Am J Transl Res 2016;8(12):5637-5645
www.ajtr.org /ISSN:1943-8141/AJTR0035657

Original Article

Reactivated astrocytes as a possible source of oligodendrocyte precursors for remyelination in remitting phase of experimental autoimmune encephalomyelitis rats

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Received July 12, 2016; Accepted October 26, 2016; Epub December 15, 2016; Published December 30, 2016

Abstract: Multiple sclerosis (MS) is ademyelinating disease in the central nervous system (CNS). Majority of the MS patients show relapsing-remitting disease course. Evidences show that oligodendrocyte precursor cells (OPCs), which remain relatively quiescent in normal adult CNS, play a key role in the remitting phase by proliferation and remyelination. In the present study, we found that spinal cord astrocytesco-expressed progenitor cell marker and oligodendroglial lineage markers in the remittance phase in adult rat experimental autoimmune encephalomyelitis (EAE) model. We suggest that activated astrocyte could de-differentiate into OPCs and re-differentiate into mature oligodendrocytes, raising the possibility that astrocytes can be a potential source of OPCs in the adult demyelinated spinal cord.

Keywords: Oligodendrocyte, encephalomyelitis, astrocytesco, multiple sclerosis (MS)

Introduction

Multiple sclerosis (MS) is one of the devastating demyelinating diseases in the central nervous system (CNS). The disease onset usually occurs in young adults. The symptoms of patients include weakness, balance problems, bladder and bowel problems, vision loss, and often progress to physical and cognitive disability. The general consensus of the pathological mechanisms of MS is related to immune attack and subsequent demyelination. The fatty myelin sheaths surrounding the axons are damaged, leading to demyelination and clinical symptoms manifestation [1]. Experimental autoimmune encephalomyelitis (EAE) is the one of the widely adopted animal models representing MS. Both MS patients and its animal model display relapsing and remitting phases in the diseases courses. Several lines of evidences showed that remyelination and functional restoration happens in remitting phase. But the mechanism contributing to auto-recovery is largely unknown [2].

Oligodendrocyte and its precursor cell are the myelin-forming cells in the CNS. Mature oligodendrocytes have lost their abilities to remyelinateaxons, whereas oligodendrocyte precursor cells (OPCs) are believed to play the remyelinating role in the MS [3-5]. Animal and post-mortem studies showed that number of OPC increased in the CNS in EAE model and in MS patients [6]. In chronic MS patient samples, OPCs were found to make contacts with demyelinated axons despite limited success to myelinate them [7]. Cultured OPCs can myelinate the axon in DRG-OPC co-culture system [8-10]. Transplanted neural stem cells (NSCs) and bone marrow stromal cells (BMSCs) can

also differentiate into OPCs and remyelinateaxonsin EAE model [11, 12].

Previous reports have shown that OPCs can be arose from cells other than from self proliferation. Armstrong reported that endogenous oligodendrocytescande-differentiate into OPCs. These de-differenated cells express classical markers of OPC [13]. Another possible source is from infiltrated macrophage which can express NG2, and transdifferentiate into oligodendrocytes [14]. These results suggest that OPCs can be generated from multiple sources [15].

Recent studies showed that reactive astrocytes play important roles in neurological diseases as supporting cells. Besides, reactive astrocytes can trans-differentiate into neurons and transmit action potential [16]. Other studies showed that astrocytes can dedifferentiate into neural progenitor cells [17-19]. Since neural progenitor cells can differentiate into oligodendrocyte precursors [20, 21], we speculated that reactive astrocyte might be a source of oligodendrocyte precursor. In the present study, we aimed to study the profile of reactive astrocytes in the remitting course of EAE.

Materials and methods

Animal use

Forty-eight adult female Lewis rats were used in the present study. All procedures carried out for the animals in the study were approved by the Committee for the Use of Live Animals in Teaching and Research at the University of Hong Kong.

Expression of recombinant MOG

For expression of recombinant rat MOG, the bacterial expression vector pRSETA (a kind gift of DrZeis) was used containing the amino acids 1-125 of the mature rat protein fused to 6 histidine residues. An overnight culture of a transformed E. Coli Bl_{21} strain was used for inoculation of a large expression culture (SOB, ampicillin, kanamycin). The OD600 was measured until it reaches 0.5 and expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside at 1 mM final concentration. After 4 hours the bacteria were harvested by centrifugation (15 min at 4000×g). The pellet was then frozen and stored until purification was performed.

Purification of his-tagged MOG

For immobilized metal ion affinity chromatography, the Talon purification system (Clontech) was used. The bacterial pellet was brought to suspension in lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8) and sonicated to disrupt the bacteria. After a further centrifugation (20 min at 10,000 g), the pellet was dissolved in lysis buffer and the centrifugation step was repeated. Both supernatants were pooled, and subjected on the immobilized metal ion affinity chromatography column for purification. After loading, the column was washed with two volumes of lysis buffer and two volumes of washing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3). The purified recombinant protein was collected by eluting the column with elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 4.5). To obtain soluble recombinant MOG, the purified protein was dialyzed four times (dilution factor 1:200 each) against 20 mM sodiumacetate buffer (pH 3.6) at 4°C. Finally, the purified and soluble protein was concentrated (Centricon, 10,000 MWCO) until the protein concentration was at least 2 mg/ml. The protein was aliquot and stored at -80°C.

Induction of EAE animal model

A total of 48 Lewis rats (10-12 weeks) were used to induce EAE [22]. 50 μg of rMOG emulsified with complete Freud's adjuvant (CFA, Difco Laboratories, Detroit, MI) was injected into the tail base. 6 rats were injected with saline, emulsified with an equal volume of CFA as control. The clinical progression of the disease was monitored daily.

Clinical disease scoring

The clinical symptoms of rats were assessed every day after immunization with MOG. The clinical signs were scored as follows: score 0 indicating no disease (S0); Score 1, complete tail paralysis (S1); Score 2, complete bilateral hind limb paralysis (S2); Score 3, complete paralysis (tetraplegia), moribund state (S3). And accordingly, the clinical signs in the remission phase were scored as: RS2; RS1 and RS0 (**Figure 1B**).

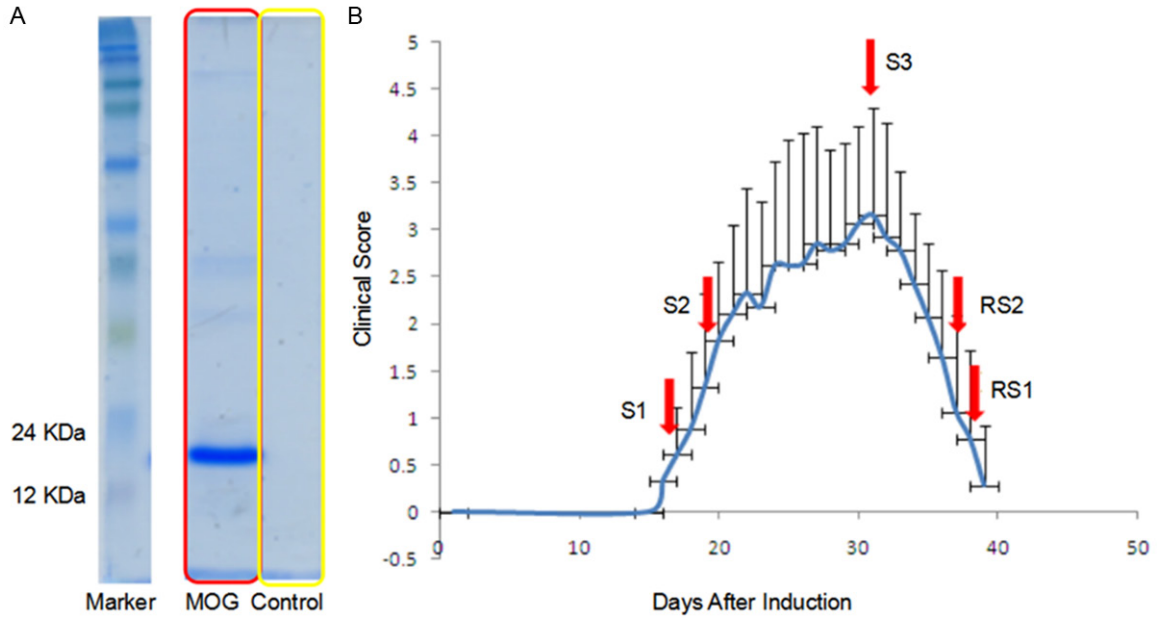

Figure 1. A: Expression of MOG 1-125. After expression in E coli and purification, there is a clear band at 15 kDa. But there is no expression band in the control lane with Commassie blue staining; B: In the selected animals, the animals developed classical clinical score trend. These animals began to display the behavior deficiency at 15 dpi and arrive at the most severe (S3) at day 35. Then the symptoms of the animals will recover until to clinical 1 (RS1) at about day 40 dpi.

Time point for the animal selection

When animals with score S3 survived and recovered, they displayed remission phase. We selected three time-points as the test groups: First was S3 animals (6 animals). Second was the animal with RS2 stage which recovered from S3 (6 animals). And the last group animals with RS1 stage which recovered from RS2 (6 animals).

Tissue processing and immunohistochemistry

At the end of the survival period, the animals were given a lethal dose of sodium pentobarbital and perfused intracardially with normal saline followed by perfusion with 200 to 300 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cords were harvested and post-fixed with fresh fixative overnight and subsequently placed in 30% sucrose. After the samples had sunk, the spinal cords were embedded in Optimum Cutting Temperature (OCT, Sakura Finetek, Torrance, CA) and cut into 10 μm-thick frozen sections on a cryostat (Leica). The sections were mounted on gelatin coated slide and stored at -20°C for later use.

For immunohistochemistry, sections were brought to room temperature and rehydrated with PBS. They were then incubated in blocking solution containing 10% normal serum for 1 hour at room temperature. The sections were subsequently incubated in primary antibodies at 4°C for 14-16 hrs. Primary antibodies included rabbit anti-GFAP (1:1000, Sigma); mouse anti-nestin (Progenitors, 1:500, Sigma); and mouse anti-OMg (OPCs marker, 1:10, from Dr X, Xu) were used to detect astrocyte, progenitor cells and oligodendrocyte precursors [23]. After extensive washes, the sections were incubated in the appropriate secondary antibodies (1:500, Molecular Probes, Eugene, OR) for 1 hour. The sections were subsequently mounted in fluorescent mounting medium (Dako, Denmark). Pictures were taken with a fluorescence microscope (Carl Zeiss, Germany).

Statistics

All the data are expressed as mean ± S.D. Behavioural scores were analysed by the Kruskal-Wallis non-parametric analysis of variance (ANOVA). Correlation between behavioural and histological outcomes was analyzed using regression analysis. Significance was set at $P<0.05$.

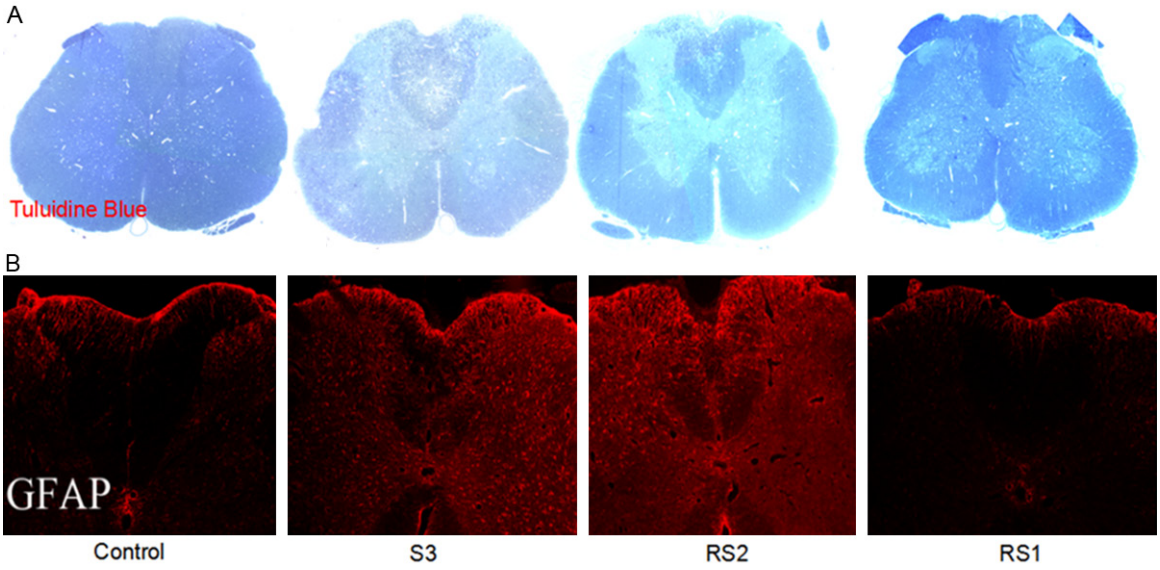

Figure 2. A: The animals were perfused with 4% PFA. And spinal cord were collected and postfixed with glutaradehyde. 1 μm-thick transections were stained with 1% toludine blue. The myelin in the red square were obvious destroyed in the S3 animals and the myelin in the RS2 displayed the recovery than that of S3 and the myelin in the RS1 was similar with that of control. (Scale bar: 1 mm); B: Distribution of astrocytes. The astrocytes were active in the S3 and RS2 animals than that in the control animals. The astrocytes in the RS1 animals were similar with that of control animals. (Scale bar: 100 μm).

Results

MOG expression and EAE induction

After emulfication with CFA, the recombinant protein can induce symptoms characterized by EAE in 34 rats. Amongst them, 22 rats developed the onset and remission phase. The other 12 rats showed primary progressive disease course and not used in the study (**Figure 1**).

The level of demyelination is synchronous with the clinical score

After induction of EAE and at different time point the spinal cord were cut the 1-μm thick semisections and stained with tolundine blue. Under the microscope, we found that the severe level of demyelination in different animals are synchronous with the clinical score of the behavior. At the animal of CS 4.0, the dorsal column of spinal cord display the most severe demyelination, and at the animal of CS 3.0, the demyelination of spinal cord is ameliorated. And at the animal of CS 1.0, the myelin is similar with the controls. These results demonstrated that when the clinical scores decrease, the pathology of demyelination is recovered and the animals display the remyelinating trend. This results also demonstrate that there is endogenous remyelinating in the remission animals (**Figures 1B** and **2A**).

Astrocytes are reactive when the spinal cords are demyelinated

We stained the sections with GFAP to demonstrate the characteristics of astrocytes in the sections from the animal at the different time point. Compare to the control sections, the number of astrocytes in the S3 increase significantly. The number of astrocytes in the sections of RS2 decreases than that in the sections of RS2 but more than that in the sections ofRS1. The number of astrocytes in the section of RS1 display no significantly difference with normal animals (**Figure 2B**). At the same time the astrocytes in the sections of S3 and RS2 display more branches than the RS1 and control. These results demonstrate that the astrocytes are reactive in the demyelinating animals.

Reactive astrocytes dedifferentiate into the progenitors

Astrocytes in the spinal cord sections were detected by staining with GFAP. It is evident that more astrocytes can be found in S3 stage than in control and remission samples (RS2

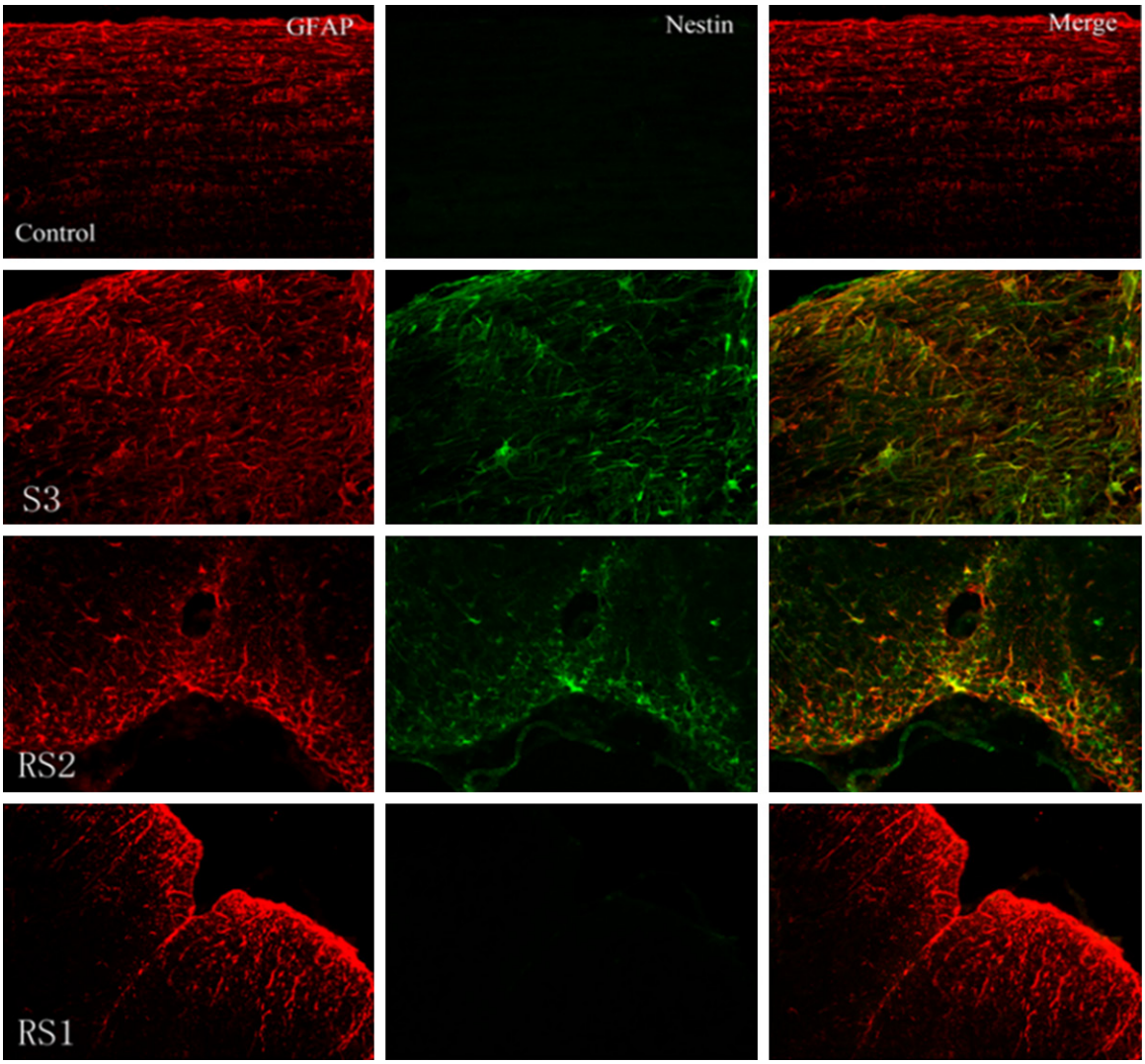

Figure 3. Almost all the reactive astrocytes in the S3 and RS2 animals showed the characteristics of progenitors. GFAP staining was co-localized with nestin staining and almost all the nestin positive cells are GFAP positive. These nestin-positive cells might be induced from GFAP positive cells and play the different role form astrocytes. And there are no nestin positive cells in the control and RS1 animals. (Scale bar: 100 μm).

and RS1). We co-stained the sections with nestin and found that GFAP positive astrocytes expressed nestin at S3 and RS2 but not in control and RS1 stage. Interestingly, most GFAP positive astrocytes expressed nestin in S3 but only a limited number of them expressed nestin in RS2 (**Figures 3** and **4**).

GFAP positive cells express oligodendrocyte marker OMg

Nestin is often considered as a progenitor cell marker, we then further asked whether these GFAP +ve/nestin +ve cells are progenitor cells, specifically, whether they showed oligodendrocyte phenotype. We then co-stained the sections with GFAP, and OMg, a marker of pro-oligodendrocyte cell. We found that in the S3 and RS2 samples, some of the cells expressed GFAP and OMg. No co-labeling of these two markers were found in control and RS1 samples. The presence of pro-oligodendrocyte markers in GFAP cells suggested that astrocyte might be able to transform into oligodendroglial lineage cells (**Figure 5**).

Discussion

The possible source of endogenous OPC in MS

Researches have demonstrated that there are endogenous OPCs in the central nervous sys-

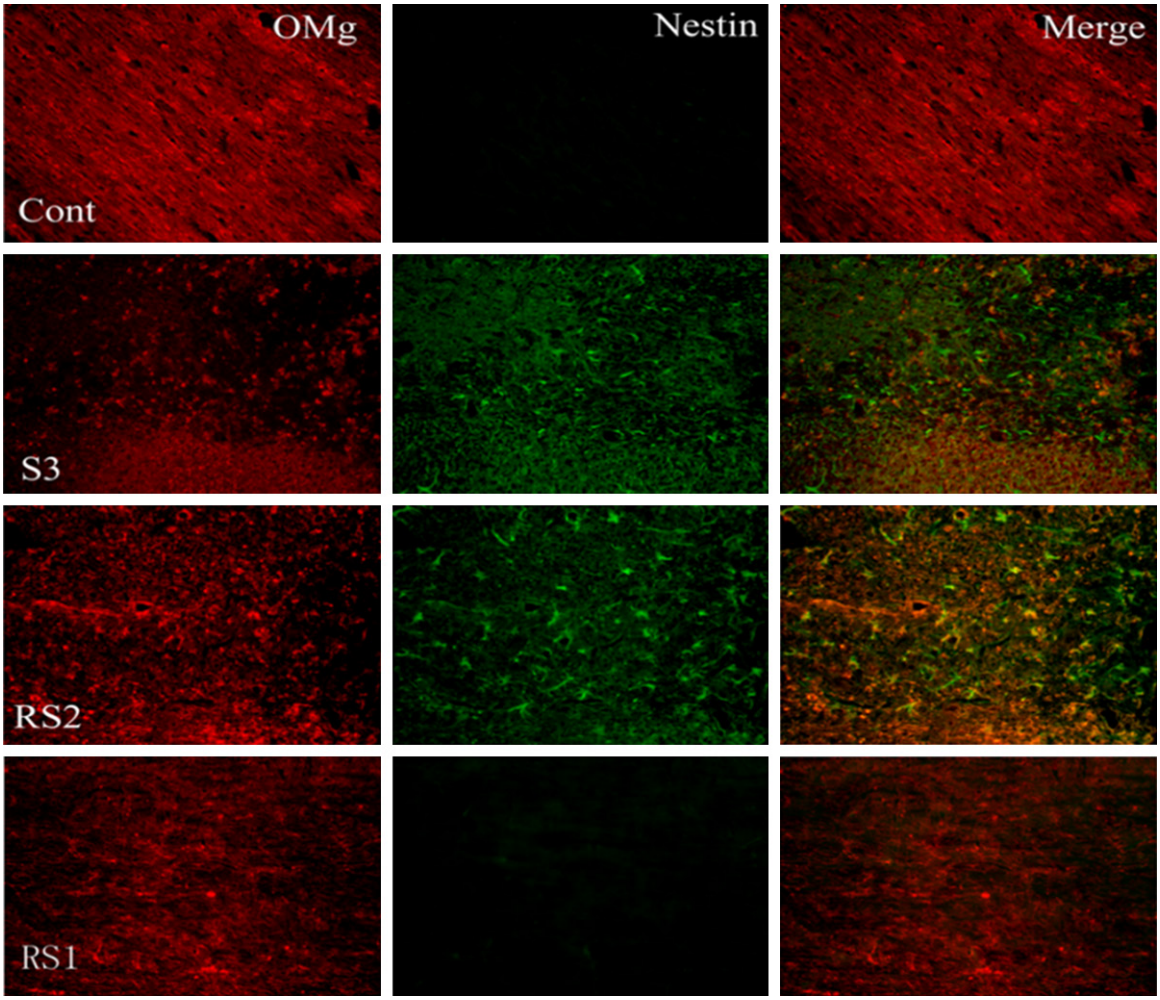

Figure 4. The characteristics of nestin positive cells. To detect the profiles of nestin-positive cells, the sections were stained with nestin (green) and OMg (red) simultaneously, which is a marker of oligodendrocyte precursors. In the S3 sections, there was no co-localized between nestin and OMg. But in the RS2 sections, parts of cells were positive with OMg and nestin simultaneously. This results means a possibility that part of nestin positive cells began to express the marker of oligodendrocyte precursors. And there was no colocalized staining between nestin and OMg in the control and RS1 animals. (Scale bar: 100 μm).

tem of patients with spinal cord injury and multiple sclerosis. These endogenous OPCs can express the marker of oligodendrocytes and promote remyelination and recovery in these diseases [6]. But the source of these endogenous OPCs is not clear. Studies showed that few OPCs exist in the normal CNS. When the brain and spinal cord are injured, endogenous OPCs can proliferate at the injury site and promote functional recovery [24, 25]. Macrophages and microglia are the principle immune cells in the central nervous system and play the significant role in the defense against the invading microorganisms [26]. These cells can express neuron/glia2 (NG2), and NG2-expressing cells are often referred to as oligodendrocyte precursor cells (OPCs), so these cells are thought to be the possible origin of OPCs [27, 28]. The third possible source for endogenous OPCs is the neural stem cells. Recent researches have demonstrated that neural stem cells exist in hippocampus and subventricular zone even at adult stage. These neural stem cells can migrate to the demyelinating sites and differentiate into OPCs [21]. The newborn OPCs can replace the degenerating oligodendrocytes and remyelinate the axons [25].

The previous view about the role of astrocyte in multiple sclerosis

Astrocytes are the major cell type in the CNS. It contributes to about 70% in the brain. It has long been thought that astrocytes just play a

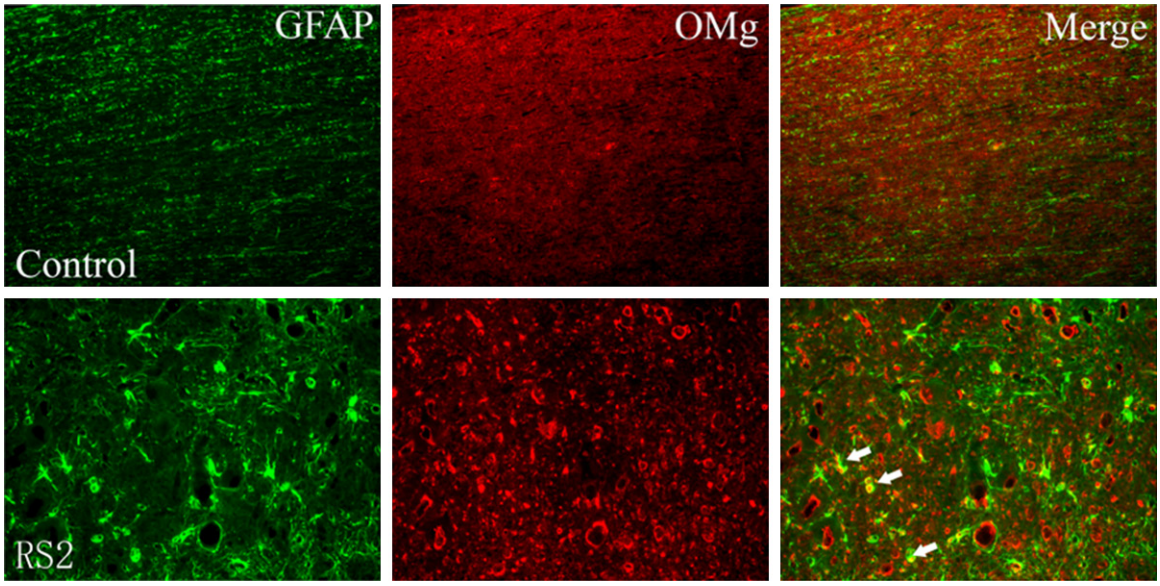

Figure 5. Part of GFAP positive cells expressed markers of OPCs. In RS2 groups, some GFAP positive cells expressed OMg, which is a marker of OPCs (arrow labeled cells). But in Control group, these double staining cells are not existed. (Scale bar: 100 μm).

supporting role for neuronal function in the CNS [29]. Recent evidences suggest that astrocytes play dual roles in demyelinating disease such as in multiple sclerosis. Most literature found that astrocytes played detrimental roles in demyelinating disease [30, 31]. Astrocytes release monocyte chemoattractant protein-1 and interferon-gamma inducible protein-10 and promote the migration of macrophages and microglia through the blood brain barrier into demyelinating area. These immune cells are responsible for demyelination in multiple sclerosis [32]. On the contrary, accumulating evidences demonstrated that astrocytes may play positive functions in MS. For example, astrocytes provide the micro-scaffold to support the progenitor cells to migrate into the demyelinating area. And they can limit the CNS inflammation and promote axonal regeneration [33]. Astrocytes can also provide a permissive environment for remyelination by promoting oligodendrocyte precursor migration, oligodendrocyte proliferation, and differentiation [34].

The possibility of astrocyte as a source of OPC

In fact, the role of astrocyte in MS has attracted attention for a long time. At 1904, Muller claimed that multiple sclerosis was a primary disease of astrocytes [34]. Conditional knockout of GFAP in astrocytes caused the animals to display more severe clinical course in EAE. Astrocytes plays an important role in the development of clinical symptoms of EAE [35].

Developmentally, astrocytes and oligodendroyctes are derived from the common glial progenitor cells [36]. These observation suggested that astrocyte have closer relationship with oligodendrocytes and the astrocyte may possess the potential to transdifferentiate into oligodendroyctes.

New experimental data show that astrocytes retained the ability to transdifferentiate into other cells in vivo and in vitro [37-39]. After being transfected with the gene neurogenin 2, astrocytes can undergo a conversion across cell lineages and generate fully differentiated neurons [40]. In vitro and in vivo experiments demonstrated that reactive astrocytes can also acquire stem cell properties via sonic hedgehog signal pathway. These findings provide a possible molecular basis for how stem cell emerge in the sites of CNS diseases [18, 39, 41].

Numerous reports have shown that OPCs are present and increased in cell number in the demyelinating site of MS, but the source is not clear, in this study, we found that astrocytes might be a source of oligodendrocyte in the remission phase of EAE. We found that astrocytes in the demyelinating regions express nes-

tin, the marker of precursors. This suggested that astrocytes may regain to a more primitive stage and has the potential to become progenitor cells. To characterize these GFAP positive cells, we stained the cells with OMg, a marker for pro-oligodendocyte. We found that some GFAP positive cells co-expressed OMg, suggesting a transdifferentiation of oligodendrocyte from astrocyte. In fact, the presence of this co-labeling can be interpreted as the opposite way, i.e. oligodendrocyte trans-differentiate into astrocyte. However, we are in favour of the first assumption because currently undergoing in vitro experiments showed that we can induce transformation of astrocyte into oligodendrocytes.

Disclosure of conflict of interest

None.

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