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## Insulin-like growth factor system in human central nervous system, multiple sclerosis and amyotrophic lateral sclerosis

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Chapter 7

**Upregulation of insulin like growth factor binding proteins -1 and -6 in oligodendrocytes in chronic multiple sclerosis lesions**

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Submitted for publication

## Abstract

Multiple sclerosis (MS) is a disease of the central nervous system in which myelin; oligodendrocytes and axons are destroyed. As the disease progresses most lesions fail to remyelinate. Insulin-like growth factor-I (IGF-I) plays a pivotal role in oligodendrocyte development, survival, and myelin synthesis. We investigated IGF-I receptors and IGF binding proteins (IGFBPs) in oligodendrocytes at the edges of chronic lesions of MS. Sections of cerebral white matter containing chronic plaques from 11 MS cases and cerebral white matter from 12 controls without neurologic disease were studied by immunohistochemistry and confocal microscopy. Oligodendrocytes in cerebral white matter from controls and normal appearing white matter in MS stained for IGFBPs-1, -2, -3, -4 and -5. Oligodendrocytes at the margins of the MS plaques displayed enhanced IGFBP-1 immunoreactivity and were also IGFBP-6 positive, while immunoreactivity for IGFBPs-2, -3, -4 and -5 was similar to that detected in oligodendrocytes of control white matter and normal appearing white matter in MS. Experiments in cultures of rat oligodendrocyte progenitor cells showed that both IGFBP-1 and IGFBP-6 reduced IGF-I mediated cell survival and formation of the myelin protein CNPase. Consistent with the observation that these IGFBPs reduce the activity of IGF-I, we found that IGF-I receptors on oligodendrocytes at the margins of MS plaques were upregulated. Our results suggest that an up-regulation of IGFBPs-1 and -6 in oligodendrocytes may contribute to the loss of oligodendrocytes and failure of remyelination in MS lesions.

## Introduction

The underlying cause of myelin destruction in multiple sclerosis (MS) is not completely understood. Recent studies suggest that different processes may be involved<sup>21, 22</sup>. In most lesions a T cell and macrophage dominant inflammatory attack could explain the myelin damage, while in others a primary disturbance of oligodendrocytes might be responsible. Remyelination of MS lesions, although often limited in its extent, can be observed during the early stages of the disease<sup>27, 28</sup>, but largely fails as the disease progresses. Most chronic lesions of MS are not remyelinated, although oligodendrocyte precursors and premyelinating oligodendrocytes are present in many demyelinated plaques<sup>7, 8, 21, 26, 32, 36, 37</sup>. This suggests that the microenvironment in chronic MS lesions does not provide or blocks the appropriate signals for remyelination. A recent study suggested that astrocytic expression of Jagged1 might be involved in the limited remyelination observed in MS<sup>15</sup>.

Another hypothesis is that a reduced trophic support to oligodendrocytes might also be implicated in oligodendrocyte apoptosis and the failure of remyelination of lesions in MS. IGF-I is an important survival factor for oligodendrocytes and their precursors<sup>1, 23, 25, 40</sup>, and it stimulates the synthesis of myelin by regulating myelin gene expression in oligodendrocytes<sup>30</sup>. Transgenic mice overexpressing IGF-I have increased myelin content in their CNS<sup>6, 38</sup>, and IGF-deficient mice show a strong reduction in myelination and the numbers of oligodendrocytes<sup>3</sup>. Cerebral white matter in human neonates, undergoing active myelination, contains a 3-fold higher density of IGF-I receptors than in adults, indicating that IGF-I also plays an important role in the myelination of the human CNS<sup>11</sup>.

The IGF system is complex and includes IGF binding proteins (IGFBPs), of which at least 6 have been identified<sup>2, 10</sup>. Circulating IGFBPs are responsible for transport of IGF-I to the target tissues. At the level of the extracellular matrix or cell surface of the target cells, IGFBPs can either inhibit or enhance the presentation of IGF-I to their receptors<sup>2, 10</sup>. The concentrations of IGF-I, and IGFBPs-1 to -3 in both serum and cerebrospinal fluid were found not to be different between patients with MS and controls<sup>34, 35</sup>. Little is known about IGF-I receptors and IGFBPs in oligodendrocytes in MS. Gveric and co-workers reported that oligodendrocytes in normal appearing white matter in MS expressed IGFBP-1, but they could not detect IGF-I receptors<sup>14</sup>. We investigated IGF-I receptors and IGFBPs in oligodendrocytes in MS periplaque white matter.

## Materials and methods

### *Post-mortem brain tissue*

Brain samples were obtained at autopsy from 11 patients with pathologically confirmed MS, and from 12 controls without evidence of neurological disease. Clinical data of the patients are summarised in the Table 1. Post-mortem intervals for the two groups were not significantly different and ranged between 4-11 hours. Tissue blocks of approximately 0.5 cm thick were frozen rapidly by immersion in liquid nitrogen, and stored at -80°C until further processing.

### *Antibodies for immunohistochemistry*

Polyclonal antibodies against IGFFBPs -1, -2, -3, -4, -5 and -6 were obtained from Gropep (Thebarton, Australia). Monoclonal antibody against human IGF-I receptor was purchased from Upstate (Lake Placid, NY). Monoclonal anti-2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNPase, a marker for oligodendrocytes) and ExtrAvidin-TRITC conjugate were purchased from Sigma (Saint Louis, Missouri). Biotine conjugated rabbit-anti-chicken (IgG) was obtained from Jackson Immunoresearch Laboratories (West Baltimore, PA). Alexa-fluor 488 goat-anti-mouse-IgG and Alexa-fluor 568-goat-anti-rabbit-IgG were purchased from Molecular probes (Leiden, the Netherlands). The specificity of the antibodies was confirmed using the antibody pre-absorbed with excess of matched recombinant human IGFFBPs-1, -2, -3, -4, -5, and -6. Specificity of the immunoreactivity was also controlled by the incubation of tissue sections in 5 % goat serum, and or 5 % sheep serum instead of primary antibodies; the immunohistochemical reactions were negative (not shown).

### *Immunohistochemistry*

The frozen brain samples were sectioned (10- $\mu$ m thick) on a freezing-sliding microtome, fixed for 10 minutes in 2 % buffered formaldehyde (pH 7.4) and washed in phosphate buffered saline (PBS) for 15 minutes. Before the addition of first and secondary antibody solution, sections were incubated for 30 min at room temperature in normal goat serum or sheep serum to suppress non-specific antibody binding. Sections were incubated with the primary antibody solution: rabbit-anti-IGFBPs-1, -2, -3, -4, -5, and -6 (1:100) and mouse-anti-CNPase (1:100) in PBS overnight at 4 °C. Thereafter, sections were incubated with the

secondary antibody solution: Alexa-fluor 488 goat-anti-mouse-IgG (FITC-conjugated) and Alexa-fluor 568-goat-anti-rabbit (TRITC-conjugated) (1:100) in PBS for 120 min at room temperature. For the double staining of IGF-I receptors and oligodendrocytes, sections were incubated with chicken-anti-human IGF-I receptor (1:100) and mouse-anti-CNPase (1:100) in PBS overnight at 4 °C. Next, sections were incubated with the secondary antibody solution: biotin-conjugated-anti-chicken (1:100) in PBS for 90 min at room temperature. The last step was the incubation in a mixture of Alexa-fluor 488 (FITC) goat-anti-mouse-IgG (1:100) and extrAvidin-TRITC (1:100) in PBS for 90 min at room temperature. Between all steps the sections were rinsed thoroughly with PBS. Sections were embedded in fluorescent mounting medium and analysed using confocal scanning laser microscopy. The images were digitised, analysed and quantified by computer-assisted densitometry using the program Image (National Institutes of Health Research Services Branch, NIMH, Bethesda, MD).

#### *Isolation of oligodendrocyte progenitor cells*

Rat oligodendrocyte progenitor (O2A) cell-enriched cultures (purity about 95%) were prepared from cerebral hemispheres of 1 day-old Wistar rats according to a modified shake-off protocol described previously<sup>24</sup>.

#### *Oligodendrocyte progenitor cell survival and myelin formation*

Cells were washed with Hank's balanced salt solution and fed with a chemically defined insulin-free medium, with or without the addition of IGF-I (10 ng/ml) or a combination of IGF-I with either IGFBP-1 (200 ng/ml) or IGFBP-6 (200 ng/ml). After 24 h and 48 h, supplementation of IGF-I without or with the respective IGFBPs was repeated. Cell survival was tested after 72 h by using a commercial MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay (Sigma, Deisenhofen, Germany). MTT is cleaved to a dark blue product by mitochondrial dehydrogenases in living but not in dead cells. The effect on the expression of CNPase, a myelin protein, was measured by Western blot. For cell lysates, SDS-PAGE was performed running 5- to 15- $\mu$ g protein/lane on 12,5 % gels. Proteins were blotted onto PVDF membranes for 1h at 350 mA. Immediately afterwards membranes were blocked for 30 min in TBS-T (6 g/l Tris-Base, 8 g/l NaCl, 0.2 g/l KCl, 0,2 % (vol/vol) tween 20, pH 7.6), containing 50 g/l dry milk powder. Incubation with the primary antibody anti-CNPase (1:1,000) was performed overnight at 4°C in TBS-T. The secondary peroxidase- or alkaline phosphatase-conjugated antibodies (1:10,000) were applied for 1 h at room temperature. Signals were detected with the enhanced chemoluminescence method

(Amersham Pharmacia Biotech, Roosendaal, The Netherlands) or by NBT/X-phosphate staining. Bands were scanned and pixel densities were measured using the National Institute of Health Image software.

**Table 1**      **Characteristics of patients with multiple sclerosis and controls without neurological disease**

No	Age/sex	Condition	Postmortem time	Cause of death
1	68/F	Relapsing secondary progressive	9 h	Pneumonia
2	79/M	Relapsing secondary progressive	7 h	Sepsis
3	47/F	Relapsing secondary progressive	6 h	Euthanasia
4	45/F	Relapsing secondary progressive	11 h	Euthanasia
5	72/F	Secondary progressive	8 h	Respiratory failure
6	57/F	Secondary progressive	6 h	Sepsis
7	58/F	Secondary progressive	8 h	Euthanasia
8	78/F	Secondary progressive	6 h	Euthanasia
9	48/M	Secondary progressive	7 h	Hepatic failure
10	81/F	Secondary progressive	4 h	Cachexia
11	56/M	Secondary progressive	6 h	Respiratory failure
12	45/M	Control	9 h	Hypovolemic shock
13	49/M	Control	7 h	Sepsis
14	51/M	Control	6 h 30 min	Liposarcoma and ileus
15	54/M	Control	7 h	Cardiac arrest
16	62/M	Control	6 h 30 min	Adenocarcinoma
17	78/M	Control	7 h	Cardiac arrhythmia
18	54 /F	Control	8 h	Acute renal failure
19	63/F	Control	6 h	Euthanasia
20	68/F	Control	10 h 30 min	Breast cancer
21	72/F	Control	9 h	Cardiac failure
22	81/F	Control	7 h	Abdominal bleeding
23	65/F	Control	5 h	Pneumonia

## Results

### *Statistical analysis*

We used the unpaired *t*-test to test for significant differences between MS patients and controls. We judged a *p* value of 0.05 or less to be significant. Statistical analyses were performed using GraphPad Instat<sup>®</sup> 3 for Macintosh. (Table 2 and 3).

### *Lesions of multiple sclerosis*

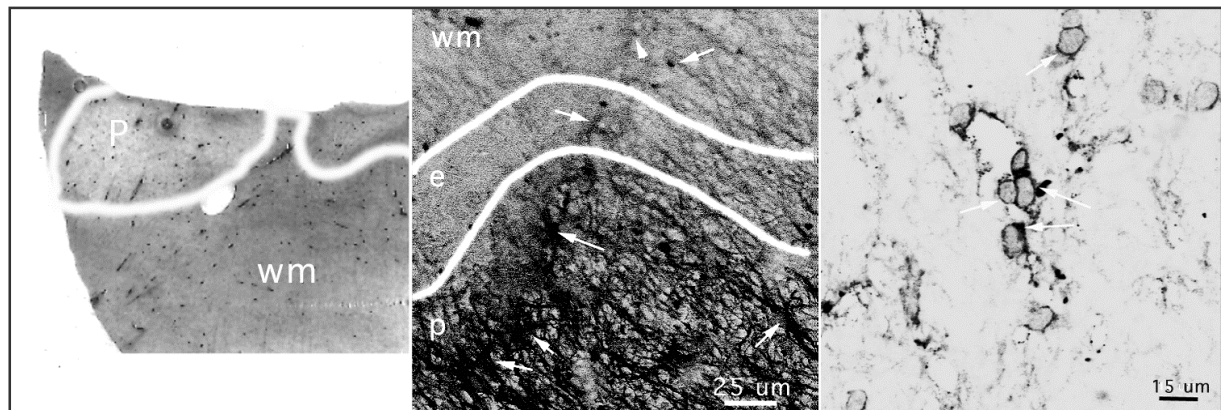
Eighteen chronic plaques from 11 MS cases (Table 1) were examined for oligodendrocytes. Lesions were identified by the presence of demyelination demonstrated with myelin staining (luxol fast blue). Reactive astrocytes at the edges of MS plaques were identified by glial fibrillary acidic protein (GFAP) staining (Figure 1). All lesions displayed central astrogliosis, and the presence of inflammatory cells (either lymphocytes or microglia/macrophages, or both) and hypertrophic astrocytes at the edges. Oligodendrocytes were identified by immunostaining with anti-CPNase (Figure 1).

Oligodendrocytes in cerebral white matter from controls and normal appearing white matter in MS expressed IGFBPs-1, -2, -3, -4 and -5 (Figure 2 and 3), and pixel densities between the two groups were not significantly by different (Table 2). Oligodendrocytes in cerebral white matter from controls and normal appearing white matter in MS were negative for IGFBP-6 (Figure 3). Oligodendrocytes at the edges of all the MS lesions that we investigated showed enhanced expression of IGFBP-1 (relative increase of 83 % compared to control patients), and were also IGFBP-6 positive (Figure 2 and 3, Table 3).

IGF-I receptors are present in oligodendrocytes, in control white matter and in normal appearing white matter from MS patients, and pixel densities between these two groups were not significantly by different (Figure 4, Table 2). Compared to control and normal appearing white matter in MS, oligodendrocytes at the edges of all MS lesions that were investigated clearly displayed increased immunoreactivity for IGF-I receptors (relative increase of 23 % compared to control patients) (Figure 4, Table 3).



Figure 1



**A:** Luxol-fast blue staining (myelin staining) of MS white matter. The pale area is a demyelinated plaque (**p**) and the surrounding non-affected area in gray is myelinated white matter (**wm**). **B:** GFAP-staining of hypertrophic astrocytes in MS plaques (**p**); at the edges of chronic plaques (**e**), and in the normal appearing white matter (**wm**). **C:** CNPase staining of oligodendrocytes at the edges of chronic MS plaques. Pictures are expressed at gray-level.

**Table 2** pixel densities of IGFBPs and IGF-I receptors measured in oligodendrocytes in control white matter and in normal appearing white matter in MS (n = 23).

	control white matter pixel density (mean, SEM)	normal appearing white matter pixel density (mean, SEM)	*P
<b>IGFBP-1</b>	130.81 ± 1.01	129,57 ± 0,81	0.1701
<b>IGFBP-2</b>	157.69 ± 2.60	157,01 ± 1.95	0.8349
<b>IGFBP-3</b>	141.98 ± 4.54	142,76 ± 4.28	0.9016
<b>IGFBP-4</b>	124.90 ± 0.79	125,51 ± 1.07	0.6470
<b>IGFBP-5</b>	134.07 ± 0.55	133.87 ± 0.45	0.3852
<b>IGFBP-6</b>	ND	ND	/
<b>IGF-IR</b>	190.73 ± 1,84	189.11 ± 1.91	0.5440

IGF = Insulin like growth factor; IGFBP = Insulin like growth factor binding protein, IGF-IR = IGF-I receptor. \*unpaired *t*-test. ND = not detectable.

**Table 3** pixel densities of IGFBPs and IGF-I receptors measured in oligodendrocytes in control cerebral white matter and at the edges of MS plaques (n =23).

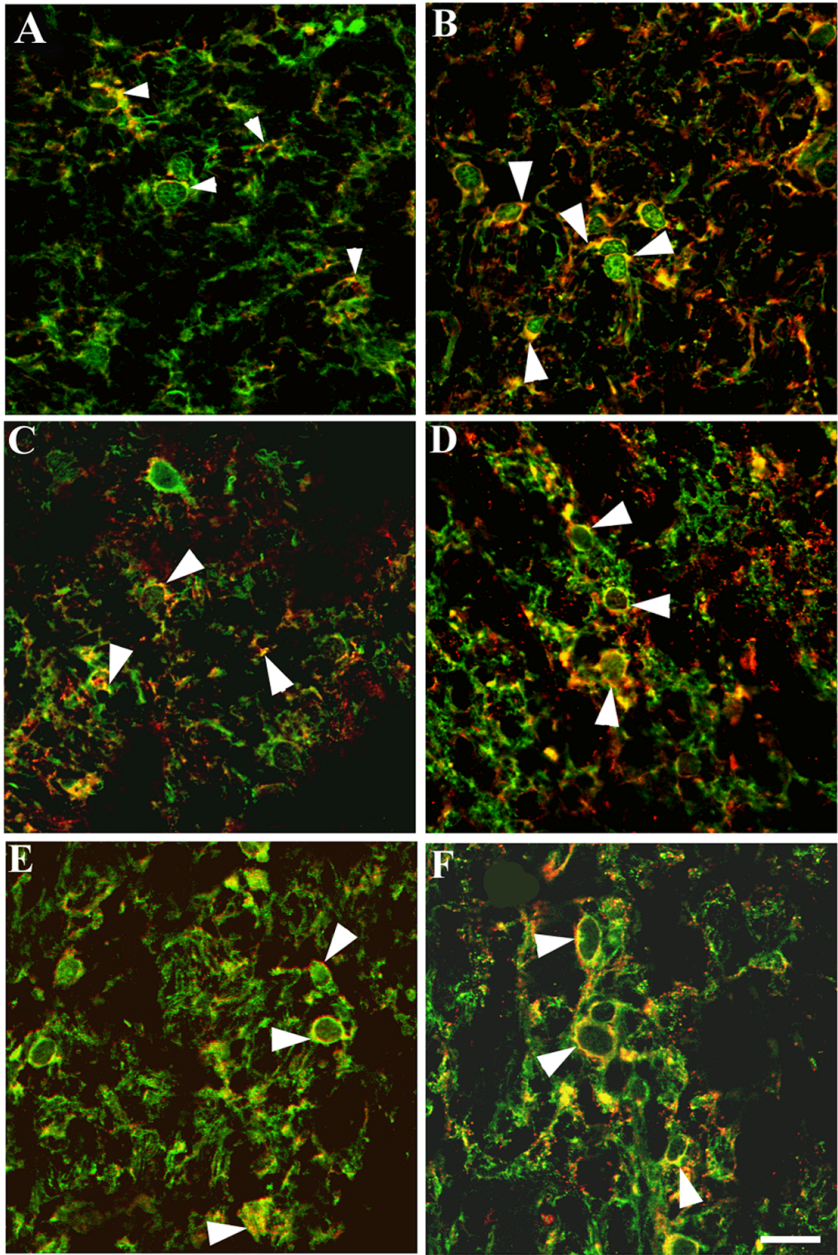
	control white matter pixel density (mean, SEM)	edges of MS lesions pixel density (mean, SEM)	*P
<b>IGFBP-1</b>	130.81± 1.01	239.59 ± 1.20	<0.0001
<b>IGFBP-2</b>	157.69 ± 2.60	158.58 ± 2.62	0.8114
<b>IGFBP-3</b>	141.98 ± 4.54	139.82 ± 4.59	0.7403
<b>IGFBP-4</b>	124.90 ± 0.79	124.23 ± 0.72	0.5312
<b>IGFBP-5</b>	134.07 ± 0.55	134.69 ± 0.49	0.2028
<b>IGFBP-6</b>	ND	193.14 ± 2.96	/
<b>IGF-IR</b>	190.73 ± 1,84	235.82 ± 1.46	<0.0001

IGF = Insulin like growth factor; IGFBP = Insulin like growth factor binding protein, IGF-IR = IGF-I receptor. \*unpaired *t*-test. ND = not detectable

#### *O2A cell survival and myelin synthesis*

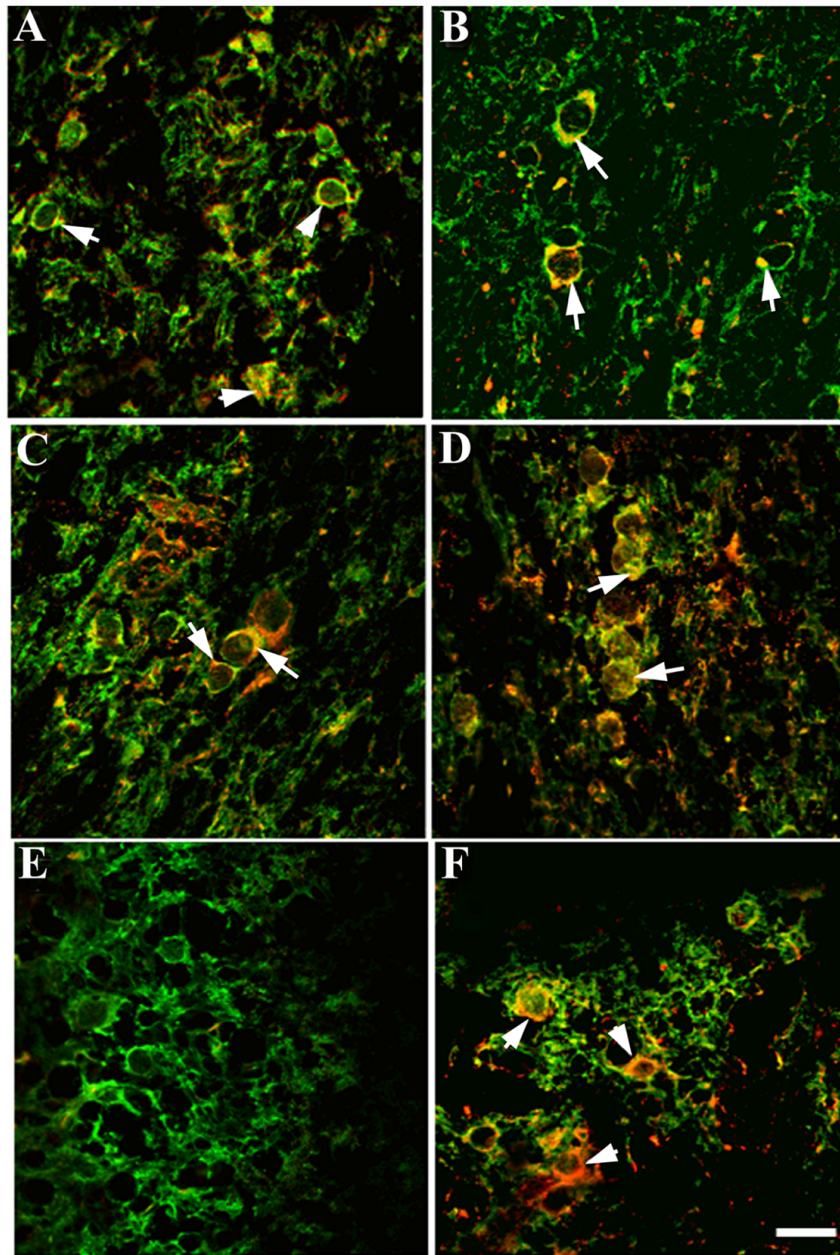
Whether enhanced levels of IGFBP-1 or IGFBP-6 inhibit or stimulate the action of IGF-I was investigated in rat O2A cell cultures. Addition of 10 ng/ml IGF-I drastically enhanced O2A cell survival (Figure 5b). This effect of IGF-I was reduced in the presence of 200 ng/ml of either IGFBP-1 (relative reduction of 25 %,  $p < 0.001$ ) or IGFBP-6 (relative reduction of 24 %,  $p < 0.001$ ; Figure 5b). IGF-I (10 ng/ml) stimulated the expression of CNPase (Figure 5a). This effect of IGF-I was also reduced in the presence of 200 ng/ml of either IGFBP-1 (relative reduction of 30 %,  $p < 0.001$ ) or IGFBP-6 (relative reduction of 26 %,  $p < 0.001$ ; Figure 5a).

Figure 2



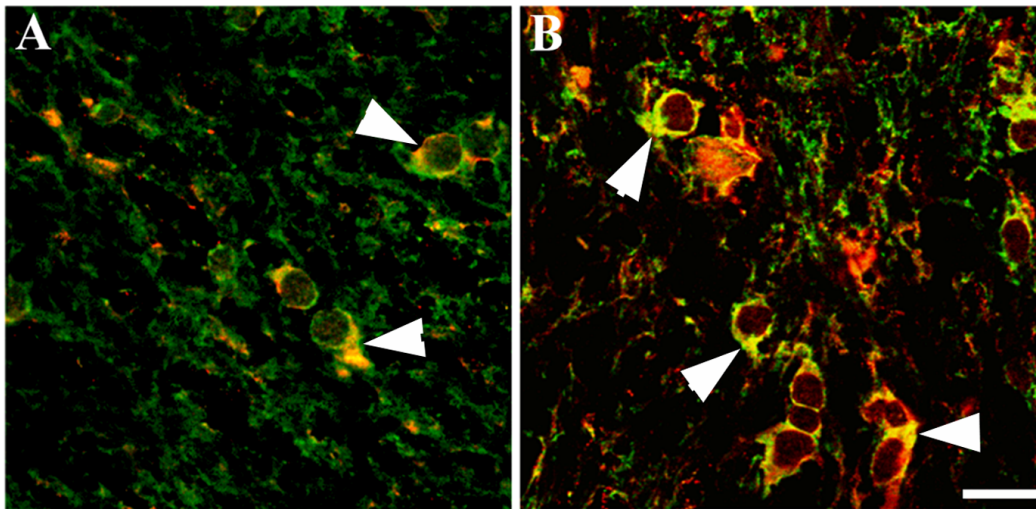
Double-staining for oligodendrocytes (anti-CNPase; green) and IGFBPs-1, -2 and -3 (red) in control white matter (**A, C, E**) and at the edges of a chronic MS plaque (periplaque) (**B, D, F**). IGFBP-1 immunoreactivity is increased in oligodendrocytes at the edges of chronic MS plaques (**B**) compared to oligodendrocytes in control white matter (**A**). IGFBP-2 (**C, D**) and IGFBP-3 (**E, F**) showed no differences in immunoreactivity between oligodendrocytes in control white matter (**C, E**) and at the edges of MS plaques (**D, F**). The scale bar represents 15  $\mu\text{m}$ .

Figure 3



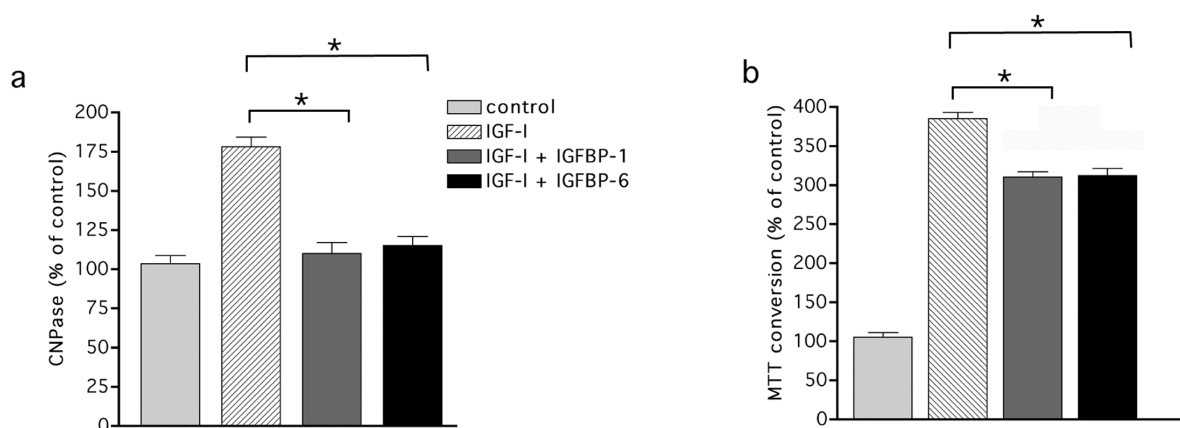
Double-staining for oligodendrocytes (anti-CNPase; green) and IGFBPs-4, -5 and -6 (red) in control white matter (**A**, **C**, **E**) and at the edges of a chronic MS plaque (periplaque) (**B**, **D**, **F**). IGFBP-6 immunoreactivity is absent in oligodendrocytes in control white matter (**E**) and present at the edges of chronic MS plaques (**F**). IGFBP-4 (**A**, **B**) and IGFBP-5 (**C**, **D**) showed no differences in immunoreactivity between oligodendrocytes in control white matter (**A**, **C**) and at the edges of MS plaques (**B**, **D**). The scale bar represents 15  $\mu\text{m}$ .

Figure 4



Double-staining for oligodendrocytes (anti-CNPase; green) and IGF-I receptors (red) in control white matter (A) and at the edges of a chronic MS plaque (periplaque) (B). IGF-I receptor immunoreactivity was enhanced on oligodendrocytes at the edges of chronic MS plaques (B) compared to oligodendrocytes in control white matter (A). Co-localisation is showed in yellow. The scale bar represents 15 μm.

Figure 5



The effects of IGFBP-1 and IGFBP-6 on IGF-I mediated (a) myelin formation assessed by quantitative evaluation of CNPase western blots and (b) cell survival measured by MTT assays, in cultured rat O2A cells. Data are expressed as percentages of control and represent means  $\pm$  SEM. \*P < 0.001.

## Discussion

Our results show a difference in the expression pattern of IGFBPs between oligodendrocytes at the edges of chronic MS plaques and oligodendrocytes in normal cerebral white matter. Oligodendrocytes in control cerebral white matter and normal appearing white matter from MS patients were immunoreactive for IGFBPs-1 to -5. Oligodendrocytes at the margins of periplaque white matter displayed a similar degree of immunoreactivity for IGFBPs-2, -3, -4 and -5 as oligodendrocytes in control cerebral white matter and normal appearing white matter in MS, but they were also IGFBP-6 positive and IGFBP-1 immunoreactivity was enhanced. Gveric and co-workers, who investigated IGFBPs-1, -2, and -3 in MS lesions, also found that oligodendrocytes surrounding plaques expressed high amounts of IGFBP-1<sup>14</sup>. IGFBPs that are present at the level of target cells regulate the presentation of IGF-I to IGF-I receptors<sup>2, 10</sup>. IGFBP-1 has an inhibitory effect on IGF-I induced oligodendrocyte survival and myelination as evidenced by a study in transgenic mice that overexpress IGFBP-1<sup>39</sup>, and experiments on rat oligodendrocyte progenitor cells<sup>16</sup>. We confirmed that IGFBP-1 inhibits IGF-I mediated oligodendrocyte survival and the formation of CNPase<sup>16</sup>, which is a myelin protein that plays a role in the synthesis and maintenance of the myelin sheath<sup>12</sup>. Little is known about the role of IGFBP-6. We found that IGFBP-6 had the same inhibitory effect on cell survival and formation of CNPase as IGFBP-1. Taken together, our data suggest that an enhanced expression of IGFBPs-1 and -6 in oligodendrocytes in periplaque white matter in MS reduce binding of IGF-I to the IGF-I receptors. This may explain why IGF-I receptors in periplaque oligodendrocytes were upregulated.

A reduction in the bioactivity of IGF-I may contribute to the loss of oligodendrocytes. IGF-I is an important survival factor for oligodendrocytes<sup>1, 23, 25, 40</sup>, and it also protects oligodendrocytes and their precursors from the deleterious effects of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a cytokine released in MS lesions<sup>40</sup>. In mouse glial cultures, TNF- $\alpha$  decreased oligodendrocyte number by approximately 40% and doubled the number of apoptotic oligodendrocytes and their precursors, and this effect was greatly reduced by IGF-I<sup>40</sup>.

In many experimental models of demyelinating disease in the CNS, remyelination of denuded axons is a relatively rapid and efficient process<sup>18</sup>. Current concepts suggest that remyelination requires generation of new oligodendrocytes from precursor cells. Oligodendrocyte progenitor cells are present in the adult CNS, and they can differentiate into premyelinating oligodendrocytes<sup>18</sup>. Both oligodendrocyte precursors and premyelinating

oligodendrocytes have been demonstrated in chronic MS lesions, but they do not induce remyelination<sup>7, 8, 21, 26, 32, 36, 37</sup>.

It appears that oligodendrocytes in MS lesions do not receive the necessary signal(s) to myelinate axons. A reduced bioactivity of IGF-I, due to an increased expression of IGFBPs-1 and -6, may play an important role in the failure of remyelination of MS lesions.

We can only speculate about the mechanism(s) underlying the increased expression of IGFBPs-1 and -6 on oligodendrocytes, but cytokines and reactive oxygen species, which are formed in MS lesions, may play a role. It has been demonstrated that the proinflammatory cytokines interleukin (IL)-1, IL-6, and TNF- $\alpha$ , and reactive oxygen species, which are present in lesions of MS<sup>5, 33</sup>, produce dose-dependent increases in IGFBP-1 expression in Hep G2 human hepatoma cell lines<sup>4, 17, 31</sup>. IL-1 $\beta$  and TNF- $\alpha$  also enhance the production of IGFBP-6 in fibroblasts<sup>19</sup>.

It is likely that a number of interrelated factors are involved in the loss of oligodendrocytes and the failure of remyelination in MS. The present study suggests that an up-regulation of IGFBPs-1 and -6 in oligodendrocytes in MS lesions may play a role. Our findings may have implications for therapeutic strategies aimed at protecting oligodendrocytes and promoting myelin repair in MS. It may explain why a pilot study with recombinant IGF-I in patients with MS had no effect on new lesion formation and remyelination<sup>13</sup>. Several options are worthwhile to explore. One could develop pharmacological approaches to reduce the expression of IGFBP-1 and IGFBP-6 in oligodendrocytes in MS. Nonpeptide small molecules have been developed that act as IGFBP ligand inhibitors and prevent binding of IGF-I to IGFBPs, resulting in an elevation of free biological available IGF-I at the level of the oligodendrocytes<sup>9, 20, 29</sup>. Another approach could be to use IGF-I analogues with high affinity for IGF-I receptors and little or no affinity for IGFBPs.

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