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Characterization of oligodendrocyte lineage precursor cells in the mouse cerebral cortex: a confocal microscopy approach to demyelinating diseases

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Summary

The identification of stem cells resident in the adult central nervous system has redirected the focus of research into demyelinating diseases, such as multiple sclerosis, mainly affecting the brain white matter. This immunocytochemical and morphometrical study was carried out by confocal microscopy in the adult mouse cerebral cortex, with the aim of analysing, in the brain grey matter, the characteristics of the oligodendrocyte lineage cells, whose capability to remyelinate is still controversial. The observations demonstrated the presence in all the cortex layers of glial restricted progenitors, reactive to A2B5 marker, oligodendrocyte precursor cells, expressing the NG2 proteoglycan, and pre-oligodendrocytes and pre-myelinating oligodendrocytes, reactive to the specific marker O4. NG2 expressing cells constitute the major immature population of the cortex, since not only oligodendrocyte precursor cells and pre-oligodendrocytes but also a part of the glial restrict progenitors express the NG2 proteoglycan. Together with the population of these immature cells, a larger population of mature oligodendrocytes was revealed by the classical oligodendrocyte and myelin markers, 2',3'-cyclic nucleotide 3'-phosphodiesterase, myelin basic protein and myelin oligodendrocyte glycoprotein. The results indicate that oligodendrocyte precursors committed to differentiate into myelin forming oligodendrocytes are present through all layers of the adult cortex and that their phenotypic features exactly recall those of the oligodendroglial lineage cells during development.

Key words

Mouse Cerebral Cortex; Oligodendrocyte Precursor Cells; A2B5; NG2; O4; Confocal Microscopy

Introduction

Among the degenerative diseases of the human central nervous system (CNS), particular attention is paid to multiple sclerosis (MS), an invalidating disease characterized by inflammation and demyelination mainly affecting the nerve fibres of the brain white matter (WM) (Noseworthy, 2000).

The recent identification and immunocytochemical characterization of stem cells resident in the adult CNS has redirected the focus of research into neurodegenerative disease therapy. In this context, great interest is now devoted to cells, oligodendrocytes and their progenitors, which provide the nerve fibres with a myelin sheath, and are thought to be involved in remyelination mechanisms (Carrol and Jennings, 1994; Baumann and Pham-Dinh, 2001; Reynolds et al., 2002).

During normal development, the maturation of the oligodendrocyte lineage cells destined to trigger myelinogenesis takes place according to sequential stages and a better knowledge of the antigenic characteristics of oligodendrocyte progenitors and precursors, pre-oligodendrocytes, non-myelinating and myelinating mature oligodendrocytes has allowed their immunocytochemical identification (Liu et al., 2002).

Oligodendrocyte immature forms have been demonstrated also in the adult brain (Armstrong et al., 1992; Dawson et al., 2000; Polito and Reynolds, 2005), although their recognition is complicated by the fact that immature oligodendrocytes express different markers at the same differentiation stage, and the passage from one differentiation stage to the next is not even and does not involve only the loss of one marker and acquisition of another one (Aloisi et al., 1992; Liu et al., 2002).

The present study was carried out in the cerebral cortex of adult mouse with the aim of analysing, from the immunocytochemical, morphological and morphometric points of view, individual characteristics of immature and mature cells of the oligodendrocyte lineage, whose capability to remyelinate is controversial. The immunocytochemical study was performed by analysing the expression of molecules considered to be markers of oligodendroglial lineage cells. The selected markers were: A2B5 antibody, a specific marker of gangliosides expressed by glial restricted progenitors (GRPs) (Rao and Mayer-Proschel, 1997); O4 antibody, a specific marker of gangliosides expressed by pre-oligodendrocytes and pre-myelinating oligodendrocytes (Sommer and Schachner, 1981); an antibody against NG2 (nerve-glial antigen 2) proteoglycan, specifically expressed by oligodendrocyte precursor cells (OPCs) (Nishiyama et al., 1996) and pre-oligodendrocytes (Baumann and Pham-Dinh, 2001; Lee et al, 2000); antibodies against CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase), MBP (myelin basic protein) and MOG (myelin oligodendrocyte glycoprotein), classical oligodendrocyte and myelin markers expressed by mature oligodendrocytes and myelinated nerve fibres (Reynolds and Wilkin, 1988; Pfeiffer, 1993; Solly et al, 1986). The immunolabelled sections were analyzed by confocal laser scanning microscopy, that has been proven to be a very efficacious technology to finely discriminate immunoreactivity and cell morphological details in the nervous tissue. The results in the normal mouse cerebral cortex were compared with those obtained in a mouse model of MS, namely experimental autoimmune encephalomyelitis (EAE), characterized by inflammation, demyelination and neurodegeneration processes similar to those described in MS lesions (Virgintino et al., 2009).

Materials and Methods

C57BL/6 wild type mice (9 weeks of age) were obtained from Harlan (Bresso, MI, Italy) and maintained in specific pathogen-free conditions. Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national laws and policies (D.L. 116/1992). Mice were anesthetized with chloral hydrate (3 μl/g, intraperitoneal injection) and transcardially perfused with 2% paraformaldehyde solution. The whole brain was rapidly removed, halved into hemispheres and washed in phosphate buffer saline (PBS) overnight a 4°C. Each hemisphere was cut into 20-μm serial sagittal sections using a vibrating microtome (Leica; Milton Keynes, UK).

Immunocytochemistry. The sections were submitted to double or triple fluorescence immunolabellings with fluorochrome conjugated secondary antibodies for confocal laser microscopy. The following primary antibodies were used: mouse IgM A2B5 (Chemicon International-Millipore Corporation, Billerica, MA; diluted 1:600), rabbit anti-NG2 (Chemicon; 1:200), biotinylated mouse IgG1 anti-NeuN, (Neuronal Nuclei; Chemicon; 1:100), mouse IgM O4 (Chemicon; 1:1000), mouse IgG1 anti-CNPase (clone 11-5B, Sigma, Sant Louis, MO; 1:60), rabbit anti-MBP (Abcam, Cambridge, UK; 1:100), goat anti-MOG (R&D Systems, Minneapolis, MN; diluted 1:300). Sections were submitted to immunofluorescence staining according to the following method: (1) tissue permeabilization was carried out either with 0.05% Triton X-100 in PBS for 30 min at room temperature (for A2B5/NG2, O4/NG2) or with 0.5% Triton X-100 in PBS for 30 min at room temperature (for CNPase/MBP/MOG); then the sections were incubated (2) with the above mentioned combination of primary antibodies diluted in blocking buffer (10% albumin, 5% foetal calf serum in PBS), overnight at 4°C, and (3) with either appropriate fluorophore-conjugated secondary antibodies (Alexa 488, Alexa 568, Alexa 633 conjugated; Invitrogen, Eugene, OR; diluted 1:300) or biotinylated goat anti-mouse IgM secondary antibodies (Invitrogen; diluted 1:500), followed by streptavidin-conjugated Alexa 488 (Invitrogen; diluted 1:300) for 45 min at room temperature. After each incubation step the sections were washed 3 times for 5 min with PBS, collected on Vectabond™ treated slides (Vector, Burlingame, CA) and coverslipped with Vectashield (Vector). Negative controls were prepared by omitting the primary antibodies. Sections were examined under a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) using a sequential scan procedure. Confocal images were taken with 40x oil lenses.

Quantitative assessment and image processing. For quantitative analyses of the number of the different cell types of the oligodendrocyte lineage, 10 randomly chosen fields for each of the above described combination of antibodies were taken from 20-μm brain sections (20 for each immunostaining) of healthy (n=5) mice. The numbers of GRPs, OPCs, pre-oligodendrocytes and oligodendrocytes were interactively counted by a computerized morphometric analysis software (Image J, NIH, Bethesda, MD). Cell bodies were identified by their nucleus on each of the selected stained sections and were counted on the projection images; then the numbers were normalized to the same volume (1 mm^3). The average density of each of the oligodendrocyte lineage cells was expressed as a mean value ± SD.

Results

A2B5 immunostaining demonstrated the presence of glial restricted progenitors (GRPs) in the adult mouse cerebral cortex (Fig.1A).

A2B5-reactive GRPs appeared as poorly ramified cells with a large nucleus and scanty cytoplasm, often barely visible, only at the nuclear periphery. Cell bodies and processes did not show a preferential localization in the cortex and were seen in all layers. A2B5-stained puncta were scattered through the neuropil and seemed to mainly correspond to transverse sections of A2B5 cell processes. Morphometric analysis demonstrated that in the cerebral cortex A2B5 glial progenitors were 4988 ±

607/mm^3. A number of cortex cells showed A2B5-stained nuclei, and these cells were identified as neurons by A2B5/NeuN double immunostaining (not shown).

NG2 immunostaining demonstrated the presence of oligodendrocyte precursor cells (OPCs) everywhere in the cortex (Fig. 1B). The population of cells reactive to the proteoglican NG2, an established marker of immature oligodendrocytes, was very large (10144 ± 1648 cells/mm^3). NG2 glial cells appeared star-shaped and their multiple, thin processes radiating from the cell body were richly ramified, so that the cortex neuropil appeared to be occupied by a dense network of NG2-stained cell bodies and processes. NG2/A2B5 double immunolabelling demonstrated the presence of a very low number of A2B5-reactive cells co-expressing NG2 (36 ± 16 cells/mm^3). These cells, 'transitional' OPCs, had a bipolar cell body and low branching processes, and were mainly localized in the subcortical WM.

O4 immunostaining demonstrated the presence in the cortex of pre-oligodendrocytes (Fig. 1C, D). The pre-oligodendrocyte population was recognized by both NG2 and O4 antibodies**:** O4 gangliosides were mainly contained in the pre-oligodendrocyte body whereas NG2 proteoglycan was preferentially localized in the cell processes. O4/NG2 positive pre-oligodendrocytes were more numerous in the superficial and intermediate layers of the cortex (6178 ± 1441 cells/mm^3) than in the deep ones (layer VI and subcortical WM: 2976 ± 1533 cells/mm^3). A population of NG2-negative/O4-positive cells was also recognizable and these cells were identified as satellite, perineuronal oligodendrocytes in cortex layers and as pre-myelinating oligodendrocytes in subcortical WM.

In addition to these immature cells of the oligodendrocyte lineage, i.e. oligodendrocyte progenitors and precursors, and pre-oligodendrocytes, which express markers of progressive differentiation, mature oligodendrocytes and myelinated nerve fibres were revealed by the classical oligodendrocyte and myelin markers, CNPase, MBP and MOG, in both the cortical layers and subcortical WM (Fig. 2A-F). CNPase immunolabelling allowed easy discrimination between NG2/O4 positive pre-oligodendrocytes and mature oligodendrocytes (9203 ± 4429 cells/mm^3 in cerebral cortex layers from I to V; 22772 ± 10390 cells/mm^3 in layer VI and WM). Oligodendrocytes were intensely reactive to the CNPase protein at both the perinuclear cytoplasm and processes that appeared laminar, less numerous and less ramified in comparison with those of the pre-oligodendrocytes (Fig. 1D; 2C). Unlike CNPase staining, which evidences the cell body and processes, MBP and MOG staining only revealed myelinated fibres where the expression of the two myelin proteins overlapped.

Discussion

Analysis carried out under the laser confocal scanning microscope of the morphology and immunoreactivity of immature oligodendrocytes resident in the cerebral cortex of the adult mouse confirmed the presence of cells that express A2B5, one of the earliest markers for oligodendroglia and astroglia differentiating cells during development (Liu et al., 2002; Polito and Reynolds, 2005), and also demonstrated that these cells constitute a minor population among the cortical glial progenitors. In addition, observations of double A2B5/NeuN stained cortex, which showed A2B5 gangliosides expressed by nuclei of a subpopulation of cortical cells, confirmed that A2B5 express-

ing cells lying in the adult cortex may be both glial and neuronal progenitors (Kondo and Raff, 2000; Saito and Sugiyama, 2002; Liu et al., 2002; Wu et al., 2002).

The results also demonstrated that a population of NG2-reactive cells, more numerous than the A2B5 cells, exists in the adult mouse cerebral cortex, where these richly ramified cells build a very dense texture intermingled with the neuronal and astroglial cell networks. NG2 proteoglycan individuates the oligodendrocyte precursor cells but is co-expressed with A2B5 in a small number of oligodendrocyte progenitors and with O4 in a high number of pre-oligodendrocytes, so that NG2 may be considered the molecule guiding, in adult brain as well as during development, the long transition from proliferating and migrating oligodendrocyte progenitors to mature, myelin-forming oligodendrocytes, which lose the NG2 expression. The high number of NG2-reactive cells in the adult brain, where they constitute the major lineage of dividing cells (Horner et al., 2000), the diversity of markers they express as well as the different functions they are attributed (e.g. involvement in nervous tissue homeostasis and in neurotransmission/neuromodulation) have suggested that NG2 cells constitute a heterogeneous population (Butt et al., 1999; Bergles et al., 2000; Dawson et al., 2000). A noteworthy finding for studies on neurodegenerative diseases is that NG2 cells react to demyelination and, following exposure of nerve fibre axons, proliferate and generate oligodendrocytes that may, in turn, be engaged in remyelination (Di Bello et al., 1999).

In conclusion, morphological and morphometric analysis of adult mouse cerebral cortex under the confocal microscope utilizing the A2B5, NG2, O4 markers and the CNPase, MBP, MOG markers, respectively, allowed us to systematically describe, for the first time, the presence and distribution, and evaluate the amount of different oligodendrocyte precursors throughout all the cortical layers, where they lie together with mature, myelin forming oligodendrocytes. The phenotypic, immunocytochemical characteristics of these immature oligodendrocytes recall those of the oligodendroglial lineage cells during brain development. In addition, the results are in agreement with the suggestion that the stem-like cells and their progeny present in the adult CNS are not only confined to the brain ventricular and subventricular zones but may also be evenly spread through the nervous tissue.

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Figures

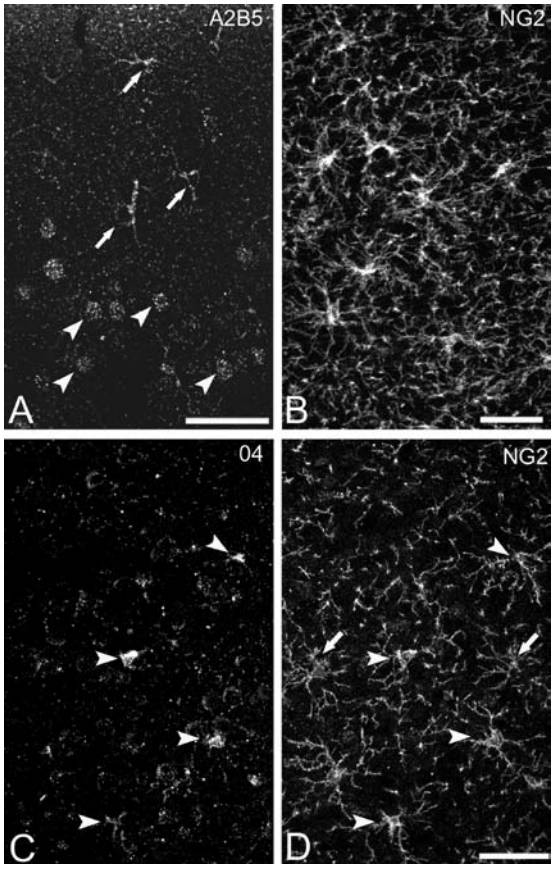

Fig. 1 - Confocal images of 9 week mouse cerebral cortex. (A) A2B5 immunostaining reveals GRPs bodies and processes (arrows) and several cell nuclei with a finely punctuate reactivity (arrowheads). (B) A2B5/NG2 double immunostaining. NG2 single channel: the dense network built by the richly ramified OPCs. (C) and (D) O4/NG2 double immunostaining. (C) O4 single channel: bodies and scanty, thin processes of pre-oligodendrocytes; (D) NG2 single channel: meshwork of bodies and processes of OPCs (arrows) and pre-oligodendrocytes (arrowheads). Pre-oligodendrocytes are discriminated from OPCs by O4/NG2 co-localization (compare C with D, arrowheads). Scale bars: 50 μm.

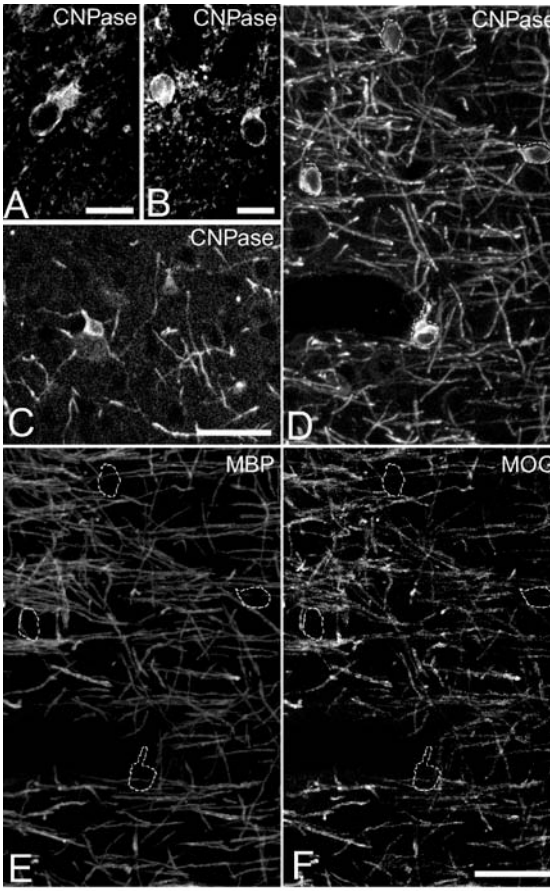

Fig. 2 - Confocal images of 9 week mouse cerebral cortex. Triple CNPase/MBP/MOG immunostaining. (A-D) CNPase single channel: oligodendrocyte bodies with laminar processes (A, B, arrowhead), and myelinated nerve fibres (C, D). MBP (E) and MOG (F) single channels: bundles of myelinated nerve fibres; MBP and MOG immunoreactivity exactly overlaps (compare E with F). The profiles of oligodendrocyte bodies are outlined (compare D with E and F). Scale bars: 10 μm in (A, B); 30 μm in (C-F).