Macrogial Alterations after Isolated Optic Nerve Sheath Fenestration in Rabbit

Max Villain,1,2 Françoise Sandillon,1 Agnès Muller,5 Emmanuel Candon,1 Gérard Alonso,4 Bernard Arnaud,2 and Alain Privat1

PURPOSE. To study the modifications undergone by the macrogial cells after meningeal breach of the optic nerve in the rabbit, without optic neuropathy.

METHODS. The optic nerve sheath fenestration technique carried out in humans was adapted to rabbit without axonal injury in the optic nerve. The effects of meningeal fenestration on glial cells were examined by immunocytochemical procedures (day 15) using the antibodies against two astrocyte markers: glial fibrillary acidic protein (GFAP) and vimentin. Proliferation of glial cells was evaluated with single 5-bromodeoxyuridine (BrdU) labeling or double GFAP and BrdU labelings. Qualitative data on glial cells were evaluated with the electron microscope.

RESULTS. Optic nerve sheath fenestration on healthy adult rabbits resulted in a decrease of volume of the subarachnoid space located at the level of the meningeal scar, with a significant increase of the optic nerve area. The meninges presented a fibrous scar. In the optic nerve parenchyma, astrocytes appeared hypertrophic in the vicinity of the fenestration. The whole nerve contains numerous BrdU-labeled mitotic cells, a number of which double-labeled for both BrdU and GFAP belong to the astrocyte line. There was no loss of optic nerve axons.

CONCLUSIONS. The inflammation produced by the surgical breach of the peri-optic meningeal sheaths induces a significant reactivity, including proliferation of astrocytes in the optic nerve. Reactive astrocytes may interact positively with axons and may modify the extracellular environment in the optic nerve. (Invest Ophthalmol Vis Sci. 2002;43:120-128)

Papilledema from idiopathic intracranial hypertension (IH) can cause an optic neuropathy, with severe loss of visual acuity and visual field.1–4 In experimental IH, Hayreh5,6 demonstrated that orbital optic nerve sheath fenestration (ONSF) provoked resolution of papilledema. Since then, many authors7–14 have reported the therapeutic efficacy of decompressing the meningeal sheath of the optic nerve in idiopathic IH with papilledema in humans, even in cases of optic atrophy postedema.11,14,15 On the contrary, ONSF is not effective and may be harmful in nonarteritic anterior ischemic optic neuropathy.16

The success of this surgical technique in optic neuropathies (ON) of Idiopathic IH was attributed to the development of a fistula or a cyst.10,15,17–18 However, the postoperative fistula was found inconstant.19,20 Moreover, the intracranial pressure was not modified by this surgical procedure.8,15,20–22 Finally, no rational explanation can account for the bilateral efficacy of the ONSF in up to 50% of cases after unilateral surgical procedure.7,8,10,14,24 Yet, significant scarring and obliteration of normal tissue planes was encountered in histologic studies6,19,24 and in all the reoperations.11,25–27 Ultrastructural studies carried out in humans after ONSF pointed to a nonspecific chronic fibrosis with the presence of fibroblasts and immature collagen in meningeal sheaths.19,28–30

In idiopathic IH with severe ON, there exist histologic modifications in the optic chiasm and the proximal portion of the optic nerve: diffuse axonal degeneration with axonal loss,19,31 myelin pallor, and diffuse mild astrogliosis.32 Glia is the major nonneuronal component of the optic nerve. Glia is made up of two components: the macroglia and the microglia. Microglial cells are the resident macrophages of the CNS.32

Macroglia consists of two cellular types: oligodendrocytes, which are responsible for myelination, and astrocytes.33 In the optic nerve astrocytes are particularly fibrous, with most of their processes constituting the glia limitans and perivascular end-feet and a few of them abutting on to the nodes of Ranvier. Astrocytes are connected by gap junctions, which are the substratum of efficient ionic homeostasis of axons.34,35 They are the only optic nerve cells in direct contact with the pia mater and constitute a glial limiting membrane known as Graefe’s peripheral layer.36–38 In the adult rat optic nerve Miller et al.39 demonstrated type 1 astrocytes as arranged mainly at the periphery of the nerve, whereas type 2 astrocytes are found mainly in the interior of the optic nerve abutting the nodes of Ranvier. Astrocytes have a large number of key functions in the central nervous system (CNS), because they are closely associated with neurons during development as a support for migration and axonal growth. In addition, they serve as key elements in nutrition and metabolic support40 and are known to play an important role in the removal of toxic metabolites in the extracellular environment.41,42 Finally as reactive cells, they play a key role in cellular remodeling after CNS insult.13,14

Reactive astrocytes are a major source of neurotrophic factors in the brain.41,42,45–47 In the adult mammalian CNS, reactive astrocytes can express nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), and brain-derived nerve factor (BDNF).42,48 Reactive astrocytes are larger than nonreactive astrocytes, extend thicker and longer processes, and upregulate astrocyte-specific intermediate filament glial fibrillary acidic protein (GFAP).42,49,50

From 1Unité INSERM 336, Développement, Plasticité et Vieillissement du Système Nerveux Central, Montpellier, France; 2Service d’Ophthalmologie, Hôpital Gué de Chauliac, Centre Hospitalo-Universitaire de Montpellier, Montpellier, France; 3Laboratoire de physiologie cellulaire, UMR-CNRS 5074, Faculté de pharmacie, Université Montpellier, France; and 4UMR-CNRS 5101, Biologie des neuroendocrines, Montpellier, France. Submitted for publication September 28, 2000; revised July 23, 2001; accepted August 16, 2001.

Commercial relationships: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked ‘advertisement’ in accordance with 18 U.S.C. §1754 solely to indicate this fact.

Corresponding author: Max Villain, Unité INSERM 336, Place Eugène Bataillon, Case courrier 106, Université des Sciences et Techniques du Languedoc, 34095 Montpellier Cedex, France; u356@univ-montp2.fr.
Given the major role of macroglial cells in the physiology and pathophysiology of the optic nerve, we developed a model of experimental ONSF in the rabbit to study the modifications undergone by the macroglial cells after meningeal breach of the optic nerve in the rabbit, without optic neuropathy.

**MATERIALS AND METHODS**

Adult male burgundy rabbits weighing 3 kg were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fundus examination performed before surgery was normal. Anesthesia combined intravenous injection of acepromazine (2 mg/kg) and xylazine (2 mg/kg) and intraperitoneal injection of urethane (4 mg/kg). The surgical procedure was carried out by one surgeon using an operative microscope and surgical asepsis. The following stages were performed: superior conjunctival peritomy and disinsertion of the superior rectus muscle and fenestration of the subarachnoid lamina in the retrobulbar segment of the superior edge of the optic nerve after penetration of the central retinal artery.

The fenestration consisted of an approximately 4-mm² rectangular block of tissue. Cautious traction was exerted on the globe with

**Figure 1.** Transversal section of the anterior optic nerve (day 15). Semi-thin section 0.5 μm, toluidine blue (A, fenestrated; B, control). There is a significant reduction in the volume of the subarachnoid space (SA S) near the meningeal scar (arrow). This reduction is strictly inversely correlated to the increase in volume of the optic nerve (opt N). Bar, 200 μm.
a silk suture applied to the corneal limbus. During the fenestration, we respected the pia mater and the fenestration was made by peeling off the fine meningeal layers rather than by direct incision. The closing procedure consisted of reinsertion of the superior rectus muscle with a 5-0 Dexon, and the conjunctiva was sutured with 7-0 Dexon.

After a fixed period of observation, the rabbits were anesthetized (10 mg/kg intramuscular ketamine, 20-30 mg/kg intravenous pentobarbital) and killed by perfusion through the aorta (2 liters of fixative: 4% paraformaldehyde in phosphate buffer for immunocytochemistry, 2.5% glutaraldehyde in phosphate buffer for the ultrastructural study). The orbital dissection was performed under a surgical microscope and the optic nerves with their meningeal sheath were excised 15 days postoperatively (12 rabbits) and 42 days postoperatively (4 rabbits) for immunocytochemical and ultrastructural studies. A nonoperated rabbit was used as a control subject.

For the immunocytochemical study, tissues were postfixed for 24 hours in 4% paraformaldehyde in phosphate buffer, rinsed in a saline swab Tris buffer (TBS, pH 7.5), and then immersed overnight in a 0.12 M phosphate and 30% sucrose buffer solution. Longitudinal sections were made using a cryostat 20-μm (thickness) and collected on slides. We used monoclonal antibodies against GFAP and vimentin (mouse IgG; Sigma, Sigma-Aldrich, Saint Quentin Fallavier, France), to visualize the astrocytic reaction. The primary antibodies were diluted 1:10 000 in TBS with 1% rabbit serum, 1% bovine serum albumin, and 0.1% Triton X-100. Immunohistochemistry was carried out at room temperature using the peroxidase antiperoxidase system (PAP) according to Sternberger et al. Sections were successively incubated with (1) rabbit antibody against mouse IgG (diluted 1:1 000; Dako, Trappes, France), (2) a mouse peroxidase antiperoxidase complex (diluted 1:1 000; Dako), and (3) 0.05% diaminobenzidine (DAB) + 0.2% H2O2. Controls for the retina and the optic nerve were made by incubating the sections without primary antibodies. An image analyzer (Alcatel Samba 900 electron microscope). The ultrastructural study was performed on 2-mm high-cylindrical blocks of optic nerve. The contralateral optic nerves were used as controls. The blocks were rinsed in a 0.12 M phosphate buffer with 8% glucose for 5 minutes and postfixed in a 2% osmic acid solution in a 0.12 M phosphate buffer with 8% glucose for 2 hours. After dehydration the blocks were embedded in Araldite. After light microscopy control of toluidine blue-stained, 0.5 μm-transverse sections of the optic nerve, 0.1 μm-sections were made by means of an ultramicrotome MT-7. The sections were collected on copper grids (300 mesh), stained with uranyl acetate and lead citrate, and examined in a Zeiss 900 electron microscope.

Finally, planimetric measurement of the subarachnoid space area, the optic nerve and pia mater surfaces were carried out on cross sections performed at the same level in eight rabbits, with digitalization of the image. Data are presented as means ± SD. Statistical comparisons were made by one-way analysis of variance and by the Mann–Whitney test. P ≤ 0.01 was considered statistically significant.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932903/) Subarachnoid space and tissular area (8 rabbits, day 15).

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932903/) Cells in fenestrated optic nerve near the meningeal scar (day 15), with microglial cell (m; small cell elongated nucleus with large masses of dark chromatin, a relatively dense cytoplasm containing long, tortuous cisternae of rough endoplasmic reticulum), oligodendrocyte (Ol; light oligodendrocyte with a large, and pale nucleus. The abundant cytoplasm is not as light as the nucleus and it contains numerous organelles), and glioblast (Gb; small cell, with a limited cytoplasm). Bar, 2.5 μm.
RESULTS

General Observations

There was no clear outflow of cerebrospinal fluid during the opening of the meningeal sheath, but a minimal flow of serous fluid could be observed at the operation site. During the fenestration, examination of the subarachnoid space revealed no fibrous link between the arachnoid and the pia mater confining the optic nerve.

During orbital dissection after perfusion fixation, we observed a thickening of the meninges at the level of the fenestration with a proliferation of fibrous material. Examination under light or electron microscopy showed that the pia mater was intact at the site of the fenestration. There were some inconstant fibrous arachnoidal adhesions in between the pia mater and the meningeal scar.

The thickening of the meningeal sheath on the side of the operation was very irregular and variable precluding quantification. However, there was no significant difference in the pia mater area, between the operated and nonoperated sides, and no difference in the total area (subarachnoid space, pia mater, optic nerve). However, on the operated side, a significant reduction of the subarachnoid space ($P < 0.001$) was associated with a significant increase of the area of the optic nerve ($P < 0.01$; Figs. 1 and 2).

There was no difference in immunostaining with CNPase between control and operated sides. Similarly, using the electron microscope, oligodendrocytes and microglial cells appeared unaltered (Fig. 3). The immunostaining with ED1 highlighting the activated microglial cells was positive only at the level of the meningeal scar, which corresponded to the site of opening of the meningeal sheaths outside the pia mater (Fig. 4).

Light and electron microscopy showed that the axonal density was identical in control and operated optic nerves of each animal (Fig. 5), and there was no evidence of axonal degeneration (loss of axoplasm and collapse of the myelin sheath).

Astroglial Reactivity

A marked increase in GFAP and vimentin immunoreactivity was apparent in the operated optic nerve near the meningeal scar with the same intensity from superior to inferior face at day 15 (Figs. 6A, 6E) but not at day 42 (Figs. 6C, 6D, 6G, 6H). There was a sharply defined boundary in immunoreactivity located at the level of the posterior part of the meningeal scar (Figs. 6A, 6E).

The anti-BrdU antibody labeled round structures, which corresponded to nuclei (Figs. 7A, 7B). This immunostaining revealed many cells at the level of the fibrous scar (Fig. 7A) and throughout the anterior 5 mm of the optic nerve and also along $>1.0$ cm behind the level of fenestration. Double-labeling anti-BrdU and anti-GFAP antibodies showed that within these regions some BrdU-labeled nuclei were associated with both GFAP-positive and GFAP-negative cell bodies (Figs. 7B, 7B').

Figure 4. Peroxidase immunostaining for ED1 (day 15). Meningeal scar with strong immunostaining (A, fenestrated optic nerve; B, control; p, optic papilla). Bar, 500 μm.

Figure 5. Axonal density in the optic nerve (day 15). Transversal section, 0.5-μm semithin section , toluidine blue (A, fenestrated optic nerve; B, control). Bar, 250 μm.
On day 15, electron microscopy showed the presence of glioblasts, paucity of cytoplasm, irregular nuclear shape, and clusters of chromatin (Figs. 8A, 8B). Electron microscopy showed that intermediate filaments were sparse or absent in these cells, indicating that they were immature. Conversely, they exhibited a high content of free ribosomes. On day 42 there were only reactive astrocytes with irregularly oval nuclei and packed organelles in the perinuclear area (Figs. 8C, 8D). The density of intermediate filaments appeared to be higher in the cell processes than in the control optic nerve. An increase in the number of gap junctions was present on astrocyte membranes.

**DISCUSSION**

The modifications induced by a meningeal breach in the rabbit optic nerve consist mainly of a nonspecific meningeal fibrosis and astroglial reactivity within the optic nerve. We found a significant reduction in the volume of the subarachnoid space near the meningeal scar in all eight eyes after ONSF. Such a feature had already been reported in operated monkey optic nerves. This reduction is inversely correlated to the increase in volume of the optic nerve, rather than to a specific feature of the meningeal scar. The reduction in subarachnoid space after ONSF had been reported in operated humans after fenestrated optic nerve.
The increase in optic nerve volume has not been described. The significant increase in the optic nerve caliber is most likely due to the increased volume of the macroglial compartment. Indeed, we noticed both hypertrophy and hyperplasia of astroglia.

Hyperplasia can be suspected from two indirect observations. First, the incorporation of BrdU indicating cell proliferation was maximal in the anterior part of the nerve but extended all over its length, suggesting either a gradient of proliferation or a migration of postmitotic cells. Second, the identification of immature cells in the operated nerve with the electron microscope is suggestive of glioblasts. Identification of precursor, partially uncommitted cells, depends mainly on morphologic characteristics because specific markers are not available.

Data suggest that astroglia retain the capacity to initiate cell division throughout life, notably in brain injuries. The glial cells can be activated by neuronal death, infection, demyelination, inflammation, trauma, or axonal degeneration. Astrocyte activation enables them to change form, migrate, acquire new molecular markers, differentiate, and sometimes phagocytose. Glioblasts are the common precursors of oligodendrocytes and astrocytes. After a CNS insult, reactive astrocytes can return to their preinjury state if the injury is minor or if they are distant from an injured area. The BrdU-positive cells detected here may correspond to mature and immature astrocytes that have proliferated. Proliferating glial precursor cells can be isolated from the adult rat optic nerve and can differentiate into astrocytes or oligodendrocytes. Immature precursor cells in adult CNS have limited intrinsic migratory properties. Proliferating precursor cells may become astrocytes, oligodendrocytes, or even constitute a new pool of immature cells. Interestingly, in operated optic nerve double immunofluorescence detected a number of BrdU+ and GFAP+ cells, indicating that indeed some proliferating cells were actually astrocytes or that they differentiated along this cell lineage from immature precursors.

Hypertrophy was detected in the anterior part of the operated optic nerve, at the level of the meningeal scar at day 15. Electron microscopy revealed an increase of gliofilaments in astrocytes in this area of anterior optic nerve, which was confirmed by the increase in GFAP and vimentin detected by immunocytochemistry. Any injury of the CNS induces an up-regulation of GFAP that is more marked in the vicinity of the lesion than in remote areas.

Astroglial reactivity is clearly associated with the localized inflammation exhibited by the meninges containing ED1+ macrophages. A plausible sequence would be the release of interleukin by phagocytes, rather than by microglial cells at the meningeal inflammatory site. These activated phagocytes could release interleukin (IL)-1, which can trigger the mitosis of the astrocytes in vitro. However, other cytokines could be involved, on the basis of the numbers of molecules released by activated macrophages during the different phases of inflammation (IL-1, IL-6, interferon-γ, tumor necrosis factor-α, and transforming growth factor-β1). It has been shown that IL-1, IL-6, tumor necrosis factor-α, and transforming growth factor-β1 contribute to astroglial reactivity by stimulating astrocyte proliferation or hypertrophy. IL-1 and IL-6 have been reported to promote astroglial activation both in vivo and in vitro. Astrocytes and microglia have been shown to produce inflammatory cytokines both in vivo and in vitro.

Thus, astrocytic hypertrophy and hyperplasia without axonal death can explain the increase in volume of the optic nerve on the operated side. Indeed, any difference in surface or volume of fixed histologic specimen must be treated cautiously because interindividual variations and fixation artifacts can be misleading. In our case, the use of control unoperated contralateral optic nerves and sample size of eight animals should eliminate such artifacts.

In conclusion, the present study brings original data that may explain the efficacy of ONSF in human idiopathic IH with papilledema. Besides the well-known mechanical theory of decompression, a more complex histopathologic process involving the cellular environment of optic axons and its main constituent, the macroglia, must be considered.
ONSF can trigger a cascade of astrocytic reactivity, which may be to some extent favorable for the survival of ON axons, through mechanisms that await further investigation. The modification of ionic homeostasis and the release of trophic factors are likely candidates for these mechanisms.

References


