RESEARCH ARTICLE

Oligodendrocyte Progenitor Cells in the Adult Rat CNS Express Myelin Oligodendrocyte Glycoprotein (MOG)

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While the effects of high dose X-irradiation on mitotically active progenitor cells and remyelination are well-documented, its effects on myelinating oligodendrocytes are less clear, due in part to divergent views on their mitotic capacity. To examine the effect of X-irradiation on oligodendrocytes, the spinal cord of rats was exposed to 40 Gy of X-irradiation and the number of oligodendrocytes and oligodendrocyte progenitors in the dorsal funiculi at T12 and L1 was determined by in situ hybridization using cRNA-probes for platelet derived growth factor alpha receptor (PDGFRα) (to identify oligodendrocyte progenitors), exon 3b of proteolipid protein (PLP) (to identify mature oligodendrocytes) and myelin oligodendrocyte glycoprotein (MOG). X-irradiation resulted in no change in the number of PLP positive cells and no loss of myelin internodes, but caused an almost complete loss of PDGFRα-expressing cells, and a reduction in the number of MOG positive cells to a number similar to that found using the PLP exon 3b probe. Importantly, the number of radiation-sensitive MOG-expressing cells was similar to the number of PDGFRα positive cells. To determine if the radiation-sensitive MOG positive cells were the same population as the radiation sensitive PDGFRα-expressing cells, MOG and PDGFRα-expressing cells were isolated from the adult CNS using antibody coated magnetic beads. Twelve to thirteen percent of MOG positive cells were PDGFRα positive and nearly all the PDGFRα isolated cells were MOG and galactocerebroside positive. Double immunofluorescence revealed colocalization of NG2 and MOG on cells in the normal adult rat spinal cord. These results show that in situ in the adult rat spinal cord white matter oligodendrocyte progenitors are MOG positive and indicates that expression of MOG cannot be regarded a marker that only identifies mature myelin-supporting oligodendrocytes in tissue.

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Introduction

Oligodendrocytes are the myelin-forming cells in the CNS. During development, and in vitro, these cells are generated from precursors via a number of stages that can be identified using either antibodies or probes to molecules that are expressed at different stages in the differentiation from precursor to fully differentiated oligodendrocytes. Thus, in the spinal cord, cells destined to give rise to oligodendrocytes are first identified in the ventral ventricular zone dorsal to the floor plate by the expression of platelet derived growth factor alpha receptor (PDGFRα), and as cells mature through the lineage they acquire sulphatide, galactocerebroside (GalC), Rip, cyclic nucleotide phosphodiesterase (CNPase), myelin proteolipid protein (PLP), myelin basic protein (MBP), and finally, myelin oligodendrocyte glycoprotein (MOG) (16, 28). PDGFRα expression is generally considered to cease with the onset of GalC expression (5, 28). A number of studies have used late expressed molecules such as Rip, CNPase, PLP, MBP, and MOG to identify mature myelin-bearing oligodendrocytes in tissue sections (3, 22, 24, 26, 27, 30, 34), and by documenting co-expression of indicators of cell division, such as cyclins, or nucleotide uptake, concluded that mature oligodendrocytes can divide to give rise to remyelinating oligodendrocytes. Using anti-leu-7 monoclonal antibodies to identify oligodendrocytes, it has also been inferred that mature oligodendrocytes are radiation sensitive and therefore capable of cell division (18, 19). In addition, in vitro studies using anti-GalC antibodies to isolate oligodendrocytes from the adult nervous system have suggested that cells bearing this molecule can undergo cell division and give rise to new oligodendrocytes (38). Such conclusions are controversial since in vitro studies using neonatal cells, and analysis of antigen expression during myelination have consistently indicated that cells that have reached the galactocerebroside-expressing stage of development do not divide, and therefore, can be considered post mitotic cells (16, 28). Moreover, a number of experimental in vivo studies investigating the origin of remyelinating oligodendrocytes have clearly implicated their origin

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from oligodendrocyte progenitors (OPCs) and have found no evidence for cell division by mature oligodendrocytes (6, 13).

Li and co-workers have shown that certain cells in the rat spinal cord (which they identified as oligodendrocytes) are extremely radiation sensitive, undergoing apoptosis within 24 hours following exposure to even low doses of X-irradiation (18, 19). We have shown that 40 Gy of X-irradiation of the rat spinal cord abolishes the ability of irradiated tissue to generate remyelinating cells (1). The same dose of X-irradiation abolishes the remyelinating capacity of glial cell cultures transplanted into areas of demyelination (8, 12) while allowing myelin forming oligodendrocytes to survive (8, 17). Since this dose of irradiation removes oligodendrocytes progenitors (dividing cells) and does not kill mature oligodendrocytes (post mitotic cells), it provides a tool that can be used to examine the phenotype of oligodendrocyte progenitors in the adult spinal cord.

The aim of this study was to resolve the controversy concerning the mitotic ability of mature oligodendrocytes by examining the effect of a dose of X-irradiation, known to completely abolish OPCs, on the number of oligodendrocytes in the dorsal funiculus of the rat spinal cord using 2 cRNA probes that have been used to identify mature myelin bearing oligodendrocytes, MOG and expression of exon 3b of PLP.

Materials and Methods

Animals, irradiation and sampling. Eight female inbred PVG rats (weight: 200 to 230 g) were used for the in situ hybridization experiments. Four rats were X-irradiated and 4 rats were not. In addition to examine if X-irradiation killed oligodendrocytes and thereby caused demyelination a large number of 1 μm thick toluidine blue stained resin sections were available from previous experiments in which the spinal cord had been exposed to 40 Gy of X-irradiation one or 2 months previously.

For X-irradiation, rats were anesthetized with an intramuscular injection of Hypnorm (Janssen Pharmaceuticals, UK) and placed in lateral recumbancy. Using radiographs to position lead shielding, a 4 cm length of the spinal cord centred on T13 was exposed to 40 Gy of X-irradiation using a Pantak 255-kV radiotherapy machine. The irradiated and control rats were killed after 2 weeks with an overdose of pentobarbitone sodium and perfused through the aorta with 4% paraformaldehyde in 0.1 M PBS. The spinal cord was removed taking care to mark the position of the T13 spinal nerve roots and post-fixed in the same fixative for 2 hours. By using the spinal nerve roots as reference points transverse tissue blocks were cut from the T12 and L1 segments of the spinal cord and placed in 30% sucrose in PBS overnight before being frozen using dry ice. 15 μm thick sections were cut using a cryostat and collected onto polylysine-coated slides in a manner such that each slide had 4 sections taken 60 mm apart. By adopting this approach slides were available in which different probes could be applied to 4 sets of sequential sections for each animal.

cRNA probe synthesis. Digoxigenin-labelled probes were used to detect PDGFRα, PLP-exon 3b and MOG mRNA. The PLP exon 3b probe was generated from a 81 bp *Sau* 96 I/*Bfa* I fragment from pBSK mouse PLP sub-cloned into pGEM 3z/ Sma I (Promega). The PDGFRα probe was generated from a 1.5 kb *Eco* RI/*Hind* III fragment encoding the rat PDGFRα sub-cloned into pGEM (Promega). The MOG probe was generated from a 780 kb *BamH*I/*Sa*l I fragment encoding mouse MOG cDNA sub-cloned into pGEM 4z (Promega). The plasmids were linearized at 37°C for 2.5 hours using endonuclease *Xba* I for producing a PLP anti-sense probe, *Eco* R1 for PLP sense probe, PDGFRα and MOG anti-sense probe, and *Hind* III for MOG sense probe and PDGFRα anti-sense probe. Following DNA extraction digoxigenin-labelled cRNA probes were synthesized from 1 μg of DNA using a DIG RNA labelling kit using T7 promoter to produce anti-sense probes for PLP, MOG and PDGFRα and SP6 promoter to produce sense probes. The size of the RNA anti-sense and sense probes to PLP, MOG, and PDGFRα was checked by agarose gel electrophoresis.

In situ hybridization. The frozen sections were thawed and air-dried in a clean fume hood for 1 hour. The PLP, MOG, and PDGFRα anti-sense probes were diluted 1:700 in hybridization buffer and denatured at 70°C for 10 minutes. The sections were placed in a chamber humidified with SSC (sodium chloride and sodium citrate, pH 7.0) and formamide and the probes applied to the sections which were then covered with sterile glass coverslips prior to hybridising overnight at 65°C. The slides were then washed 3 times for 30 minutes with washing buffer (SSC, 50% formamide, 0.1% Tween) at 65°C and twice for 30 minutes in MABT buffer (100 mM maleic acid, pH7.5, 150 mM NaCl, 0.1 Tween) at room temperature. The sections were blocked with blocking-solution (Roche) for 1 hour at room temperature prior to incubation with anti-DIG AP-conjugated antibody

(1:1500, Roche) in blocking solution overnight at 4°C in a humidified chamber. The slides were then washed in 1× MABT 5 times for 10 minutes and then in prestaining buffer (1M Tris-HCl, pH 9.5, 1M NaCl, 0.5M $MgCl_2$) twice for 10 minutes. The reaction products were visualized by incubating the slides in staining buffer containing NBT+BCIP (Roche) at 37°C for 3.5 hours. The slides were dehydrated and mounted in XAM.

Cell counting and statistics. The slides were coded and the absolute number of PLP, MOG, and PDGFRα probe labelled cells in the dorsal funiculi were counted using a ×40 objective. The criteria for cells to be counted were the presence of clear cytoplasmic labelling within which the outline of a nucleus could be detected. Also included in the counts were some large, darkly stained cells with few processes, in which a nuclear outline could not be resolved. Labelled cells located on the border of the white matter and grey matter were excluded. The labelled cells were counted on 4 sections each separated by 60 μm from each segment for the 4 X-irradiated and 4 normal rats.

For statistical purpose, software Prism 3.0 was used. The one-way ANOVA were used to compare multiple means between groups. Values in figures are given as the mean ± SEM. Differences with a P-value <0.05 were considered significant.

Isolation of cells from adult CNS tissue. Midbrain and cerebellum were dissected to exclude the entire forebrain from 2 adult (>6 months) female PVG rats in MEM-HEPES containing 20 mM N-acetyl cysteine and 215 units per ml catalase (isolation buffer). Meninges and surface blood vessels were carefully removed under a dissecting microscope before the tissue was chopped and then triturated by passage through a luer syringe tip in 5 ml isolation medium. Tissue was collected following centrifugation at 500 g for 5 minutes and resuspended in 30 ml isolation medium containing 0.25% porcine trypsin (Sigma) and 167 units per ml DNAse-1 (Roche) and then incubated for 40 minutes at 37°C with continuous gentle shaking. Digested tissue was recovered following centrifugation at 800 g for 5 minutes, the supernatant was removed and 500 μl soybean trypsin inhibitor (5 mg/ml, Sigma), 120 μl DNAse (20 000 units/ml) and 6 ml isolation buffer were added prior to tritutation through 19 g, 21 g, 23 g, and 25 g syringe needles. Dissociated material was then filtered through 40 μm and 20 μm mesh and OptiPrep (60%, w/v, NycoMed Pharm AS, Oslo, Norway) was added to give a final concentration of 9%; this suspension was divided between two 25 ml universal containers. Following centrifugation at 800 g for 20 minutes the myelin was removed and the cellular fractions collected and washed by re-suspension in 50 ml MEM-HEPES followed by at 500 g for 5 minutes. The sediment was re-suspended in 1 ml isolation buffer containing 0.1% BSA and 1×10^7 washed Cellection Dynabeads Pan Mouse IgG and incubated with slow rotation (Dynal MX1 Sample Mixer, Dynal [UK] Ltd.) at 4°C for 30 minutes. Dynabeads with attached cells were removed using a Dynal MPC-L magnetic particle concentrator (MPC) and the negative fraction collected and incubated with 1×10^7 Dynabeads with bound anti-MOG mAb (either 8-18C5 [20] or Y10 [2]) or PDGFRα polyclonal antibody 3980 to an external part of the receptor (15) at 4 °C for 30 minutes. Dynabeads with bound cells were collected using the MPC and washed by 3 cycles of resuspension and magnetic concentration in 1 ml isolation buffer containing 0.1% BSA. Cells were then detached from the beads by incubation at 37°C for 30 minutes in 200 μl isolation buffer containing 10 mM $MgSO_4$, 1 mM $CaCl_2$ and 200 units DNAse followed by 10 passes through a 1 ml pipettor tip, the beads were removed using the MPC and the cells recovered by centrifugation at 500 g for 5 minutes.

Immunocytochemistry. Isolated adult CNS cells were re-suspended in MEM-HEPES and 1 μl samples were plated onto slides coated with poly-D-lysine and air dried. Cell phenotypes were investigated using monoclonal antibody O1, which identifies galactocerebroside (36), anti-PDGFRα antibodies-polyclonal rabbit to the carboxy terminus (4), polyclonal rabbit to an external epitope (15) and anti-MOG monoclonal antibodies 8-18C5 (20) and Y10 (2) using appropriate secondary antibodies. Cell nuclei were visualized by staining with HO 33342 (10 micrograms per ml).

Cryostat sections from normal adult rat spinal cord, perfusion fixed with 4% paraformaldehyde and cryoprotected with 20% sucrose prior to rapid freezing, were exposed overnight at 4°C to rabbit polyclonal antibodies to NG2 proteoglycan (Chemicon, United States) 1:200 to identify oligodendrocyte progenitors (6, 9, 25) and the monoclonal 8-18C5 1:8000 to identify MOG. Secondary antibodies for NG2 were goat-anti rabbit Biotin 1:200 (Vector Labs, UK) visualized with Streptavidin FITC (Serotec, UK) 1:100 and for MOG goat-anti mouse CY3 (Jackson Immunochemicals, United States) 1:750. Primary antibodies were blocked with 10% normal goat serum. Sections were mounted in Vector shield (Vector Labs, UK) and examined by normal and confocal microscopy.

	T-12 Dorsal funiculi		L-1 Dorsal funiculi	
	Normal	Irradiated	Normal	Irradiated
PLP exon 3b	145.5 ± 1.5†	142.2 ± 1.4	166.3 ± 1.2†	164.1 ± 1.2
MOG	181.4 ± 2.4	142.8 ± 2.2*	206.4 ± 3.0	172.2 ± 2.2*
PDGFRα	28.9 + 0.8	0.0*	29.4 ± 0.7	0.1 ± 0.15*
% radiation sensitive MOG cells	21.3		16.6	
PDGFRα labelled cells as % of MOG cells	15.9		14.2	

Table 1. Number of PLP exon 3b, MOG and PDGFRα labelled cells in dorsal funiculi of the spinal cord at levels T-12 and L-1.
* significant difference (P<0.001) between number in irradiated and normal tissue.
† significant difference (P<0.001) between PLP and MOG labelled cells in normal tissue.

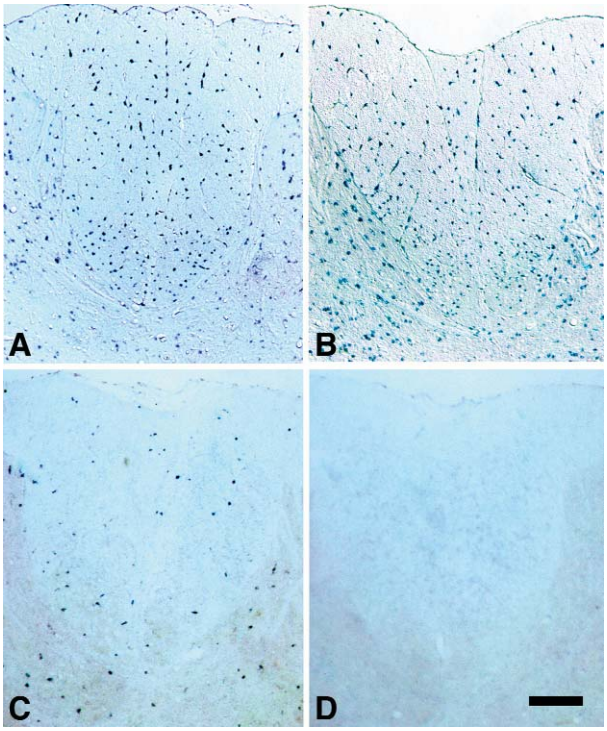

Figure 1. A. In situ hybridization using a probe to detect MOG mRNA in the normal dorsal funiculi of the rat spinal cord at T12 and **B** the appearance following exposure to 40 Gy of X-irradiation. **C.** In adjacent sections the distribution of cells labelled with a probe to PDGFRα mRNA in the normal tissue and **D** the complete absence of labelled cells following exposure to 40 Gy of X-irradiation. Bar = 200 μm

Results

All probes labelled cells in white and grey matter and with the exception of the PDGFRα probe the density of staining correlated with cell size. There was no labelling with any of the sense probes. The number of cells in the dorsal funiculi labelled with each probe in sections from T12 and L1 is presented in Table 1. When further sets of sections were reacted and counted there was no significant difference in the number of labelled cells between the two experiments.

In normal tissue there were significantly more MOG-labelled cells than PLP-labelled cells and following X-irradiation the number of MOG-labelled cells was significantly reduced (Figure 1A, B) to a number that was not significantly different from the number of PLP-labelled cells. X-irradiation had no effect on the number of PLP-labelled cells. X-irradiation resulted in almost complete loss of PDGFRα staining cells (Figure 1C, D).

These results indicate that all the PDGFRα positive cells and a proportion of the MOG positive cells are radiation sensitive, while the PLP exon 3b expressing cells are not. Significantly, the number of radiation resistant MOG positive cells was similar to the number of PLP exon 3b expressing cells. This suggests that the radiation-resistant MOG expressing cells are the same population of cells as those labelled by the exon 3b PLP probe and that the PDGFRα positive cells may be the same cells as the radiation sensitive MOG positive cells. To investigate this, we isolated cells from the adult CNS using anti-MOG or anti-PDGFRα -antibody coated magnetic beads. Immediately after isolation, all the MOG-isolated cells were GalC positive and 12 to 13% were clearly positive for PDGFRα (Figure 2A-C), whilst almost all of the PDGFRα-isolated cells were MOG and GalC positive (Figure 2D-G). These observations indicate that MOG and PDGFRα can be expressed by the same cell. Since MOG positive cells could also be PDGFRα positive, we compared the dif-

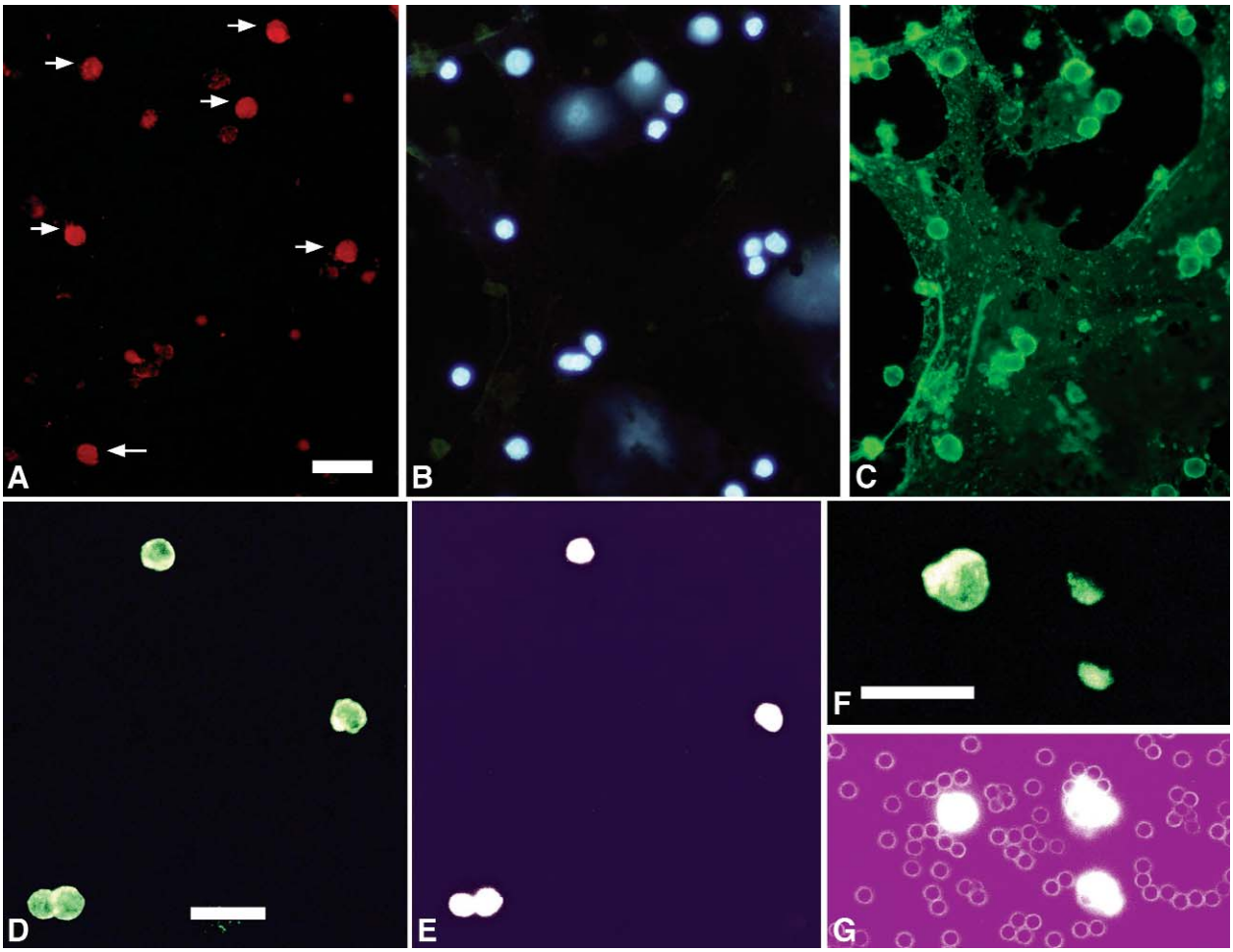

Figure 2. A-C. Freshly isolated MOG-antibody sorted cells stained with O1 monoclonal antibody (**C**), antibodies to PDGFRα (**A**) and HO33342 (**B**). Approximately 15 % of MOG-sorted cells co-express PDGFRα (**A**). There is some non-specific staining with this PDGFRα antibody. Weaker O1-staining also indicates some myelin membranous material that co-purifies with the cells (**C**). **D-E.** Freshly isolated PDGFRα-antibody sorted cells stained with anti-MOG monoclonal antibody Y10 (**D**) and HO33342 (**E**). Virtually all cells sorted from adult brain using DynaBeads coated with 3980 rabbit antibody to PDGFRα external domain are MOG-positive (**D**). **F-G.** Demonstration of co-expression of PDGFRα and MOG. MOG-positive cells in a preparation of freshly isolated PDGFRα-antibody sorted cells prior to bead detachment are surrounded by DynaBeads. These are visualized in (**G**) by HO33342 fluorescence and dark-field illumination to demonstrate cell nuclei and PDGFRα antibody coated DynaBeads and shows that the two cells on the right of the field in (**G**) are heavily coated by beads which has interfered with the MOG immuno-fluorescence signal over more than half of the underlying cells (**F**). **A-G.** Bar = 10 μm.

ference between the mean number of PLP and MOG labelled cells in normal tissue to the reduction in the number of MOG labelled cells caused by X-irradiation. At T12 there were 36 more MOG labelled cells than PLP labelled cells in normal tissue and irradiation depleted the mean number of MOG cells by 38; at L1 the numbers were 40 and 34 respectively. These values are very similar to each other, but slightly larger than the mean number of PDGFRα labelled cells, which suggests that although there may be a small number of MOG+ PDGFRα– radiation sensitive cells, most of the radiation sensitive cells in white matter could co-express PDGFRα and MOG.

Oligodendrocytes myelinate more than one axon so evidence of loss of oligodendrocytes would take the form of small groups of demyelinated axons. Careful examination of 40 toluidine blue stained sections from white matter that had been exposed to 40 Gy of X-irradiation 1 or 2 months previously revealed no evidence of demyelination in any white matter tract (Figure 3).

In order to examine whether oligodendrocyte progenitors expressed MOG in situ in the adult rat spinal

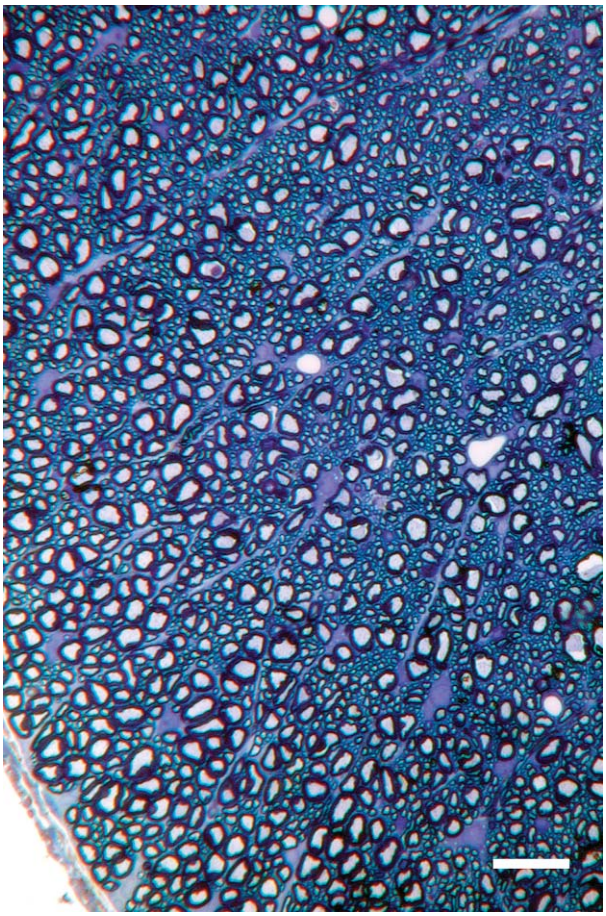

Figure 3. One micron toluidine blue stained section of white matter exposed to 40 Gy of X-irradiation 2 months previously. The tissue appears normal and there is no evidence of demyelination that would occur if oligodendrocytes were killed by X-irradiation. Bar = 80 μm.

cord, cryostat sections of normal adult rat spinal cord were exposed to antibodies to NG2 proteoglycan—a well accepted marker for oligodendrocyte progenitor cells (6, 9, 25)—and MOG. Confocal imaging revealed that the majority of NG2 positive cells were also MOG positive (Figure 4).

Discussion

Following 40 Gy of X-irradiation, the number of MOG labelled cells in the dorsal funiculi was reduced by 16.6 to 21.3 %, a figure not dissimilar to that found by Li and co-workers who reported reductions of leu-7 positive cells in white matter 24 hours after X-irradiation of 21% after 30 Gy and 28% after 20 Gy (19). In our study the number of radiation-resistant MOG labelled cells was not significantly different from the total number of PLP labelled cells in both X-irradiated and normal tissue. This indicates that some of the MOG labelled cells are radiation sensitive, while few if any of the cells labelled by our PLP probe are radiation sensitive. The PLP probe we used identifies mRNA that contains exon 3b of the PLP gene (10). By alternative splicing, the PLP gene codes for 2 molecules PLP and DM-20, these differ in that exon 3b is spliced out for DM-20 (23). The smaller form of PLP, DM-20, is expressed early in development, while the longer form of PLP is only expressed when oligodendrocytes start to make myelin associated proteins such as MBP (14). The presence of exon 3b can therefore be used to distinguish between cells early in the oligodendrocyte lineage that express the DM-20 form of PLP but not the exon 3b containing form of PLP, from differentiated oligodendrocytes that express both products of the PLP gene (10, 14). Our data indicate that mature oligodendrocytes are not radiation sensitive and therefore are non-dividing cells. Furthermore, we found no demyelinated axons in white matter examined 2 months after X-irradiation indicating that this treatment caused no degeneration of myelin-supporting oligodendrocytes. The radiation sensitive cells expressing MOG in our study and those detected with leu-7 in previous studies (18, 19) are not therefore myelin supporting oligodendrocytes. This would indicate that there is no turnover of myelin bearing oligodendrocytes in normal tissue.

Oligodendrocyte progenitor cells express PDGFRα and NG2 in the adult CNS (25) and most evidence indicates that these cells generate oligodendrocytes during development and following demyelination (9). Our current experiments show that in the adult rat PDGFRα and NG2 expressing cells can co-express MOG, and that in the dorsal funiculi the number of radiation-sensitive MOG expressing cells is very similar to the number of radiation-sensitive PDGFRα expressing cells, indicating that OPCs in situ in the adult CNS express a marker thought to be restricted to mature oligodendrocytes. This was an unexpected finding since MOG is generally considered to be one of the last oligodendrocyte-associated proteins to be expressed (7, 21, 31, 33, 35) and the consensus view is that PDGFRα expression is lost by cells of the oligodendrocyte lineage at around the time cells became committed to an oligodendrocyte fate, as indicated by the appearance of galactocerebroside expression (28). This view was based on investigations involving both in vitro culture of neonatal cells and observation on the sequence of expression of developmentally regulated oligodendrocyte expressed molecules, which clearly indicated that cells expressing markers associated with mature oligodendrocytes did

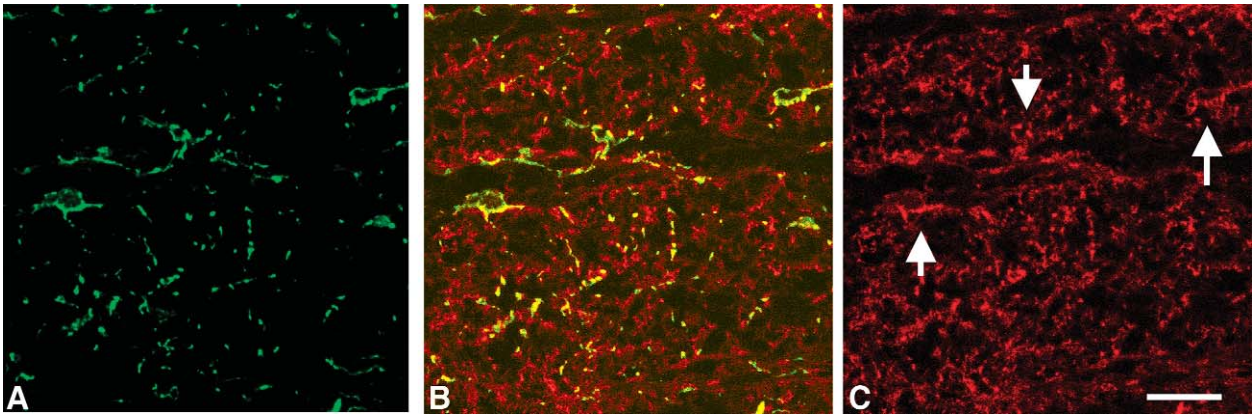

Figure 4. Confocal images from normal adult rat spinal cord white matter labelled with to anti-NG2 antibodies (green) and anti-MOG antibodies (red). In (**A**) NG2 labelling of oligodendrocyte progenitors is readily apparent while identification of individual MOG labelled cells (arrows) (**C**) is more difficult due to labelling of myelin sheaths; however, the merged image (**B**) clearly indicates colocalisation (yellow) of MOG with NG2. Bar = 10 μm.

not express PDGFRα (5). Based on this understanding, investigators used markers expressed later than GalC to investigate the mitotic potential of mature oligodendrocytes following loss of their myelin sheaths during demyelination (3, 22, 24, 26, 27, 30, 34) and concluded that mature oligodendrocytes could divide and therefore contribute to remyelination. Our observations question such a conclusion and are in agreement with recent studies showing that freshly isolated A2B5 expressing cells from the optic nerves of adult rats label with anti-GalC antibodies (32). Thus, the phenotype of OPCs in neonatal and adult tissue is more different than previously recognized, with OPCs in the adult CNS expressing molecules that are restricted to mature oligodendrocytes during development and following culture of neonatal cells. The demonstration of co-expression of PDGFRα with GalC also helps to resolves the controversy that surrounds experiments in which oligodendrocytes isolated from the adult nervous system were demonstrated to be able to generate new oligodendrocytes (11, 29, 37-39). In such experiments the freshly isolated cells were immunosorted using anti-GalC antibodies. It is clear from the results of our current study and that of Shi and co-workers (32), that GalC selection would have resulted in isolating both mature oligodendrocytes and OPCs and not just oligodendrocytes as the investigators thought. It is noteworthy that Wood and Bunge noted that, just as Shi and co-workers found, that cells lost GalC expression prior to cell division. In addition, we have preliminary data that also shows that a proportion of cells isolated form the adult CNS using MOG as the selection marker down-regulate MOG and GalC prior to cell division. With the knowledge that OPCs in the adult nervous system (defined as PDGFRα expressing cells) also express GalC and MOG, it is now possible to understand how previous investigators could conclude that mature oligodendrocyte in the adult nervous system could be a cell that had the potential to generate new oligodendrocytes following demyelination and a cell that was radiation sensitive.

Acknowledgments

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