INHIBITING CELL PROLIFERATION DURING FORMATION OF THE GLIAL SCAR: EFFECTS ON AXON REGENERATION IN THE CNS

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Abstract—Following a CNS lesion many glial cell types proliferate and/or migrate to the lesion site, forming the glial scar. The majority of these cells express chondroitin sulphate proteoglycans (CS-PGs), previously shown to inhibit axonal growth. In this study, in an attempt to diminish glial scar formation and improve axonal regeneration, proliferating cells were eliminated from the lesion site. Adult rats received a continuous infusion of 2% cytosine-D-arabinofuranoside (araC) or saline for 7 days over the lesion site, immediately following a unilateral transection of the right medial forebrain bundle. Additional groups of rats that received subdural infusions prior to the lesion, and lesioned rats which received no infusion, were also compared in the analyses. Animals were killed at 4, 7, 12 or 18 days post-lesion (dpl) and immunohistochemistry was used to determine the effects of these treatments on tyrosine hydroxylase (TH)-lesioned axons, and on the injury response of glial cells. Almost complete elimination of NG2 oligodendrocyte progenitor cells from the lesion site was seen up to 7 dpl in araC-infused animals; reduced numbers of reactive CD11b microglia were also seen but no effects were seen on the injury response of GFAP astrocytes. Significantly more TH axons were seen distal to the lesion in araC-treated brains, but these numbers dwindled by 18 dpl. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: NG2, chondroitin sulphate proteoglycans, oligodendrocyte precursor cells, araC, microglia, nigrostriatal lesion.

A lesion to the CNS provokes reactive behaviour amongst glia resulting in the formation of the glial scar. Astrocytes form the bulk of the mature glial scar, responding to injuries by changing their cellular phenotype and behaviour (for review see Ridet et al., 1997). Astrocytes divide and/or migrate to the lesioned area, hypertrophy, up-regulate intermediate filament proteins glial fibillary acidic protein (GFAP) and vimentin, and up-regulate a number of cell-surface and extracellular matrix (ECM) molecules, including proteoglycans (McKeon et al., 1991, 1995; Haas et al., 1999; Asher et al., 2000). These changes develop over about 10 days leading to the classic appearance of the glial scar, which eventually consists of highly branched astrocytic processes attached to one another by junctional complexes, with plentiful ECM.

Microglia respond rapidly to injury, with cell division and transition from a resting to an amoeboid state (for review see Kreutzberg, 1996). Reactive microglia (and blood-borne monocytes where the blood–brain barrier is interrupted) are seen in large numbers in the glial scar where they phagocytose debris and secrete a variety of cytokines (Morshead and van der Kooy, 1990; Fitch et al., 1999; Jensen et al., 1999).

Recently another form of glia has been identified with immunoreactivity to antibodies against the chondroitin sulphate proteoglycan (CS-PG) NG2 (Levine, 1994), believed to be an immature cell of the oligodendrocyte lineage (Espinosa de los Monteros et al., 1993) which is probably equivalent to the adult oligodendrocyte precursor (OP) that may be cultured from the adult CNS (Wolswijk and Noble, 1989). OP cells are seen throughout the adult rat brain and respond rapidly to CNS injury by dividing, increasing NG2 immunoreactivity, hypertrophy, and accumulating around the lesion beginning within 24 h, with large numbers of cells by 7 days post-lesion (dpl; Levine, 1994; Ong and Levine, 1999; Bu et al., 2001; Levine et al., 2001; Jones et al., 2002).

Most adult mammalian CNS axons will not regenerate after a lesion: this is attributed to a limited intrinsic ability of axons to regenerate, combined with the presence of the dense glial scar and its many associated inhibitory molecules (for review see Fawcett and Asher, 1999), and also to inhibitory molecules produced by oligodendrocytes (Coroni and Schwab, 1988a, 1988b; McKerracher et al., 1994; Mukhopadhyay et al., 1994). Various CS-PGs are up-regulated in the glial scar (Levine, 1994; Davies et al., 1997; Asher et al., 1999, 2000; Fitch et al., 1999; Jones et al., 2002; Tang et al., 2003), and both in vivo and in vitro experiments have demonstrated inhibitory effects of CS-PGs on axonal growth (e.g. Snow et al., 1990; McKeon et al., 1991, 1995; Dou and Levine, 1994; Smith-Thomas et al., 1994, 1995; Fidler et al., 1999; Niederöst et al., 1999; Moon et al., 2001; Bradbury et al., 2002; Chen et al., 2002; Ughrin et al., 2003). Some inhibitory CS-PGs are expressed by astrocytes, including brevican (Yamada et al., 1997), neurocan (Friedlander et al., 1994; Haas et al., 1999; Asher et al., 2000) and phosphacan (McKeon et al., 2003).
the lesion using immunohistochemistry. and glial cells were examined at various time points after (including the nigrostriatal tract); effects on lesioned axons immediately following, a unilateral transection of the MFB glia. The antimitotic, cytosine-D-arabinofuranoside (araC), lesion, by reducing the number of rapidly dividing reactive formation, and improve axonal regeneration after a CNS literature (for review see Eddleston and Mucke, 1993). In previously GFAP-negative cells, has been much debated in the proliferation, migration or up-regulation of GFAP in previ-

numbers in the glial scar occur as a result of astrocytic CNS lesion is not due largely to proliferation of these cells (e.g. Miyake et al., 1988; Mitchell et al., 1993; Di Prospero Reynolds, 1999; McKeon et al., 1995), a possible approach to reducing inhibition of CNS-PGs comprise a protein core with a varying number of glycosaminoglycan (GAG) side chains attached: these highly charged sugar chains are often inhibitory toward axon growth (for review see Fawcett and Asher, 1999), although some CS-PGs can exert their inhibition via the protein core (for example, versican). Thus, treatments which prevent either the sulphation of GAG chains (e.g. chlorate), or the attachment of GAG chains to the protein core (e.g. β-D-xylosides) (Smith-Thomas et al., 1994, 1995), or which digest away the GAGs (e.g. chondroitinase-ABC; Snow et al., 1990; McKeon et al., 1995) can enhance axon growth on, or through, CNS glia in vitro. In vivo, it has been shown that sensory neurons microinjected into adult white matter tracts, thereby minimising glial scar production, are capable of substantial growth until they reach an injury site with increased CS-PG expression (Davies et al., 1997, 1999). In addition, it has been demonstrated more recently that injections of chondroitinase-ABC into the site of a lesion of the medial forebrain bundle (MBF), or the dorsal column of the spinal cord, allow robust regeneration of the lesioned axons (Moon et al., 2001; Bradbury et al., 2002).

Since a proportion of the cells which produce inhibitory CS-PGs have been reported to undergo cell division following CNS injury (i.e. OPs (Levine 1994; Levine and Reynolds, 1999; McGlade et al., 2001) and some astrocytes (Janeczko, 1989, 1993; Amat et al., 1996; Eclancher et al., 1996)), a possible approach to reducing inhibition of axon regeneration in the damaged CNS would be to kill dividing cells. However, other studies have shown that the increase in numbers of GFAP astrocytes surrounding a CNS lesion is not due largely to proliferation of these cells (e.g. Miyake et al., 1988; Mitchell et al., 1993; Di Prospero et al., 1998); thus the question of whether their increased numbers in the glial scar occur as a result of astrocitary proliferation, migration or up-regulation of GFAP in previously GFAP-negative cells, has been much debated in the literature (for review see Eddleston and Mucke, 1993). In this study, an attempt was made to diminish glial scar formation, and improve axonal regeneration after a CNS lesion, by reducing the number of rapidly dividing reactive glia. The antimitotic, cytokine-D-arabinofuranoside (araC), was delivered to rat brains for 7 days either prior to, or immediately following, a unilateral transection of the MFB (including the nigrostriatal tract); effects on lesioned axons and glial cells were examined at various time points after the lesion using immunohistochemistry.

**EXPERIMENTAL PROCEDURES**

**Surgical procedures**

General procedures and animal care. All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act (1986); all efforts were made to minimise the number of animals used, and their suffering. Adult male Sprague–Dawley rats (200–260 g; n=76) were anaesthetized for 5 min with 5% halothane and 2 L/min O₂ prior to surgery. Each animal was positioned into a stereotactic frame where anaesthesia was maintained at 1–2% halothane during surgery (with 0.6 L/min O₂ and N₂O); the incisor bar was set 2.3 mm below the interaural line. A midline incision was made, the skin retracted and the peristium cleared to expose the bregma; surgical procedures were carried out accordingly (as below). Wounds were sutured (Vicryl 4/0 absorbable sutures); animals received an injection of 4% glucose–0.18% saline (Aquapharm, UK) sc (5 ml per flank) and were kept in a heated recovery box until fully conscious. Paracetamol (1 mg/ml) was administered in the drinking water for 3 days after surgery; soggy chow was supplied.

**MBF lesion.** The MFB/nigrostriatal tract was unilaterally transected (through the right hemisphere) using a steel wire re-tractable blade Scuten Knife (David Kopf Instruments, Harvard Apparatus, UK; see Fig. 1A). A dental drill was used to remove a small piece of skull at −3.0 mm anterior (A) and −3.0 mm lateral (L) relative to the bregma. The dura was referenced, gently pierced and the Scutten knife lowered through the hole to −8.0 mm ventral (V) below the dura. The Scutten knife blade was extruded 3 mm and the knife raised +3 mm (V), the blade was closed then extruded again 3 mm, the knife lowered −3 mm (V) and the blade closed before removing the apparatus from the brain.

**Insertion of s.c. minipumps.** Alzet osmotic minipumps (Alza Corporation) containing 100 µl of either 2% araC (Sigma) in 0.9% sterile saline (Aquapharm), or sterile saline only, were preincu-

bated overnight in sterile saline at 37 °C for flow to begin. For pre-lesion infused animals (n=32), the lambda was referenced and a hole was drilled −1.0 mm (A) relative to the lambda. Three further holes were drilled into the skull surrounding the first hole, and steel screws (Semat Technical UK) were inserted into these for increased stability. A minipump was placed subcutaneously along the animal’s back; the pump was attached to a 2 mm Alzet infusion cannula that was placed into the hole behind the lambda so the cannula tip was situated between the dura and the surface of the brain. Infusion was expected to begin immediately and continue for 7 day at 0.5 µl/h. The cannula and screws were cemented into place (Simplex Rapid powder and Simplex Rapid solvent, Claudish Ash, UK). For post-lesion infusions (n=32), minipumps containing 100 µl either araC or saline were inserted subcutaneously as previously, but each pump was attached to a 5 mm infusion cannula that was placed into the same hole as was used for the MFB transection (~3.0 mm A and −3.0 mm L relative to the bregma), immediately following the lesion (see Fig. 1A). The cannula was cemented into place with 3 surrounding screws as previously.

**Tissue preparation**

Animals were killed with pentobarbitone sodium (Euthatal; Rhone Merieux) at 2, 4, 7, 12 or 18 dpi (n=3 or 4 per group per time point:

<table>
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<th>Abbreviations used in the figures</th>
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<td>LO lesion only</td>
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<td>n nigra</td>
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<td>Sal saline</td>
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<td>t tissue</td>
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Fig. 1. (Caption overleaf).
Each animal was perfused through the heart with 300 ml cold phosphate-buffered saline prewash (pH 7.4) followed by 300 ml cold 4% paraformaldehyde (pH 7.4). The brain was removed and postfixed for 24 h (4 °C) then transferred to 30% sucrose (4 °C) until cryoprotected. The right hemisphere of each brain was sliced into 10 series of 40 μm sagittal sections using a freezing sledge microtome, and stored at 4 °C in Trizma (Sigma)-buffered saline (TBS) with 0.05% sodium azide.

**Immunohistochemistry**

Each antibody was used to stain sections (free-floating), taken from all brains from each group, simultaneously. Sections were quenched (10% methanol and 3% H2O2 in distilled water) for 5 min, washed with TBS (pH 7.4) then blocked in 3% normal goat serum (NGS; Dako) with TBS containing 0.2% Triton X-100 (Sigma; TXTBS) for 1 h, room temperature (RT). Sections were immediately incubated overnight with the primary antibody in TXTBS with 1% NGS, RT. Antibodies to the following molecules were used: tyrosine hydroxylase (TH; specific to MFB neurons; rabbit polyclonal, 1:4000; Jacques Boy Institute); growth-associated protein (GAP)-43 (specific to growing neurons; rabbit polyclonal, 1:500; gift from G. Wilkin, Imperial College, London); NG2 (marker of OP cells; mouse monoclonal immunoglobulin G, 1:2; from D31-10 cell line (gift from J. Levine, SUNY); GFAP (specific to astrocytes; rabbit polyclonal, 1:25,000; Dako); CD11b (marker of microglia and macrophages; clone OX42, mouse monoclonal, 1:400; Serotec); and thrombospondin (TSP; mouse monoclonal, 1:200; Neomarkers TSP ab-1). After washing in TBS, sections were incubated in secondary antibodies for 3 h (RT) in TXTBS with 1% NGS. Secondary antibodies were biotinylated anti-mouse IgG (1:200, rat-adsorbed; Vector), biotinylated rabbit immunoglobulins (1:200; Dako) or FITC-conjugated goat anti-mouse (1:200; Jackson ImmunoResearch Laboratories). After washing in TBS, sections were incubated in a streptavidin-biotinylated horse-radish peroxidase complex (Dako ABC Kit) for 2 h (RT), washed in TBS then in Trizma-non saline (TNS) prior to incubating in the chromagen diamino benzadine (0.5 mg/ml; BDH) in TNS with 0.3 μl/ml 30% H2O2 for visualisation. Sections were washed in TNS, mounted onto 1% gelatine-coated slides, air-dried overnight (RT), dehydrated through alcohols and cleared with xylene before coverslipping with DPX (BDH). For fluorescence, sections were incubated in Cy3-streptavidin (1:5000; Amersham) for 2 h (RT) instead of Dako ABC kit, washed in TNS, mounted onto slides and coverslipped immediately with DABCO (Fluka) mounting medium.

### RESULTS

**Cell and axon counts**

Numbers of TH axons were counted from three consecutive sections from each brain at 100, 500 and (for technical reasons) at either 1500 (GAP43/TH double-stains), 2000 (all pre-lesion infused brains) or 2500 (all post-lesion infused brains) μm from the distal edge of the lesion, using a light microscope at 400× magnification. Counts included all intact axons crossing an imaginary line taken across a 1 mm dorsal-ventral field at each distance examined. Statistical tests (two-way ANOVAs) were performed using SigmaStat for Windows, version 2.03 (Jandel).

The numbers of NG2, GFAP, CD11b, and TSP cells were counted using a graticule under light microscopy at 200× magnification, in each alternate 0.1×0.1 mm box on both the distal and proximal sides of the lesion, giving cell densities at increasing distances from the lesion up to a distance of 2.5 mm at 1.0 mm dorsal to the base of the lesion. Objects were classed as a cell on the basis of immunolabelled cell bodies and processes, as appropriate. Counts were taken from three consecutive sections (400 μm apart) from each brain.

**Cell and axon counts**

Table 1. Experimental design for each of five different treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Infusion and lesion</th>
<th>Killed (dpl): (n) per time point</th>
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<tbody>
<tr>
<td>Pre-lesion saline</td>
<td>Saline infused onto brain surface 6 days prior to lesion of R MFB</td>
<td>2, 4, 7, 12 n=4</td>
</tr>
<tr>
<td>Pre-lesion araC</td>
<td>araC infused onto brain surface 6 days prior to lesion of R MFB</td>
<td>2, 4, 7, 12 n=4</td>
</tr>
<tr>
<td>Lesion only</td>
<td>No infusion, lesion only to R MFB</td>
<td>4, 7, 12, 18 n=3</td>
</tr>
<tr>
<td>Post-lesion saline</td>
<td>Saline infused over lesion site immediately after lesion to R MFB</td>
<td>4, 7, 12, 18 n=4</td>
</tr>
<tr>
<td>Post-lesion araC</td>
<td>araC infused over lesion site immediately after lesion to R MFB</td>
<td>4, 7, 12, 18 n=4</td>
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R, right MFB (including nigrostriatal tract).

**Fig. 1.** (B–H): TH-labelled sections. (A) Schematic of MFB lesion and pump insertion for post-lesion infused animals (N=nigra; str=striatum; ×000=lesion site; ‘inf’ and arrow indicate entry of infusion); not to scale. (B) Lesion (l) with proximal axons and nigra; str; distal edge of the lesion, using a light microscope at 400× magnification. Counts included all intact axons crossing an imaginary line taken across a 1 mm dorsal-ventral field at each distance examined. Statistical tests (two-way ANOVAs) were performed using SigmaStat for Windows, version 2.03 (Jandel).

The numbers of NG2, GFAP, CD11b, and TSP cells were counted using a graticule under light microscopy at 200× magnification, in each alternate 0.1×0.1 mm box on both the distal and proximal sides of the lesion, giving cell densities at increasing distances from the lesion up to a distance of 2.5 mm at 1.0 mm dorsal to the base of the lesion. Objects were classed as a cell on the basis of immunolabelled cell bodies and processes, as appropriate. Counts were taken from three consecutive sections (400 μm apart) from each brain.

**RESULTS**

Groups of animals were examined after five different treatments (see Table 1):

1. **pre-lesion saline**: animals received a continuous subarachnoid infusion of saline for 7 days, prior to transection of the MFB/nigrostriatal tract;
2. **pre-lesion araC**: animals received a continuous subarachnoid infusion of araC for 7 days (after Doetsch et al., 1989), prior to MFB transection;
3. **lesion only**: MFB transection without infusion;
4. **post-lesion saline**: MFB transection immediately followed by infusion of saline for 7 days over the lesion site;
5. **post-lesion araC**: MFB transection immediately followed by infusion of araC for 7 days over the lesion site.

Animals were killed at various time points post-lesion and brains were processed using immunohistochemistry to determine the effects of the treatments on glial cells participating in the glial scar formation, and lesioned axons of the nigrostriatal tract.
Transection of the nigrostriatal tract

Neurons and axons of the nigrostriatal tract were identified using antibodies recognising tyrosine hydroxylase (TH): bundles of TH axons running from the substantia nigra to the striatum were clearly seen in unlesioned animals (data not shown). In lesioned animals, the fibre tract was transected approximately 600 μm rostral to the nigra; large numbers of TH axons could be seen coursing from the substantia nigra to the proximal edge of the lesion (Fig. 1B).

Effects on TH axons

In non-infused ‘lesion only’ animals, the majority of TH material distal to the lesion had disappeared by 4 dpl; that which remained was in the form of punctuate staining that presumably represented axonal debris; continuous axons were not seen (Fig. 1C). Similarly few axons were seen distal from the lesion in both pre-lesion araC and pre-lesion saline groups (data not shown).

In animals infused post-lesion with araC, and to a much lesser extent in those infused with saline, a small number of fine, TH axons was seen distal to the lesion (Fig. 1D, E). These axons were thinner than unlesioned axons; they tended to follow a more tortuous path and were not restricted to the fascicles that contain the axons of the unlesioned tract. In addition, sprouted processes could be seen around the borders of the lesion (Fig. 1F), and some axons could be followed through the lesion and on into the brain (Fig. 1G, H). These axons were not seen in non-infused lesioned animals, and because of their features described above and their immunoreactivity for GAP43 (see later) they are believed to represent regenerating axons.

The number of TH axons at three distances distal to the lesion was quantified in each animal (see Fig. 2). Axon numbers in pre-lesion araC-infused animals were similar to those in pre-lesion saline- and non-infused animals; araC-treatment prior to the lesion did not affect axonal growth (not shown).

As shown in Fig. 2, there were considerably greater numbers of TH axons distal to the lesion in post-lesion araC-infused brains compared with saline-infused controls, at all distances examined at 4 dpl. At later time points, there were similarly more TH axons distal to the lesion in araC-treated brains, at all three distances, compared with saline- and non-infused controls, but with each time point overall numbers of axons reduced considerably, by 18 dpl very few axons remained distal to the lesion.

Two-way ANOVAs were performed on these data for each distance examined to determine whether there were significant differences in axon numbers as a result of treatment. Numbers of axons in araC-treated animals were significantly greater (P<0.001) when compared with saline- or non-infused animals, at most time points and distances examined (see Fig. 2). In some cases numbers of axons in saline-infused animals were significantly higher than those in non-infused animals (Fig. 2): this implies that...
infusion of saline alone over the injury site provokes some local sprouting and growth of axons.

It is noticeable that all graphs of axon counts demonstrate a higher number of axons further from the lesion: this was due to the path taken by the majority of regenerating axons. There was much sprouting around the edges of the lesion and many of these axons converged within the tract as they grew on toward the striatum; these axons would have escaped the central 1 mm area used for counts at 100 μm distal from the lesion but are included within counts at further distances from the lesion.

TH and GAP43 double-staining

Double-staining of sections from all post-lesion infused brains was carried out to determine whether TH axons distal to the lesion would also demonstrate expression of GAP43, a marker previously shown to be expressed by regenerating axons (Aigner and Caroni, 1995; Aigner et al., 1995; Frey et al., 2000).

At 4 and 7 dpl in both araC- and saline-infused brains, axons expressing TH and GAP43 simultaneously could be seen on the distal as well as proximal side of the lesion (Fig. 3; proximal side not shown). Small numbers of double-stained axons were also seen at 12 dpl but none were found at 18 dpl. The number of TH axons expressing GAP43 was counted in all brains at 100, 500 and 1500 μm distal from the lesion and average numbers for each group were calculated (Fig. 4). Two-way ANOVAs were performed on these data for each distance examined, to determine whether there were differences in the number of double-stained axons as a result of treatment, after allowing for effects of time. There were significantly more double-stained axons in araC-infused animals when compared with saline-infused animals at 7 dpl (P<0.05, see Fig. 4). It is concluded from these data that the post-lesion araC-infused environment was more conducive for axonal growth than the post-lesion saline-infused environment.

Effects on NG2 OP cells

The effects of araC infusions on the NG2 OP cell’s injury response were examined and compared with those seen in saline- and non-infused control animals. Staining of sections with an antibody to NG2 demonstrated a uniform distribution of lightly stained OP cells throughout the brain. NG2-immunoreactivity was not observed on cells with the morphology of macrophages or other glia; in some cases small blood vessels were NG2; these were not included in the quantitative analyses.

In all non-infused and saline-infused animals there was a greatly increased number of reactive, hypertrophied, more strongly NG2-immunoreactive OPs around the lesion from 2 and 4 dpl; this was not seen in post-lesion araC-infused brains (Fig. 5). This increase in numbers of reactive cells in both saline and non-infused control animals
was seen to extend for approximately 1–2 mm on either side of the lesion, with the greatest number of cells at the borders of the lesion: numbers slowly reduced to ‘baseline’ (i.e. levels of non-reactive NG2 cells present in normal brain) with increasing distance on both proximal and distal sides of the lesion (Fig. 6). The number of cells around the lesion reached a peak at 7 days and began to reduce at 18 dpl.

NG2 cell numbers in brains infused with araC pre-lesion were only different at 2 dpl, where cell counts extending 0.5 mm on either side of the lesion were significantly reduced ($0.05 > P > 0.001$ when compared with pre-lesion saline using two-tailed $t$-test); data not shown. These results suggest that antimitotic treatment prior to the lesion was only able to affect the recruitment of OP cells at 2 dpl.

In post-lesion araC-infused brains very few NG2 cells could be seen around the lesion at 4 and 7 dpl: recruitment of these cells was almost completely prevented (Fig. 6), but numbers of NG2 cells increased after the araC infusion ended.

These results show that 2% araC delivered over the lesion site for 7 days immediately following transection of the nigrostriatal tract almost completely inhibited the accumulation of NG2 cells around the lesion site during the period of infusion, but after the infusion ceased cell numbers increased, leading to an increased cell count at 12 dpl. In addition, infusion of saline over the lesion site did not significantly affect the number of NG2 cells that accumulated around the lesion, at any time point.

Effects on CD11b cells

The injury response of microglia was examined using the OX42 antibody to the CD11b marker (complement receptor 3). From 4 dpl a large number of CD11b cells could be seen within the lesion walls in all treatment groups: the majority of these cells had the appearance of large, round, blood-borne monocytes/macrophages. Smaller, process-bearing microglia were seen evenly distributed throughout the brain, and from 7 dpl in saline- and non-infused brains their numbers were greatly increased, their staining more intense and their morphology hypertrophied within 2–3 mm of the lesion (see Fig. 7). Similarly reactive microglia were seen in the nigra, the striatum and along the knife track (data not shown).

Cell counts were made of all CD11b cells (Fig. 8). No differences were seen in CD11b cell numbers in pre-lesion saline animals compared with pre-lesion araC animals; data not shown.

Differences were seen however when post-lesion araC-infused animals were compared with post-lesion saline groups. At 4 dpl there were similar numbers of CD11b cells in all non-infused and post-lesion infused animals. CD11b cells at the lesion borders (forming the peak of the graphs) mainly had the morphology of blood-derived monocytes; some reactive microglia were seen further from the lesion. At 7 dpl numbers of CD11b cells were increased considerably in control animals as many more cells with the morphology of reactive microglia were seen...
around the lesion (Fig. 7). AraC-infused animals did not demonstrate this increase in numbers of reactive microglia (see Fig. 7) until 12 dpl (after the end of the infusion); at 18 dpl, numbers of CD11b cells in control animals increased further (Fig. 8, araC-treated animals showed little change from 12 dpl).

These results suggest that post-lesion infusion of araC for 7 dpl significantly reduced accumulation of CD11b cells around the lesion.

### Effects on TSP expression

Levels of the ECM protein, TSP, were assessed in brains which received post-lesion infusions of araC or saline. It is thought that TSP is produced by reactive microglia and/or macrophages; TSP cells in this study had the appearance and distribution of blood-borne monocytes (Fig. 9A), although at later time points many had developed processes (Fig. 9B).

Counts of TSP cells were carried out on sections from all infused animals, and averages calculated for each treatment group at each time point as previously (Fig. 10). A small number of TSP cells could be seen in both araC and saline-infused animals from 4 dpl, close to the lesion borders; the numbers remained similar over time. The distribution of these cells and apparent lack of effect of the antimitotic suggests that TSP cells seen in this study were blood-derived monocytes/macrophages.

### Effects on GFAP cells

Brain sections were examined for GFAP astrocytes to determine whether post-lesion infusions of araC or saline affected the injury response of these cells when compared with non-infused controls. (Pre-lesion infused animals were not included.)

Staining with an antibody to GFAP demonstrated a substantial increase in GFAP staining around the lesion from 4 dpl; the up-regulation appeared to extend up to 3 mm on either side of the lesion (Fig. 11A). Reactive astrocytes were distinguishable from non-reactive cells by their greater staining intensity and enlarged cell bodies and processes (Fig. 11B).

Cell counts were made of all GFAP cells around the lesion in post-lesion infused and non-infused brains (Fig. 12). In all three groups at 4 dpl, the numbers of cells were approximately doubled close to the lesion but only increased around the lesion borders in non-infused animals (Figs. 11 and 12). At 7 dpl the numbers of GFAP cells increased in all three groups; GFAP cells were also seen close to the lesion borders in both post-lesion araC- and saline-infused brains (Figs. 11 and 12). At 18 dpl cell numbers in both groups showed a slight reduction (data not shown). It was also noted that in general there appeared to be greater numbers of GFAP cells along the path of degenerating axons on the distal side of the lesion, and

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**Fig. 6.** Quantification of NG2<sup>+</sup> cells around the lesion in post-lesion infused brains. Graph showing the average numbers of NG2<sup>+</sup> cells counted at increasing distances from the lesion in post-lesion saline- and post-lesion araC-infused brains at 4, 7, 12 and 18 dpl. Numbers in lesion only brains were similar to saline (not shown); S.E.s are shown.

**Fig. 5.** NG2<sup>+</sup> staining in post-lesion infused and lesion only brains. (A, B) Reactive NG2<sup>+</sup> cells (e.g. arrow in B) characterised by their hypertrophied cell bodies and processes compared with NG2<sup>+</sup> cells in normal brain (A, e.g. arrowheads; 600× magnification), scale bars=20 μm. (C–H) Up-regulation of NG2 around the lesion site at 4 dpl (C, non-infused) and 7 dpl (E, post-lesion saline) is not seen in post-lesion araC<sup>-</sup> (H) though not in post-lesion saline-infused brain (G). Magnification 40×, scale bars=200 μm.
Fig. 7.
the overall increase in cell numbers was only approximately two-fold, as opposed to the three- or four-fold increase demonstrated by NG2 and CD11b cells.

These results show that infusion of araC immediately after a lesion of the nigrostriatal tract did not significantly affect the injury response of GFAP cells when compared with that seen in saline- or non-infused animals. This implies that the majority of up-regulated GFAP expression around the lesion does not occur by cell division, or in response to signals released from dividing cells.

**DISCUSSION**

**Injury response of OP cells**

As in previous studies (Levine, 1994; Ong and Levine, 1999; Bu et al., 2001; McTigue et al., 2001; Jones et al., 2002; Tang et al., 2003) we have shown a substantial increase in the number of NG2 OP cells around CNS lesions, and a large increase in the intensity of NG2 staining on those cells. Expression of NG2 on cells with the morphology of inflammatory cells or astrocytes was not observed in this study (see also below).

Our first experiment was to infuse the antimitotic, araC, over the surface of the brain for 7 days prior to a CNS injury. This resulted in a reduction of the number of NG2 cells accumulating very locally around the lesion at 2 dpl, although the number was the same as those seen in vehicle-infused controls from 4 days onwards. This dose and mode of araC administration had been previously shown to be effective in killing periventricular stem cells (Doetsch et al., 1999); either the penetration of the araC or the rate of cell division in the OP population in the unlesioned brain is not, therefore, sufficiently high that the majority of cells divide during a 7 day period, although these cells can be killed by araC - in keeping with Levine et al. (1993) who demonstrated a constant turnover of these cells in the adult rat CNS. Moreover, these findings show that cells remaining after 7 days of subarachnoid araC infusion are fully capable of division in response to injury. The lack of effect on other cell types is unsurprising given that little turnover of other glia might be expected in the mature and uninjured rat brain.

We next infused araC from the time of the injury for 7 days. This substantially reduced the number of NG2

**Fig. 8.** Quantification of CD11b<sup>+</sup> cells around the lesion in post-lesion infused brains. Graph showing the average numbers of cells counted at increasing distances from the lesion in post-lesion saline- and post-lesion araC-infused brains at 4, 7, 12 and 18 dpl. Cell numbers in lesion-only brains were similar to post-lesion saline, (not shown); S.E.s are shown.
OP cells which accumulated around the lesion, implying that OP cells undergo very rapid cell division within 2 mm of a lesion, and that their recruitment is by cell division rather than migration. Whilst no cell division marker was employed in this study, these findings are in keeping with previous studies where (H)thymidine (Levine, 1994) or bromodeoxyuridine (Levine and Reynolds, 1999; McTigue et al., 2001) have confirmed that this increase in cell number is due, at least in part, to proliferation.

However, OP cells started to accumulate around the lesion after the antimitotic infusion ceased. The cells were therefore still receiving sufficient stimulus to divide in response to the injury even 12 days after the lesion.

### Macrophages and microglia

Resident CNS microglia are known to react rapidly to brain injuries by cell division. In addition, blood-borne macrophages/monocytes enter CNS lesions that damage blood vessels. The cells produce various cytokines and chemokines (for review see Raivich et al., 1999) which have effects on local glia and neurons.

In our experiments, araC had little effect on recruitment of these cells up to 4 days after injury, although almost all these cells had the appearance of blood-borne monocytes, and were seen very close to the lesion. At 7 dpl a marked up-regulation of reactive CD11b cells with the classic appearance of process-bearing microglia was seen around

![Image](image_url)

**Fig. 9.** TSP⁺ cells in post-lesion saline-infused brains. Small numbers of TSP⁺ cells were seen at the lesion borders with the morphology of monocytes/macrophages (e.g. arrowheads in A) at 4 dpl; at later time points these cells were process-bearing (e.g. arrows in B, 7 dpl). Scale bars= 50 μm.

**Fig. 10.** Quantification of TSP⁺ cells around the lesion in post-lesion infused brains. Graph showing the average numbers of cells counted at increasing distances from the lesion in post-lesion saline- and post-lesion araC-infused brains at 4, 7, 12 and 18 dpl; S.E.s are shown.
the lesion in control animals, but not in araC-infused brains. This suggests that most of the recruitment of microglia to injuries is by cell division, rather than migration; recruitment of blood-borne monocytes occurs via a separate mechanism and these cells did not appear to be affected by araC treatment. It is interesting to note that, as with the NG2 cells, numbers of microglia around the lesion site still increased after the antimitotic infusion ceased, implying that injury-related cytokines and chemokines continue to be released for more than 7 days after the injury.

Expression of TSP was up-regulated after the lesion: this protein is thought to be produced by reactive microglia and macrophages (Chamak et al., 1995; Möller et al., 1996; Rai-vich et al., 1998). Levels of expression were similar in all treatment groups, suggesting that the protein is expressed by non-dividing cells at the lesion site. It is striking that the number of TSP-expressing cells was only a fraction of the number of CD11b cells seen to be up-regulated around the lesion, and all of them had the morphology and distribution of blood-derived monocytes/macrophages.

Previous studies have demonstrated through double-labelling that neither GFAP nor CD11b cells coexpress NG2 (e.g. Levine et al., 1993; Levine, 1994; Nishiyama et al., 1997; Reynolds and Hardy, 1997), although recent publications have demonstrated expression of NG2 by macrophage-like cells (Bu et al., 2001; McTigue et al., 2001; Jones et al., 2002; Tang et al., 2003). Such inflammatory cell expression of NG2 was not observed in this study; the discrepancy may be explained by the fact that other studies (e.g. Bu et al., 2001; McTigue et al., 2001; Tang et al., 2003) used polyclonal antibodies to NG2 whilst a monoclonal antibody was used here.

**Astrocytes**

In this study, counts of GFAP cells demonstrated that by 4 dpl an increase in number of these cells could be seen around the lesion in all three treatment groups examined. The distributions obtained by cell counts demonstrated a different injury response of these cells, compared with that of the NG2 OP cells. GFAP cell numbers did not increase by much more than two- or three-fold overall, and the increase was much more widespread. These results suggest that the increase in number of GFAP cells around the lesion site in this model was due mainly to up-regulation of GFAP in astrocytes that normally express little; thus, in keeping with previous studies demonstrating little co-expression of reactive astrocytes with markers of cell division (e.g. Miyake et al., 1988; Mitchell et al., 1993; Di Prospero et al., 1998), infusion of the antimitotic did not appear to influence their injury response. The presence of astrocytes probably contributed to the limited ability of axons to re-
generate in this environment: Moon et al. (2001) demonstrated more robust axon regeneration following enzymatic removal of all CS-PGs from the lesioned nigrostriatal tract.

The non-infused animals showed a greater up-regulation of GFAP cells than infused animals, particularly close to the lesion borders, suggesting that the region of scar tissue surrounding the lesion is slightly modified by the infusion (consistent with previous findings from our laboratory; Moon et al., 2002).

**Axon regeneration and retraction**

Previous studies have demonstrated that the CS-PGs produced by OPs—NG2, versican and phosphacan—are inhibitory toward axonal growth (Dou and Levine, 1994; Asher et al., 1999; Fidler et al., 1999; McKeon et al., 1999; Chen et al., 2002; Ughrin et al., 2003): in keeping with these findings, this study showed a greater number of regenerating axons in an environment depleted of NG2 OP cells.

The lesion model employed resulted in very rapid degeneration of lesioned axons, so that by 4 dpl essentially all the TH axons distal to the lesion had degenerated in non-infused control animals. Any remaining axons were therefore clearly distinguished and quantifiable. Significantly more axons were seen distal to the lesion in araC-infused animals compared with saline- and non-infused controls at all time points and distances examined. The morphology of these axons (fine, tortuous and not bundled into fascicles) and their GAP43 immunoreactivity imply that these were regenerating axons. Additionally, saline-infused animals had significantly greater numbers of sprouts around the edge of the lesion at 4 dpl only, when compared with non-infused controls: this is thought to be due to the infusion-induced modifications to the environment, as discussed above.

The regenerated axons proved to be transient: almost all had retracted in araC-infused brains by 18 dpl. The retraction of regenerated axons after the first 7 dpl coincided with increased numbers of OP cells and astrocytes around the lesion, and their associated axon-inhibitory CS-PGs. A temporal correlation between lack of increase in regeneration and reappearance of reactive OP cells and astrocytes has been shown previously (Moon et al., 2000), where injection of ethidium bromide solution between the site of a knife cut lesion of the nigrostriatal tract and the ipsilateral striatum created a completely glial-free region allowing robust axon regeneration up to the posterior border of the striatum. The glial cells reappeared from around 7 dpl and axon regeneration ceased; however the regeneration that had occurred was more robust than that of this study, perhaps due to the more extensive glial ablation. Axon retraction might also have occurred in this study as a result of the continuing axotomy-induced cell death of TH neurons in the substantia nigra which has been previously documented (Brecknell et al., 1995). Additionally, recent evidence has suggested a toxic effect of araC on neurons (Dessi et al., 1995; Sanz-Rodriguez et al., 1997).

**Fig. 12.** Quantification of GFAP + cells in post-lesion-infused and non-infused brains. Graph showing the average numbers of cells counted at increasing distances from the lesion in post-lesion saline- and post-lesion araC-infused brains at 4, 7 and 12 dpl. Data from non-infused lesion only are only shown for 4 dpl since numbers are similar at later time points. S.E.s are shown.
CONCLUSIONS
Infusion of the antimitotic, araC, over the lesion site for 7 days immediately following a unilateral lesion of the nigrostriatal tract significantly reduced the number of OP cells able to participate in the scar formation. The number of CD11b microglia around the lesion was also considerably reduced but GFAP astrocytes were not affected. The findings imply that the OP cell population was increased around the lesion by intensive local proliferation whilst the astrocyte population was mainly increased around the lesion by expression of GFAP in cells that normally express little, and possibly by cell migration. The effects of the antimitotic on microglia suggest that this population was recruited by local proliferation, but there was also recruitment of blood-borne monocytes. Axon regeneration was transiently enhanced in this environment until CS-PG expression increased.

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REFERENCES


