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An electron microscopic study of neuronal degeneration and glial cell reaction in the retina of glaucomatous rats

X. Wang, S.S.-W. Tay and Y.-K. Ng

Department of Anatomy, Faculty of Medicine, National University of Singapore, Medical Drive, Singapore

Summary. The present investigation was focused on the ultrastructural changes in the neurons and glial cells in the retina of rats with experimentally-induced glaucoma. An experimental glaucoma model was created by limbal-derived vein cauterization. Animals were sacrificed at 1, 3 weeks and 3 months post-operation. Retinae were dissected and processed for electron microscopy. Neuronal degeneration was observed in all the different layers of the retina at both 1 and 3 weeks post-operation. Some degenerating neurons were found in the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL). And the dying neurons presented apoptotic-like more than necrotic neurons. Many degenerating axons and axon terminals were observed between neurons in the GCL, inner plexiform layer (IPL), INL, and outer plexiform layer (OPL). Activated astrocytes and microglial cells were present in close association with degenerating neurons and axons. The Müller cells in the INL also presented longer and darker processes with more microfilaments than in normal cells. Degenerating neuronal debris, degenerating axonal profiles and electron-dense bodies were often found in the cytoplasm of macrophages. The results suggest that both microglial cells and astrocytes are activated in the process of neuronal degeneration in the retina of experimentally-induced glaucomatous rats. It is hypothesized that they may play a protective role in removing degenerating neuronal elements in the retina after the onset of glaucoma.

Key words: Glaucoma, Ultrastructure, Neurons, Müller cells, Microglia

Introduction

Glaucoma is best defined as an optic neuropathy with characteristic optic nerve head and associated visual field changes (Thomas and Liesegang, 1996). The most characteristic pathological changes reported in the glaucomatous retina were the reduction of retinal thickness and the significant loss of retinal ganglion cells (Garcia-Valenzuela et al., 1995; Kerrigan et al., 1997; Laquis et al., 1998). In recent studies, it was found that nuclei of dying ganglion cells in the rat during elevated intraocular pressure (IOP) undergo fragmentation of their chromatin, as described in classical apoptosis (Garcia-Valenzuela et al., 1995; Kerrigan et al., 1997; Laquis et al., 1998). Glaucoma secondary to blockage of the venous return may involve apoptosis as the main mechanism for cellular death (Garcia-Valenzuela et al., 1995).

It is well established that astrocytes and microglia play important roles to protect the integrity of the nervous system during the developmental period as well as throughout lifetime (Merrill, 1994). As surveillant cells, these glial cells intervene in acute destabilizing situations to repair small traumas and protect against imbalances (Merrill, 1994). In the normal retina, astrocytes are reported to perform many functions like wrapping around ganglion cell axons, contacting other glial cells (e.g. Müller cells) to form adherent junctions and also play a role in potassium buffering as well as in neuronal signalling (Lam et al., 1995). Microglia, highly reactive and mobile cells, are thought to play universal role in defence of the CNS (Kreutzberg, 1995). In recent years, microglia have also been recognised to play an important role in the field of neuroimmunology (Ling et al., 1996; Ng and Ling, 1997). There are many reports on the roles of microglia in normal and abnormal retinae (Pearson et al., 1993; Battisti et al., 1995; Kreutzberg, 1995; Zhang et al., 1997). Our previous study using light microscopy has shown that glaucoma damages both ganglion cells and some neurons in the inner nuclear layer (INL) of the retina. Microglia, astrocytes and Müller cells are activated synchronously in the retina shortly after elevation of IOP and their reactivities are closely correlated to the neuronal down-regulation in the glaucomatous retina (Wang et al., 2000). The reactions of these glial cells are most probably directly linked to the neuronal degeneration in the glaucomatous retina. But so far, little research on the ultrastructural changes has been done on glial cells after the onset of glaucoma.

Offprint requests to: Samuel Sam-Wah Tay, Department of Anatomy, Faculty of Medicine, National University of Singapore, MD10, 4 Medical Drive, Singapore 117597. Fax: +65-778-7643. e-mail: anttaysw@nus.edu.sg

The aim of the present study is to investigate the ultrastructural changes in the neurons and glial cells after the onset of glaucoma, and the mode of neuron death, as well as the possible roles of reactive glial cells in the glaucomatous retina.

Materials and methods

Animals

A total of 23 adult male Wistar rats (weighing 250-320g) was used in this study. The rats were divided into three groups: normal (n=3), sham operated (n=9) and experimental (n=9). For experimental group, only those showed sustained elevation of IOP (the IOP of operated right eye is at least 25% higher than that of the controlled left eye) were sacrificed for further processing (n=9). Those rats whose eyes with IOP readings reverted back to almost normal values were not sacrificed for further processing as glaucomatous rats (n=2).

Surgical procedure

Rats were anaesthetized by an intraperitoneal injection of 7% chloral hydrate (0.35g/kg of body weight). Limbus-draining veins were exposed and three of the four veins were cauterized using a small vessel cauterizer (Fine Science Tools, Canada). The detailed surgical procedures have been reported previously (Garcia-Valenzuela et al., 1995; Wang et al., 2000). Sham-operated rats were subjected to the same operation procedures as described above, but short of vessel cauterization. Only the right eye of each rat was used for operation, the left eye was used as control.

Measurement of intraocular pressure (IOP)

The IOP of both eyes was measured by using a factory-calibrated Tono-Pen XL tonometer (Mentor, Norwell, USA). Each IOP registered was an average of three consecutive measurements. IOP measurements were recorded before and half an hour after cauterization, and then once every week, as well as immediately just before killing by perfusion. Both glaucomatous and sham-operated rats were sacrificed at 1, 3 weeks and 3 months post-operation (n=3).

TUNEL detection

Following deep anaesthesia, all rats were perfused first with Ringer's solution followed by 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). After perfusion, eyeballs were removed and post-fixed in the same fixative for 2-4 h before they were transferred into 0.1M PB containing 15% sucrose and kept overnight at 4 °C. Frozen sagittal sections of the eye were cut at 20 μm thickness and mounted on chrome alum-gelatin coated slides. Apoptotic cells in frozen sections on slides, were detected by the commercially available Tdt Frag ELtm DNA Fragmentation Detection kit (Oncogene Research Products, Cambridge, USA) using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Briefly, sections were incubated with proteinase K (20 μg/ml) in 10 mM Tris pH 8 at room temperature for 20 minutes. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide. Following the application of the equilibration buffer, the sections were incubated in working strength terminal deoxynucleotidyl transferse (TdT) enzymes (containing dUTP – digoxigenin uridine triphosphate) at 37 °C for 90 minutes. The reaction was stopped by a pre-warmed working strength stop buffer. After washing in distilled water, an antidigoxigenin antibody fragment carrying enzyme peroxidase was applied in a humidified chamber for 30 minutes at room temperature. The localized peroxidase enzyme catalytically generated an intense signal from the chromogenic DAB substrate. The sections were counterstained with methyl green, dehydrated and mounted with permount before viewed under a light microscopy. Control sections were processed as above except without incubating in TdT enzymes.

Transmission electron microscopic (TEM) study

After deep anesthesia, rats were perfused first with Ringer's solution followed by a fixative containing 2% paraformaldehyde and 3% glutaraldehyde in 0.1M phosphate buffer (PB; pH 7.4). The retinae were removed and post-fixed in the same fixative for 24 h before being transferred into 0.1 M PB containing 5% sucrose and kept overnight at 4 ºC. Each retina was cut into small pieces (2 mm^3) of tissue samples under a dissecting microscope. The tissue samples were postfixed in 1% OsO_4 in 0.1M PB (pH 7.4) for 1 hour, and stained with 0.2% uranyl acetate for half an hour. Then, the sections were dehydrated in a graded series of ethanol and finally embedded in Araldite. Ultrathin sections were double-stained with lead citrate and uranyl acetate and viewed under a JEOL 1200EX electron microscope.

Results

Intraocular pressure

In the normal and sham-operated control eyes, the average IOP was 22.04±0.69 mmHg. The mean IOP of the experimental eyes increased immediately after operation, and remained at a significantly elevated level throughout the entire length of the experiment ($p<0.001$) (Table 1; Fig. 1). The ocular tissues, including cornea, lens and sclera, appeared normal throughout the experiment.

Detection of apoptotic neurons - TUNEL staining

In the retina of normal and sham-operated control rats, no TUNEL positive neurons were observed (Fig.

2a). Nuclei of apoptotic neurons were stained brown in colour by TUNEL. A small number of TUNEL positive neurons were found in glaucomatous retinae at 1 week post-operation. These neurons located not only in the ganglion cell layer (GCL) but also in the INL and outer nuclear layer (ONL) ONL of glaucomatous retinae (Fig. 2b). The number of TUNEL positive neurons was increased at 3 weeks post-operation. The increased apoptotic neurons were mainly present in the GCL. And a significant increase in number of apoptotic neurons was also observed in the INL and ONL (Fig. 2c).

Ultrastructure of neurons and glial cells in the retina of normal and sham-operated rats

In the normal and sham-operated rats, all the neurons and glial cells in the retina appeared normal. The cytoplasm of ganglion cells contained the usual organelles, including the cisternae of rough endoplasmic reticulum, a Golgi complex and slender mitochondria (Fig. 3). Microglia were commonly seen amongst the widely spaced axons in the nerve fiber layer (NFL),

Table 1. Average of IOP (mean ± SEM) on normal, sham-operated and glaucomatous rats.

GROUP	DURATION OF POST-OPERATION	NUMBER OF RATS (n)	IOP (Mean ± SEM) (mmHg)
Normal	not applicable	3	21.67±0.58
Sham-operated	1 week	3	22.67±1.15
	3 weeks	3	21.67±1.15
	3 months	3	23.11±1.02
Experimental	1 week	3	36.00±1.20
	3 weeks	3	33.89±1.17
	3 months	3	31.06±0.92

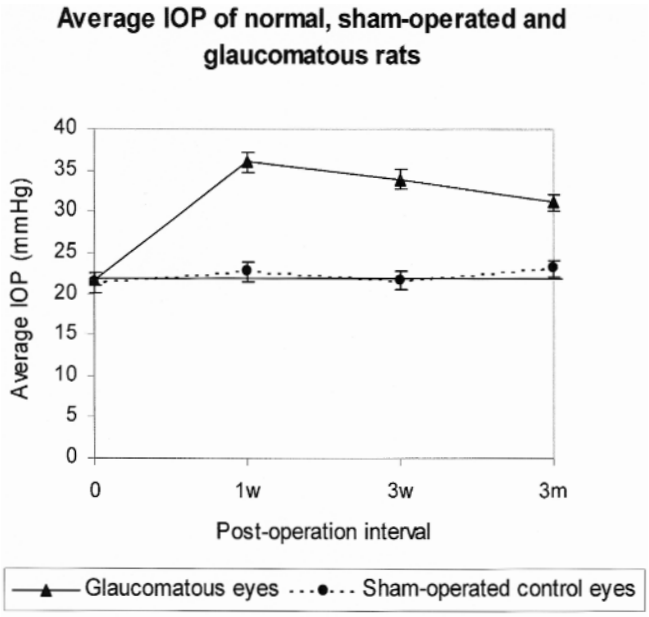

Fig. 1. Time distribution of average IOP values for normal, sham-operated and glaucomatous eyes. The IOP of the operated eye is obviously higher than that of the normal eye at each time point after operation ($p < 0.001$). The horizontal line indicates the average IOP level from normal and sham-operated control eyes.

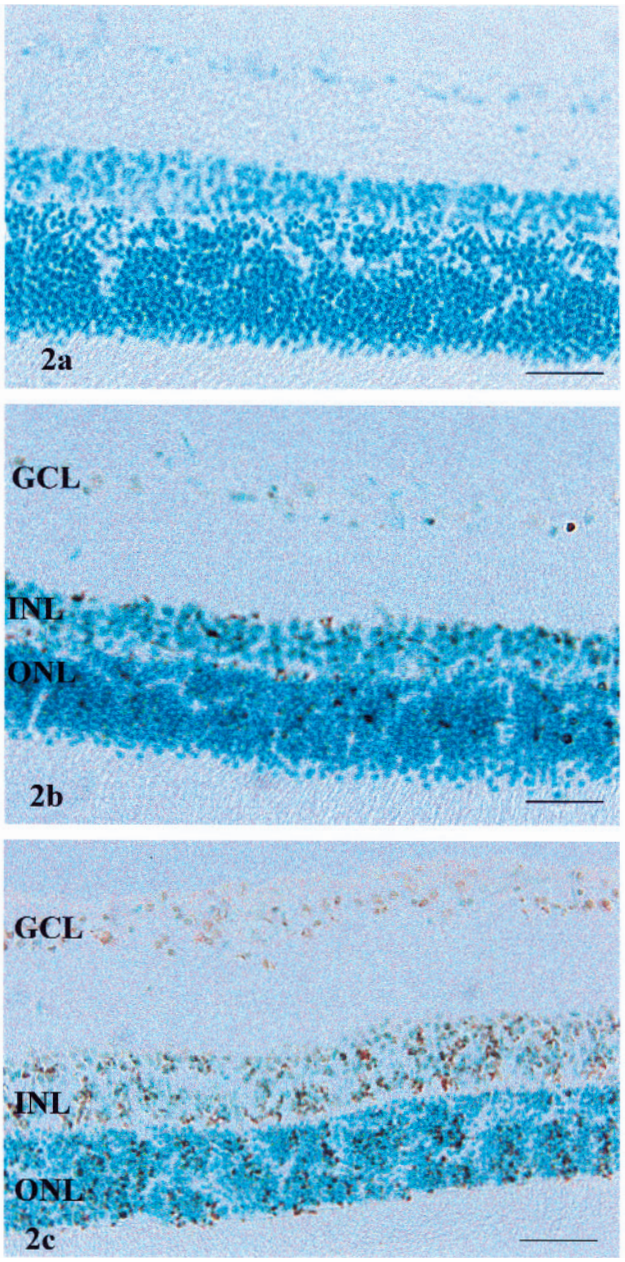

Fig. 2. Photomicrographs showing sections of retina of normal and glaucomatous rats stain with TUNEL method. TUNEL detects apoptotic cells by staining the nuclei of affected cells brown. No TUNEL positive neuron is present in the normal retina **(a)**. A small number of TUNEL positive neurons is present in GCL, INL and ONL in the glaucomatous retina at 1-week post-operation **(b)**. The number of TUNEL positive neurons is increased at 3-weeks post-operation **(c)**. Scale bars: 40 μm.

GCL and inner plexiform layer (IPL), they presented with a slim, small cell body and a few lysosomes in the cytoplasm (Fig. 4). No amoeboidic and macrophage-like cells were observed. Müller cells could be identified easily by their moderate electron-dense cell body and processes extending between neurons. They were localized in the middle of the INL. Few astrocytes and their processes containing microfilaments were found in the normal retina.

Ultrastructural changes in the neurons and glial cells of glaucomatous retinae

Neuronal degeneration was observed in all the

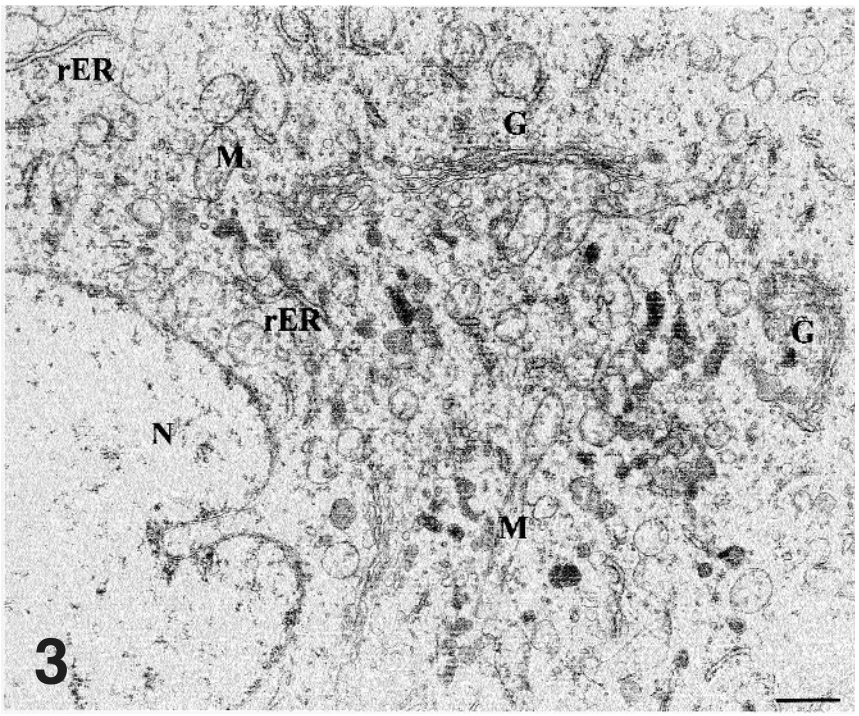

Fig. 3. Portion of a retina ganglion cell in the GCL of sham-operated rat retina. The cytoplasm contains the usual organelles: mitochondria (M), cisternae of rough endoplasmic reticulum (rER), a Golgi complex (G) and a nucleus (N). Scale bar: 1 μm.

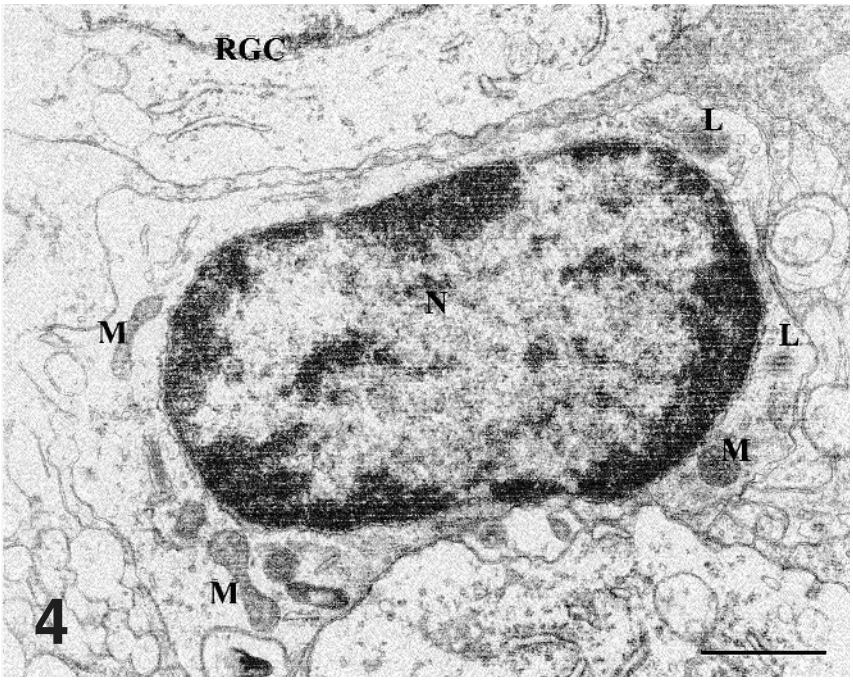

Fig. 4. Microglia in the GCL of a sham-operated rat. These cells show slim and small cell bodies, and a few lysosomes (L) in their cytoplasm. RGC: retina ganglion cell; M: mitochondria. Scale bar: 1 μm.

different layers of the retina at both 1 and 3 weeks post-operation. Some apoptotic-like degenerating neurons were found in the GCL, INL and ONL, characterized by a fragmented nucleus, darkened cell cytoplasm, membrane encapsulated apoptotic bodies, as well as relatively intact cell membrane and organelles (Fig. 5a-c). Many degenerating axons and axon terminals (with darkening of axoplasm and clumping of synaptic vesicles) were observed between the neurons and axons in the GCL, IPL, INL, and outer plexiform layer (OPL) (Fig. 5d). In the glaucomatous retinae at 3 months post-operation, few degenerating neurons were present in the retina. Many axons and axon terminals in the retina especially in the GCL and IPL were found to be abnormal, including those containing less synaptic vesicles, disintegration of axonal membrane and even depletion of axonal content (Fig. 6a-c). In some surviving ganglion cells, disorder of organelles was observed, such as rupture of the nuclear and plasma membranes, swollen and degenerating mitochondria as well as detachment of polysomes from the rough-surfaced endoplasmic reticulum (Fig. 6a).

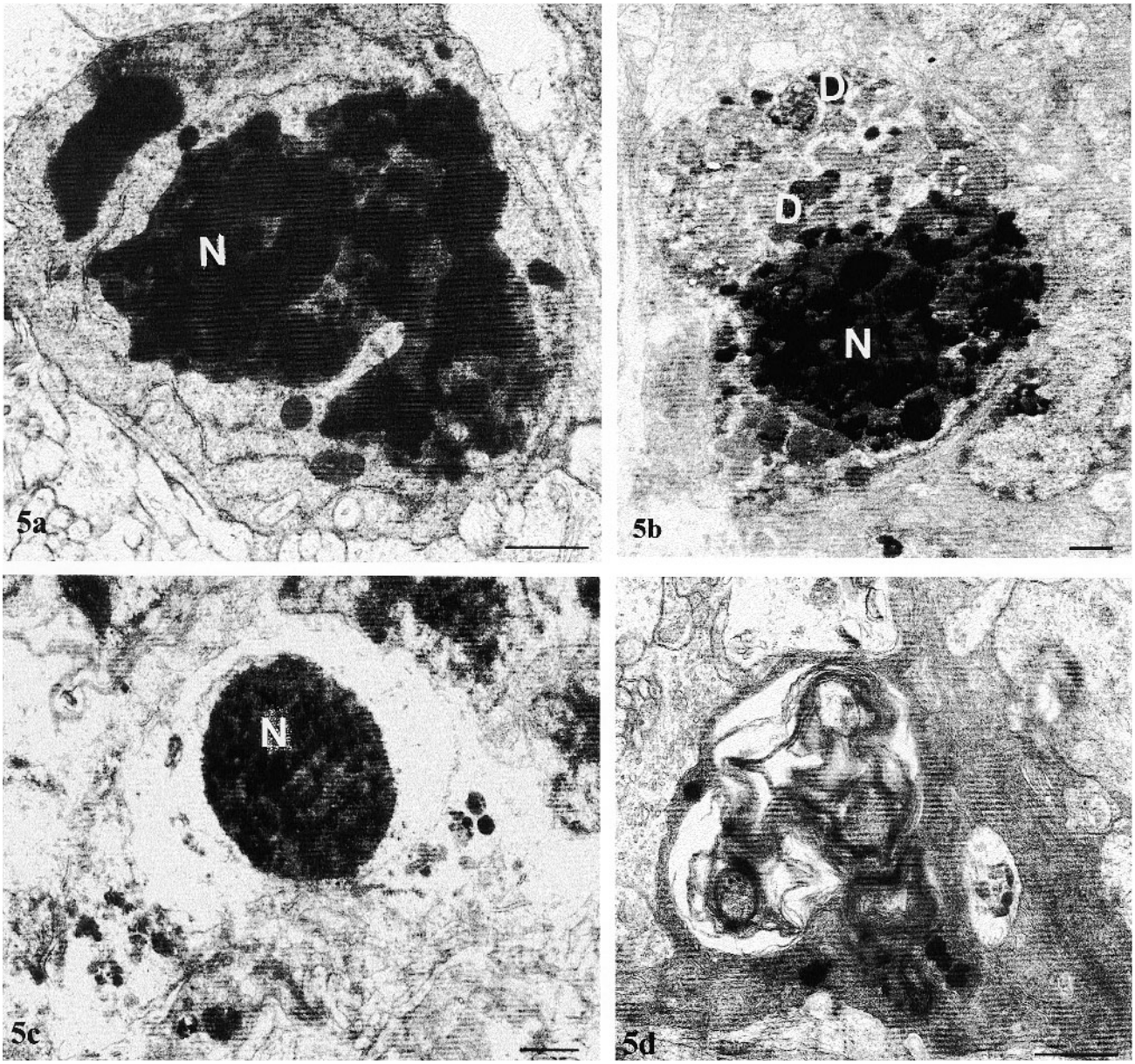

Fig. 5a-d. Electron micrographs showing neuronal degeneration at 3 weeks after onset of glaucoma. Degenerating neurons are seen in the GCL **(a)**, INL **(b)** and ONL **(c)**. Degenerating axons and axon terminals are observed between neurons in the GCL **(d)**. N: nucleus; D: degenerating debris. Scale bars. 1 μm.

Activated astrocytes, Müller cells and microglial cells were observed, especially in the GCL, IPL and INL in the glaucomatous retina at both 1 and 3 weeks post-operation. Some microglial cells were hypertrophic, and some even assumed amoeboidic appearance. They were present in close association with degenerating neurons and axons (Fig. 7a). Degenerating neuronal debris, degenerating axonal profiles and electron-dense bodies were often found in the cytoplasm of activating microglia or macrophage. Some macrophages were observed in the NFL near the inner limiting membrane of the retina as well as in the vitreous body (Fig. 7c). These cells contained a rich cytoplasm carrying large phagosomes, some of which were engulfed degenerating cells with discernible condensed nuclei (Fig. 7b,c). At the cell surface, pseudopodial processes containing vacuoles projected into the wide interstitial space (Fig. 7c). Some blood monocytes were also observed within the lumina of blood vessels. Many processes of either astrocytes or Müller cells containing numerous dark microfilaments were observed in the NFL, GCL and IPL, surrounding the degenerating neurons and axons (Fig. 7a,d). The Müller cells in the INL also presented longer and darker processes with more microfilaments

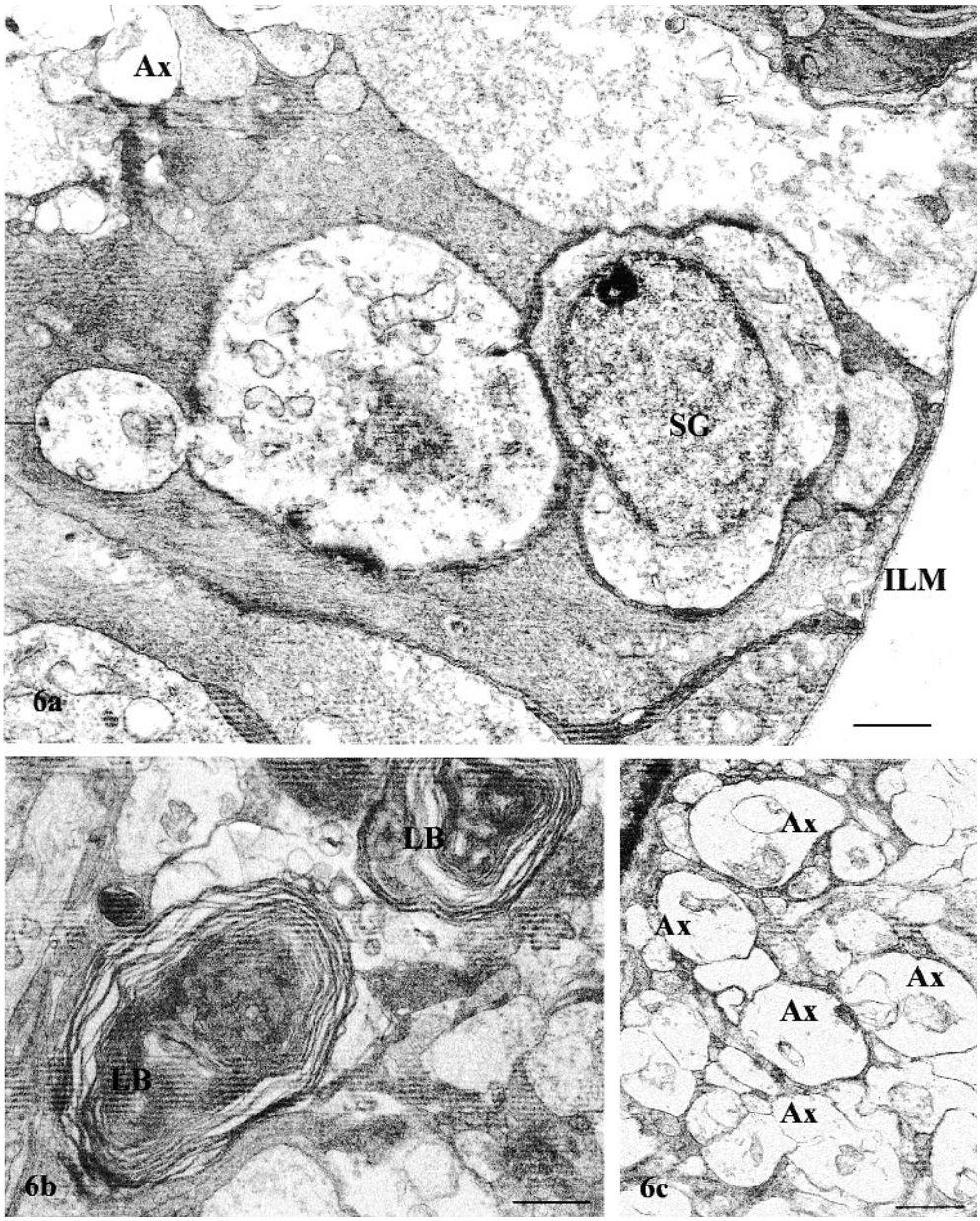

Fig. 6a-c. In 3-months post-operated glaucomatous retina, many neurons and their axons as well as axon terminals (Ax) appear abnormal in both the GCL **(a)** and IPL **(c)**. In some surviving ganglion cells (SG), organelles present disorder **(a)**, including rupture of the nuclear and plasma membranes, swollen and degenerating mitochondria as well as detachment of polysomes from the rough-surfaced endoplasmic reticulum. Some electron dense lamellar bodies (LB) are also present in the GCL (**b**). ILM: inner limit membrane. Scale bars: 1 μm.

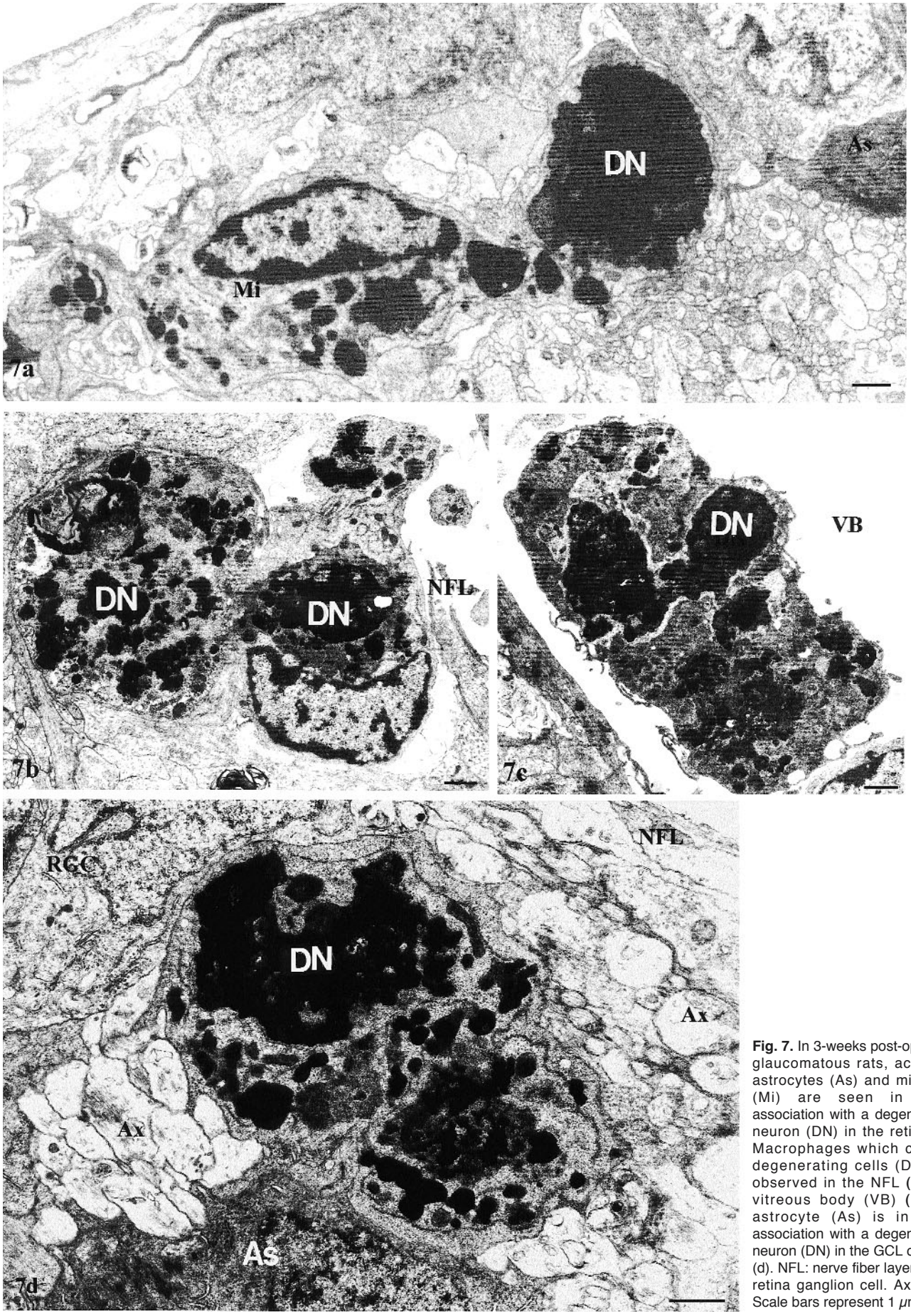

Fig. 7. In 3-weeks post-operated glaucomatous rats, activated astrocytes (As) and microglia (Mi) are seen in close association with a degenerating neuron (DN) in the retina **(a)**. Macrophages which contain degenerating cells (DN) are observed in the NFL **(b)** and vitreous body (VB) **(c)**. An astrocyte (As) is in close association with a degenerating neuron (DN) in the GCL of retina (d). NFL: nerve fiber layer, RGC: retina ganglion cell. Ax: axon. Scale bars represent 1 μm.

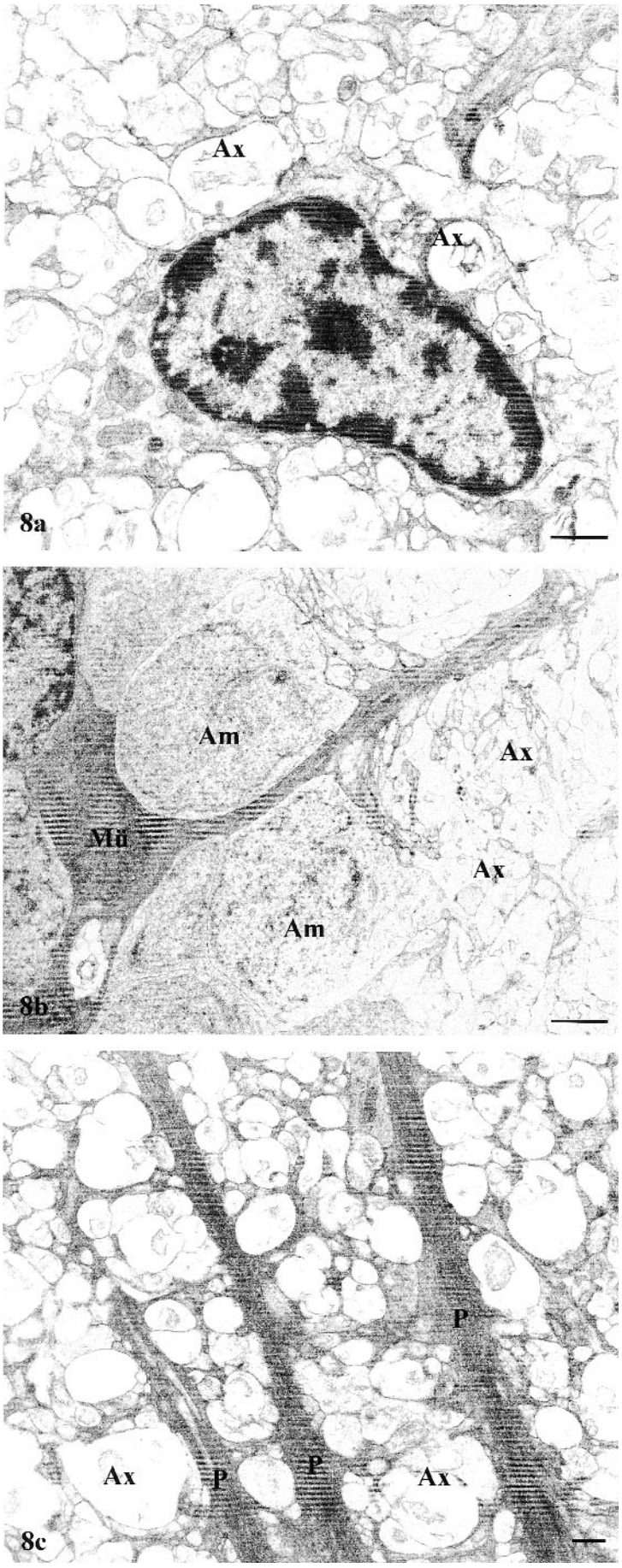

Fig. 8. In 3-months post-operated glaucomatous retina, most of the microglial cells appear normal (a). Müller cell (Mü) processes containing numerous dark microfilaments, extending between neurons in the INL and spanning further into the other retinal layers **(b)**, and some of these processes (P) are found spanning through the IPL **(c)**. Am: amacrine cell; Ax : axon. Scale bars. . a,c, 1 μm; b 2 μm.

than normal and their dark endfeet containing numerous microfilaments were observed clearly at the inner limiting membrane of the retina. In the glaucomatous retinae at 3 months post-operation, the glial cells, however, were observed to be not as active as that in the short-term. Most of the microglial cells appeared normal (Fig. 8a). The processes of astrocytes or Müller cells containing numerous dark microfilaments were still observable in the GCL and IPL, and some processes of Müller cells were found spanning through the IPL (Fig. 8b,c), which were not observed in the sham-operated retinae.

Discussion

Damage of the inner retinal layers leading to the loss of ganglion cells and their axons in glaucomatous models has been demonstrated by using various conventional histological methods as well as apoptotic markers (Glovinsky et al., 1993; Garcia-Valenzuela et al., 1995). Significant ganglion cell death was reported as early as 1 week after the elevation of IOP (Garcia-Valenzuela et al., 1995; Laquis et al., 1998). But the issue of whether the photoreceptor cells are affected in addition to the inner retinal neurons is critical and still on debate. Results from some previous studies, particularly with regard to the normal electroretinogram (ERG) recordings obtained in glaucomatous patients, indicate that photoreceptors may be unscathed by glaucoma (Trick, 1987; Korth et al., 1989; Osborne et al., 1999; Hare et al., 2001). Quigley et al. (1979, 1981) have examined many glaucomatous eyes by using light and electron microscopy during the past 25 years and have provided clear evidence that RGCs and their axons die, whereas no other neurons are visibly affected (Kendell et al., 1995; Wygnanski et al., 1995). However, studies by Panda and Jonas (1992) suggest that the photoreceptors are affected in human with advanced glaucoma, and a recent electrophysiological result implies that the outer retinal structures, especially the photoreceptors, may be affected by glaucomatous damage (Velten et al., 2001). Astrocytes, Müller cells and microglial cells are the major types of non-neuronal cells in the vertebrate retina; their activation has been reported in response to neuronal injury, degeneration and regeneration (Eisenfeld et al., 1984; Battisti et al., 1995; Kreutzberg, 1995) as well as in blind human eyes with various underlying diseases (Thale et al., 1996; Dithmar et al., 1997). In general, microglia are thought to initiate and regulate other glial cell activities in the retina and the CNS (Merrill, 1994; Battisti et al., 1995).

We have previously reported that glaucoma damages both ganglion cells and some neurons in the INL of the retina. Microglia, Müller cells and astrocytes are activated synchronously in the retina shortly after elevation of IOP and their reactivities are closely correlated to the neuronal down-regulation labelled by neuronal nuclei (NeuN) marker in the glaucomatous retina (Wang et al., 2000). Our current study showed that TUNEL positive neurons were observed in not only the GCL but also the INL and ONL of the glaucomatous retina at both 1 and 3 weeks post-operation. And the result of TEM study further confirmed that the mode of neuronal cell death in the GCL, INL and ONL, appeared to be mainly via apoptosis. Furthermore, the present results of TUNEL staining and TEM are well matched in both cell location and time interval. Although technique used in present study could not distinguish different types of neurons (amacrine cell, bipolar cell and horizontal cell) in the INL from each other, it at least provides evidences that besides RGCs, photoreceptors and INL neurons are also affected in experimentally-induced glaucomatous rats.

In the present study, astrocytes and Müller cells present longer and darker processes with more microfilaments surrounding degenerating neurons, and microglia were in close association with degenerating neurons. All these glial cells appear to participate in the pathological process of neuronal death in the retina of glaucomatous rats. With degenerating neuronal debris, degenerating axonal profiles and electron-dense bodies found in the cytoplasm of activating microglia and macrophages, it confirms that the activated microglia might respond to remove the degenerating neuronal elements. Macrophage is thought as a constituent element present mainly in the vitreal side of retina in the normal postnatal rats, yet it is rarely found in the normal retina of adult rat (Ling, 1981). The origin of macrophages in the pathological conditions is still debatable. There are several reports that microglial cells can be transformed into macrophages (resting – activating – phagocytic stages) (Jordan and Thomas, 1988; Nakajima and Kohsaka, 1996). The present study shows that blood monocytes are frequently observed in the lumina of blood vessels of the glaucomatous retina and activated macrophages are found in the GCL and vitreous chamber of the galucomatous rat. It is hypothesized that blood monocytes might transform into macrophages and the vitreous chamber might also be an extrinsic source of retinal macrophages after the onset of glaucoma. This is supported by other studies which show that the transformation of blood monocytes into macrophages occurred in wounds of neural tissues and in postnatal development of the rat brain (Ling, 1981). The present results confirm that of our previous study in that the expression of microglia is up-regulated in the retina of the glaucomatous rat and the increased glial cells may be activated to remove the neuronal degeneration products (Wang et al., 2000).

Thus, the current study demonstrates that in experimentally-induced glaucomatous retina of the rat, not only retinal ganglion cells died mainly via apoptosis, but neurons in the INL and ONL were also affected and similarly died via apoptosis. Astrocytes and Müller cells were involved in the pathological process of neuronal death, and microglia appeared to play an important role in removing degenerating neuronal elements in the retina after the onset of glaucoma.

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