Increased intrinsic neuronal vulnerability and decreased beneficial reaction of macrophages on axonal regeneration in aged rats

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Abstract

Previously we showed that macrophage activation in the eye by intravitreal application of zymosan increased retinal ganglion cell (RGC) survival and axonal regeneration after optic nerve injury. It is known that the intrinsic ability of CNS neurons to survive and to regrow axons after optic nerve injury differs between developing and adult mammals. However, whether aged animals also differ in their ability to survive and regrow injured axons are not known. In this study we investigated whether the abilities of RGCs to survive and to regrow injured axons differed between rats aged 6–8, 60 and over 96 weeks, and whether macrophage responses in the eye were different at different ages. We found that the intrinsic viability of RGCs, as shown in vitro, was reduced in aged rats, but RGC viability after optic nerve injury in vivo was similar among rats of the different ages. The ability of RGCs to regrow injured axons into a peripheral nerve graft also remained similar between young and aged rats. Macrophage activation in the eye was confirmed to be beneficial and provided the basis for zymosan treatment-dependent RGC protection. However, reduced activation of macrophages in zymosan-treated eyes was seen in aged rats. Importantly, this reduced macrophage activation in aged rats led to a decreased level of RGC axonal regeneration when compared with that in young rats of the same treatment. Thus age influences the intrinsic viability of RGCs and the beneficial impact of macrophages on RGC axonal regeneration after optic nerve injury.

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1. Introduction

It is well known that neurons of the adult mammalian central nervous system (CNS) have limited ability to spontaneously regrow axons after injury, whereas those of developing animals do. These differences are due not only to the low intrinsic axon-regrowth ability of CNS neurons, but also to the hostile CNS environment to which adult neurons are exposed. Over the past two to three decades, numerous studies have shown that adult neurons have low axon-regrowth ability and the adult CNS environment possesses axon-growth inhibitory molecules such as Nogo, MAG and OMgp that account for the majority of the inhibitory activity in CNS myelin (Filbin, 2003; He and Koprivica, 2004). Apart from these two factors, scar formation and lack of appropriate trophic supply also hinder axon regeneration after injury.

Brain aging is associated with a number of progressive structural and functional changes. These include the changes in neuronal/white matter size, blood–brain barrier (BBB) permeability, and cognitive and memory functions (Mooradian, 1988; Sowell et al., 2004). Whereas cognitive and memory functions decline, inflammatory activity, closely associated with activation of microglial, dendritic and T cells and the expression of certain cytokines and their receptors, appears
to increase (Lu et al., 2004; Sloane et al., 1999; Stichel and Luebbert, 2007; Streit et al., 2004). Thus in aged animals, different reactions of certain cellular populations may influence the extent of neural recovery after injury.

Microglia/macrophages have been well documented to play a detrimental role in certain neurodegenerative diseases (Hendriks et al., 2005; Minghetti et al., 2005; Moore and Thanos, 1996). However, microglia/macrophages, perhaps depending on the levels of certain cytokine and trophic factor production, can also be neuro-protective (Hanisch, 2002; Streit, 2002). Earlier we showed that macrophage activation in the eye by intravitreal application of zymosan, a yeast wall extract, could significantly enhance retinal ganglion cell (RGC) survival and axonal regeneration (Yin et al., 2003). Macrophage activation also participated in ciliary neurotrophic factor (CNTF)-induced RGC protection (Cen et al., 2007). Recently, we characterised the interaction between macrophages and T cells in RGC survival and axonal regeneration after optic nerve (ON) injury (Luo et al., 2007b). In this study, we investigated whether aged Fischer 344 (F344) rats are more susceptible to traumatic ON injury and whether differences in macrophage activation/recruitment in the eye across the rat lifespan resulted in different levels of RGC survival and axonal regeneration after ON injury.

2. Materials and methods

F344 rats aged 6–8, 60 and over 96 weeks were obtained from Charles River Laboratory, USA. They were housed in controlled conditions of 22°C, 12 h light–dark cycle from 0600 to 1800 h and from 1800 to 0600 h at Shantou University Medical College (SUMC) with access to food and water ad libitum. All experiments performed were approved by the SUMC Animal Experimentation Ethics Committee. The range of ages up to 96-week old has often been used to study various aspects of aging across F344 rat lifespan, and age of 96-week old is regarded as aged rats in this strain (Bizon et al., 2008; Foster et al., 2008), thus age of 60-week old should be middle-aged, and 6–8-week old as young. All surgery was carried out under anaesthesia of 1:1 mixture (1.5 ml/kg) of ketamine (100 mg/ml) and xylazine (20 mg/ml). For retinal explant experiments, retinas were obtained from 8, 60 and over 96-week-old intact rats (n = 10, 4 and 4, respectively) or rats that underwent ON transection 1.5 mm behind the globe 3 days earlier (n = 5, 6 and 5, respectively). The retinas were dissected out in cold HBSS and mounted onto nitrocellulose filter paper with the RGC layer uppermost. Each retina was cut into halves, and each half was cultured in one well in neurobasal A + B27 (GIBCO) medium supplemented with glutamine and penicillin/streptomycin for 7 days. Cultured retinas were fixed with 4% paraformaldehyde for 1 h and immunoreacted with TU1 antibody (1:400; anti-βIII tubulin, BabCo, Richmond, CA, USA), followed by cy3 secondary antibody (1:400; Jackson ImmunoResearch, West Grove, PA, USA) to identify viable RGCs in a protocol as described previously (Cen et al., 2007; Cui et al., 2003; Luo et al., 2007b). βIII tubulin has been shown to be an RGC-specific marker in the retina. We (Cen et al., 2007; Luo et al., 2007a,b; Park et al., 2004; Yin et al., 2003) and others (Fischer et al., 2004a,b; Koprivica et al., 2005; Yin et al., 2006) have used this antibody to identify surviving RGCs in the retina. Under a fluorescence microscope, the number of βIII tubulin positive (+) RGCs in each field (0.25 mm × 0.25 mm) at a fixed distance from one another, in a pattern of grid intersections, were counted throughout the whole retina (Cui et al., 2003). A total of 70–80 fields, about 8–10% of the total retinal area, were sampled per retina. The average density of viable RGCs was calculated and the total number of RGCs estimated by multiplying the density by the size of the retina. This approach avoided problems associated with uneven distribution of RGCs in the retina. To avoid bias, the experimental conditions of the retinas were only revealed to the examiner after completion of the counting.

For in vivo experiments, normal intact rats aged 8 weeks (n = 9), 60 weeks (n = 4) and over 96 weeks (n = 5) were used as controls. RGCs underwent retrograde labelling by application of 4% FluoroGold (FG, Fluorochrome Inc., Denver, USA) in gelfoam at the ON stump 1.5 mm posterior to the optic disc 40 h prior to sacrifice. Note that RGCs only start to die 5 days after ON axotomy in adult rats (Berkelaar et al., 1994).

For the operated rats, the PN–ON surgical procedure has been described previously (Berkelaar et al., 1994; Cen et al., 2007; Yin et al., 2003). Briefly, under anaesthesia the left ON was transected about 1.5 mm behind the optic disc. A 1.5 cm piece of autologous peroneal nerve (PN) was dissected out and sutured onto the proximal ON stumps to provide a permissive environment for injured axons to regrow. The distal part of the PN was secured onto the skull. PN–ON grafted animals were divided into six different groups (n = 5 each group except zymosan group in young rats; see Fig. 4): intravitreal saline (3 μL) treatment groups in young (8-week-old), middle-aged (60-week-old) and aged (over 96-week-old) rats, and zymosan (3.75 mg in 3 μL) treatment groups in young and aged rats. To confirm macrophage activation in zymosan-induced RGC protection, we used clonodrate liposomes intravenously to deplete monocytes in the blood, thus preventing macrophages from entering the eye, and examined whether zymosan-induced RGC protection was macrophage-dependent. Liposome-encapsulated clonodrate liposomes were prepared as previously described (van Rooijen and Sanders, 1994). It is known that both clonodrate and liposomes (if prepared of phosphatidylcholine and cholesterol) are not toxic. Recently, we showed that repeated intravenous applications of clonodrate liposomes or control PBS liposomes themselves did not affect RGC survival and axonal regeneration (Cen et al., 2007; Luo et al., 2007a). Intravenous applications of clonodrate liposomes (0.5 ml per 100 g bodyweight) were administered via tail vein right before the PN–ON procedure, and repeated on days 5
and 10 whereas intravitreal injection of saline or zymosan was applied 3 days after the PN–ON procedure (Cen et al., 2007; Luo et al., 2007a).

To label axon-regenerating RGCs in retrograde fashion, 0.2 μL of 4% FG was slowly injected into the distal end of the PN graft. Animals remain alive for another 3 days in order to maximize the retrograde transport of the dye. The number of FG-labelled axon-regenerating RGCs was counted in the same fashion as above. The survival time was 3 weeks for both intact and PN–ON operated rats.

Afterwards, retinas were used for immunostaining of viable RGCs and macrophages. After blocking with 10% normal goat serum (NGS) and 0.2% Triton, halves of the retinas of similar regions were immunostained with TUJ1 antibody (1:400) and the other halves with ED1 to identify macrophages (1:200, Serotec, Oxford, UK; Luo et al., 2007a,b; Yin et al., 2003). Conjugated cy3 (1:400) and FITC (Sigma) were used as secondary antibodies. Counting of immunostained surviving RGCs and macrophages were done in the same way as above. Retrograde labelling rather than immunostaining of surviving RGCs in intact rats was used because the latter often yields poor immunostaining results of RGCs owing to the thick nerve fiber layer (NFL) in the normal retina.

Data from all groups in vivo and in vitro were pooled and analysed using the Bonferroni test (Edwards and Berry, 1987) following two-way analysis of variance (ANOVA). We and others routinely use the Bonferroni test to compare mean values among all intra-groups (Cen et al., 2007; Cui et al., 2003; Luo et al., 2007a,b; Pearse et al., 2004; Yin et al., 2003).

3. Results

The general appearance of photomicrographs of βIII tubulin+ surviving RGCs in retinal explants of different ages...
It appears that the size of the retina increases as the rat ages. The average size (±S.D.) of the normal retinas is 55.5 ± 4.5 mm<sup>2</sup> in young, 66.7 ± 6.2 mm<sup>2</sup> in middle-aged and 72.1 ± 4.8 mm<sup>2</sup> in aged rats (Fig. 3A). Compared with young rats, the increases in the size of the retina in the middle-aged (p < 0.01) and the aged (p < 0.001) rats are statistically significant (Fig. 3A).

There are no significant differences in the numbers of normal RGCs among rats of different ages (97078 ± 15074 per retina in young, 98286 ± 13717 per retina in middle-aged and 108964 ± 21444 per retina in aged rats; Fig. 3B). Three weeks after the PN–ON procedure, the average numbers (±S.D.) of βIII tubulin<sup>+</sup> surviving RGCs were 8927 ± 454 per retina in the young adult, 10871 ± 899 per retina in the middle-aged and 9127 ± 1281 per retina in the aged rats after intravitreal injection of saline (Fig. 4A). There are no significant differences among these three groups. These were thus in contrast to what were seen in vitro, suggesting a possible compensatory mechanism of the in vivo system for the reduced intrinsic viability of RGCs in the aged rats.

The average numbers (±S.D.) of axon-regenerating RGCs also remained at a similar level among the different age groups (1198 ± 248 per retina in the middle-aged and 1519 ± 266 per retina in the aged rats; Fig. 4A). There are slight decreases in the average number of ED1<sup>+</sup> macrophages in the middle-aged (1710 ± 533 per retina) and aged (1630 ± 281 per retina) retinas when compared with the young ones (2192 ± 249 per retina). However, the decreases are not statistically significant (Fig. 4A).

The general appearance of photomicrographs of βIII tubulin<sup>+</sup> surviving RGCs (left column) and FG-labelled axon-regenerating RGCs (middle column) and ED1<sup>+</sup> macrophages (right column) under different conditions in vivo are shown in Fig. 1D–O. The morphology of invading macrophages appears to be uniform, in a round activated shape without apparent processes. They are located on top of RGCs in the NFL. Note that blood-borne macrophages were shown only to invade NFL in the retina after ON axotomy (Cen et al., 2007; Garcia-Valenzuela and Sharma, 1999).
As expected, intravitreal injection of zymosan significantly ($p<0.001$) increased the number of macrophages in the eyes of both young adult (27453 ± 1218 per retina) and aged (19566 ± 2524 per retina) rats (Fig. 4B). However, compared with the young adult, the extent of zymosan-induced macrophage activation in the aged eyes was significantly lower ($p<0.001$; Fig. 4B). This reduction in macrophage activation in the aged eye was accompanied by a significant ($p<0.001$) decline in the number of axon-regenerating RGCs (4853 ± 621 per retina versus 7296 ± 1910 per retina in the young) although the number of surviving RGCs in the aged (13004 ± 1056 per retina) was slightly but not significantly lower than the one (14995 ± 2260 per retina) in the young rats (Fig. 4B). These data thus suggest that age affects the beneficial impact of macrophages on RGC axonal regeneration but not RGC survival.

The underlying beneficial role of macrophages in zymosan-induced RGC protection was confirmed by the experiment in which clodronate liposomes were applied to prevent macrophage recruitment into the eye. After removal of macrophages (2891 ± 249 per retina) in the young adult eye by clodronate liposomes, significant decreases in RGC survival (11728 ± 1250 per retina; $p<0.01$) and axon regeneration (1257 ± 177 per retina; $p<0.001$) were seen (Fig. 4B).

4. Discussion

In this study we observed an age-related decline in the intrinsic viability of RGCs and in the response of macrophages in the eye. The reduced macrophage response to zymosan treatment in aged eye resulted in decreased axonal regeneration, but did not affect RGC survival after ON injury. In addition, the beneficial activity of macrophages activated by zymosan in the eye under in vivo experimental conditions is confirmed in this study.

An important finding in this study is that the intrinsic viability of RGCs, as reflected in the in vitro data obtained from intact and ON-injured rats of different ages, deteriorates in aged rats. It is surprising to see the differences in RGC viability between in vitro (Fig. 2) and in vivo (Fig. 3B) results. These data may suggest a possible compensatory mechanism of the in vivo system to cover the reduced intrinsic viability of RGCs in the aged rats after ON injury.

The beneficial activity of macrophages activated by zymosan in the eye under in vivo experimental conditions has been documented earlier (Luo et al., 2007b; Yin et al., 2003). Recently, a macrophage-derived factor, oncomodulin, has been shown to be responsible for macrophage activation-induced enhancement in RGC axon regeneration (Yin et al., 2006). In this study, the data from zymosan-treated rats that were depleted of monocytes in the blood by repeated intravenous applications of clodronate liposomes, thus preventing macrophage invasion into the eye, confirmed that macrophage activation is responsible for zymosan-induced RGC protection (Fig. 4).

Though increased cell penetration into neuronal parenchyma in aged animals has been suggested to be a result of altered structure of the BBB (Mooradian, 1988; Sowell et al., 2004), the permeability of the BBB is unlikely to increase as a function of age alone (Bechmann et al., 2007). In addition, ON axotomy may result in a similar degree of BBB disruption, and macrophage recruitment in the ON-injured eye after zymosan stimulation may reflect the capability of immune response. The decreased beneficial activity of macrophages is likely to result only from reduced macrophage recruitment in the aged rats. Thus, the immune response appears also to be a victim of aging in neural protection.

Macrophage functions are closely related to cytokines and trophic factors (Hailer, 2008; Hanisch, 2002), and macrophage activation is influenced by cytokines (Gordon, 2003). Recently we showed that CNTF is a chemotactic factor for macrophages (Cen et al., 2007). The reduced beneficial recruitment of macrophages in the aged eye may result from differential production of certain molecules by glial cells and
recruited macrophages in the eye. Differential production of detrimental pro-inflammatory cytokines (IL-1α, IL-1β and IL-6) between young and aged astrocytes and microglia has been shown, and this differential production of cytokines has been postulated to be responsible for the reduced ability of aged astrocytes and microglia to attenuate toxin-induced neuronal injury (Yu et al., 2002). However, it is also possible that the macrophages of aged rats produce different amounts of other molecules that also contribute to reduced RGC survival and axonal regeneration. Invasion of blood-derived macrophages at the injury site also occurs after spinal cord injury; these cytokine-producing macrophages participate in neuroprotection, axonal regeneration and functional recovery following spinal cord injury (Donnelly and Popovich, 2008; Longbrake et al., 2007).

In conclusion, besides various lines of evidence pointing to an important role of immune system in the development of age-related neurodegeneration, the present study demonstrates that aging influences the intrinsic viability of RGCs and affects the beneficial activity of macrophages in neural repair.

Disclosure statement

The authors declare that they have no conflict of interest, financial or otherwise, related to the present work, and that all surgical procedures carried out have been approved by Shantou University Medical College Animal Experimentation Ethics Committee.

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