated ED1 immunolabeling at 7d post-infarct (Fig. 4C). ED1(+) cells (arrowhead, Fig. C) are also Iba1(+) (arrowhead, Fig. C). Scale bar=50 μ m

Fig. 4. GAP-43 immunolabeling in control ON and retina, and following ON ischemia

A. Control section. **B.** ON/Retina section 3d post ON ischemia. **C.** ON/Retina section 7d post ON ischemia. **D.** ON/Retina section 14d post ON ischemia. In control and 3 day sections (A and B), there is an absence of GAP-43 immunoreactivity in the ON and retina. At 7d (C) and 14d (D) post-stroke, intense GAP-43 immunolabeling is identifiable on ON head areas (arrows). This is present predominantly in the anterior portion of the ON, proximal to the region of the primary infarct, but not present posterior to the intrascleral region (C, asterisk). Scattered staining is seen in the intrascleral ON region (Fig.D, short arrows; Inset: high power view of Fig.D). No GAP-43 immunostaining is identifiable in the nerve distal to the primary ischemic insult, which might be due to the significant loss of axons in this region. **E.** 1 month post-ON infarct, minimal GAP-43 staining (arrows) is seen in the anterior and intrascleral ON regions. Ve: Blood vessel. Scale bar=50 μ m

Fig. 5. GAP43 (red) and neurofilament (green, detected by SMI 312 immunostaining) expression in control (A-C) and in 14 day post-raAION-induced retinæ and optic nerves in 4 animals (D-I; Two eyes in 2 animals with ON stroke are shown)

Nuclei stained with DAPI in merged photos (merged + DAPI; C,F,I). A-C: GAP43 expression is undetectable in control retina and optic nerve, while axonal neurofilaments fill the optic nerve. D&G: Strong regional GAP43 expression (arrows) is seen 14d post-raAION, but only minimal GAP43 expression is seen beyond the intrascleral ON portion. E&H: SMI 312 immunostaining also reveals residual intact neurofilament labeling (arrows and arrowheads), as well as focal area of axonal loss (6E&6H; asterisks). F&I: There are areas of GAP43 expression in areas of SMI312 activity (arrows, D-I). However, in some areas (arrowheads), SMI312(+) residual neurofilaments are negative for GAP43 labeling. Scale bar=50 μm

Fig. 6. Laminin immunostaining of the optic nerve 14d following infarct

A. Control (contralateral eye-unstroked) section. There is laminin staining surrounding the large intraretinal vessels, internal limiting membrane and choroidal vessels, but only slight reactivity in the small vessels inside the optic nerve (arrows). B. ON section 14d post-infarct. Laminin staining is prominent in the area of the primary infarct lesion (arrows), suggesting vascularized glial scar formation post-infarct. Scale bar=50 μ m