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COMPLEMENT C5 REGULATES THE EXPRESSION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN CHRONIC EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Abstract

Complement activation plays a central role in autoimmune demyelination. To explore the possible effects of C5 on post-inflammatory tissue repair, we investigated the transcriptional profile induced by C5 in chronic experimental allergic encephalomyelitis (EAE) using oligonucleotide arrays. We used C5-deficient (C5-d) and C5-sufficient (C5-s) mice to compare the gene expression profile and we found that 390 genes were differentially regulated in C5-s mice as compared to C5-d mice during chronic EAE. Among them, a group of genes belonging to the family of insulin-like growth factor binding proteins (IGFBP) and transforming growth factor (TGF)- β 3 were found most significantly differentially regulated by C5. The dysregulation of these genes suggests that these proteins might be responsible for the gliosis and lack of remyelination seen in C5-d mice with chronic EAE.

Keywords

Experimental allergic encephalomyelitis; Complement; C5b-9; Insulin-like growth factor binding proteins; Connective tissue growth factor

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INTRODUCTION

Complement activation and the assembly of the terminal complement complex C5b-9, consisting of the C5b, C6, C7, C8, and C9 proteins, play a significant role in tissue damage and repair in a variety of central nervous system (CNS) disorders, including multiple sclerosis (MS) (reviewed in (Rus and Niculescu 2001)). By forming pores in the plasma membrane, C5b-9 can cause cell death and also induce apoptosis (Cragg et al. 2000; Papadimitriou et al. 1994; Papadimitriou et al. 1991). However, nucleated cells, including oligodendrocytes (OLG), can survive limited C5b-9 complement attack through the protection provided by complement-inhibitory membrane proteins and by eliminating membranes carrying C5b-9 complexes (Carney et al. 1985; Scolding et al. 1989). In the absence of the complement inhibitory protein CD55 (Koski et al. 1996), myelin is vulnerable to C5b-9 attack. C5b-9 can have both detrimental and beneficial effects on inflammatory demyelination (Cudrici et al. 2005). It has a detrimental effect on myelin by forming membrane pores (Shirazi et al. 1987; Vanguri and Shin 1988) and inducing myelin vesiculation in cerebellar explants (Liu et al. 1983), and demyelination is more prominent in C6-sufficient rats than in C6-deficient rats during acute EAE (Mead et al. 2002). The beneficial role of C5b-9, primarily seen at sublytic doses in OLG, involves the ability of this complement complex to rescue OLG from apoptotic death (Soane et al. 2001).

We have recently shown that C5b-9 at sublytic doses inhibits Fas/FasL-induced OLG apoptosis (Cudrici et al. 2006) as well as the mitochondrial pathway of apoptosis (Soane et al. 2001; Soane et al. 1999). Sublytic C5b-9 effectively rescues OLG from apoptosis, and this process is mediated by Gi-dependent and G β γ -mediated activation of the ERK1 and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Rus et al. 1997; Rus et al. 1996; Soane et al. 2001).

This protective effect of C5b-9 has also been demonstrated *in vivo* during EAE. In the chronic phase of EAE, we found that C5-s mice showed extensive remyelination that was associated with axon preservation, in contrast to the severe Wallerian degeneration and axonal depletion associated with severe gliosis that were seen in C5-d mice (Weerth et al. 2003). Our studies have indicated that the presence of C5, most likely in the form of C5b-9 complexes, may be responsible for producing more efficient remyelination, axon survival, and less scarring in chronic EAE. In subsequent studies, we have demonstrated a reduction in OLG apoptosis in C5-s when compared to C5-d mice during EAE (Niculescu et al. 2004).

Although it is likely that the enhanced remyelination reflects the ability of C5b-9 to enhance OLG survival, very little is known about the molecular events involved in the remyelination mediated by OLG that survive sublethal C5b-9 attack or the factors involved in protection from gliosis. In order to understand these processes, we undertook a gene pattern analysis in C5-d and C5-s mice with and without EAE.

In the present report we demonstrate that complement C5 regulates many genes involved in cell cycle activation and signal transduction. We found that in chronic EAE, some of the most differentially regulated genes by complement C5 belonged to the insulin-like growth factor proteins and TGF- β 3, which are known to be involved in the regulation of myelination and fibrosis. These findings suggest that complement C5, by regulating these genes, might not only improve the survival of OLG but also help prevent the gliosis seen in demyelinating CNS disorders.

MATERIALS AND METHODS

Induction of EAE

Adult female mice of a congenic outbred strain deficient in C5 (D10.D2/0SnJ) and C5-s controls (B10.D2/nSnJ) were generated by backcrossing C5-d for 7 generations and C5-s for 17 generations (Jackson Laboratories, Bar Harbor, ME). The mice were maintained in a barrier facility according to NIH guidelines. To induce chronic relapsing EAE (Weerth et al. 2003), the mice were immunized at 7–8 weeks of age with an subcutaneous injection of purified guinea pig myelin in an equal volume of incomplete Freund's adjuvant containing 70 µg of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI), and with pertussis toxin (100 ng) (List Biologicals, Campbell, CA) given intravenously on the same day. Mice were weighed and observed daily for clinical signs of EAE and graded in a blinded manner for neurological deficits on a scale of 0–5, as follows: 0.5 = tail weakness; 1.0 = tail paralysis; 2.0 = hind limb weakness and abnormal gait; 3.0 = paraplegia; 3.5 = tetraplegia; 4.0 = quadriplegia; 5.0 = moribund state or death. Mice, under terminal anesthesia, were sacrificed 11–14 days, 21–25 days, or 90 days post-immunization (acute EAE, recovery, and chronic phase, respectively) by transcardial perfusion with cold 4% PFA in PBS in the case of mice used for immunocytochemical analysis, or with cold sucrose in PBS in those used for total RNA isolation.

RNA purification, cRNA synthesis, and hybridization

Control uninjected mice, mice with early acute EAE (11–14 days post-immunization), recovery phase (21–25 days p.i.), or with chronic EAE (60–90 days p.i.) were perfused with cold PBS and dissected. Spinal cords were frozen in liquid nitrogen and stored at –80°C until analyzed. Samples were pooled from four mice for each time point in the case of both C5-s and C5-d mice. Total RNA was purified using Trizol extraction and RNeasy cleanup (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. The quality of the total RNA preparation was assessed by determining the A₂₆₀/A₂₈₀ ratio by electrophoresis on an Agilent Bioanalyzer (Agilent, Foster City, CA). Only samples with intact 28S and 18S ribosomal RNA peaks with ratios above 1.9 were used for microarray analysis. For each experimental condition, 10 µg of very high quality total RNA was provided to the UCLA Genome Center (Los Angeles, CA) as part of an agreement with the NINDS/NIMH Microarray Consortium, which carried out the cRNA labeling, hybridization, and data analysis as described below. The Affymetrix mouse expression set 430 2.0 array chip (Affymetrix, Santa Clara, CA) was used for hybridization. This two-array set GeneChip is comprised of over 45,000 probe sets, including over 34,000 known mouse genes. Preparation of labeled cRNA for hybridization to Affymetrix GeneChips followed the recommended Affymetrix protocol. Double-stranded cDNA was synthesized from 5 µg of total RNA using the Superscript Choice System (Invitrogen), with an HPLC-purified oligo (dT)₂₄ primer containing a T7 RNA polymerase promoter sequence at its 5'-end (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄-3'), (Genset Corp., La Jolla, CA). The second cDNA strand was synthesized by using *E. coli* DNA polymerase I, RNase H, and DNA ligase. After second-strand synthesis, the reactions were cleaned, and the cDNA was eluted with the GeneChip Cleanup Module. Labeled cRNA was generated from cDNA by *in vitro* transcription using a GeneChip IVT Labeling Kit according to the manufacturer's instructions and incorporating biotinylated CTP and UTP. Biotin-labeled cRNA was purified using the GeneChip Cleanup Module prior to fragmentation to a size of 35–200 bases by incubation at 94° C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1, with 100 mM potassium acetate, 30 mM magnesium acetate). The integrity of the cDNAs, cRNAs, and fragmented cRNAs was assessed by running aliquots on the bioanalyzer. After prehybridization, the solution was removed and replaced with 80 µl of hybridization mixture containing hybridization buffer, fragmented cRNA (0.05 µg/µl), and herring sperm DNA (0.1 µg/µl; Promega). The hybridization buffer also included acetylated

BSA (0.5 µg/µl, Invitrogen) and four control bacterial and phage cRNAs (1.5 pM BioB, 5 pM Bio C, 25 pM BioD, and 100 pM Cre) as internal controls for hybridization efficiency. Arrays were hybridized for 16 h at 45° C, then washed using an Affymetrix fluidics station and stained with streptavidin-phycoerythrin (10 µg/ml, Molecular Probes) according to the Affymetrix technical manual. Washed arrays were scanned on an Affymetrix GeneChip Scanner 3000.

Affymetrix GeneChip analysis and data mining

Robust complement C5 gene profiles were obtained using pooled spinal cord samples from EAE mice. Raw data (CEL files) (available at <http://microarray.genetics.ucla.edu/geneexp/public/>) were computed and normalized using the Bioconductor R package Affy (www.bioconductor.org) and GCRMA software (Wu 2004). For each comparison, the differentially expressed genes were identified using an ANOVA approach, implemented in the Bioconductor R package LIMMA (Gentleman), or non-parametric methods (e.g., Kruskal-Wallis- or Mann-Whitney U-tests) if the expression data were not normally distributed. We considered genes to be differentially expressed only if they met our criterion values for fold change and p-value. These differentially regulated genes were further characterized according to their role in biological processes using the Gene Ontology (GO) database. To determine whether the biological processes we identified were significantly overexpressed, the online version of Expression Analysis Systematic Explorer (EASE) (Hosack et al. 2003) (available at <http://david.niaid.nih.gov/david>) was used.

Real-time PCR

RNA samples (2 µg) were purified as described above and used to synthesize single-stranded cDNA. As an independent means of validating the microarray expression data, real-time quantitative PCR was performed using a LightCycler 2.0 system (Roche, Indianapolis, IN). Forward and reverse primers and hybridization probe sequences were provided by TIB Mol Biol LLC (Adelphia, NJ). Table 1 summarizes the primers and hybridization probes used as well as the sizes of the expected amplicons. Hybridization probes were labeled at the 3' end with fluorescein dye (FITC) or at the 5' end with LightCycler-Red 640 dye (LC640). Primer pairs were chosen to span one exon and yield 178- to 283-bp products (Table 1). Real-time PCR was performed according to the manufacturer's protocol using a LightCycler FastStart DNA MasterHybProbe (Roche) and Real-Time Light Cycle System (Roche Biochemicals). Real-time PCR was performed in glass capillaries with a final volume of 20 µl containing 50 ng cDNA, 10pM of each primer, 3pM of each hybridization probe, 2 µl LightCycler Fast-Start DNA Master HybProbe (Roche), 3mM MgCl₂, and sterile distilled water. Primers for the housekeeping gene 18S were designed by TIB Mol Biol LLC (Adelphia, NJ) and used in conjunction with LightCycler FastStart DNA Master SYBR Green I (Roche). The PCR reaction consisted of a 10-min denaturation step at 95°C, followed by 40 amplification cycles at the temperature appropriate for the genes selected, a 20-sec incubation at 95°C, and a cooling cycle at 40°C. As a negative control for each real-time PCR assay, the same reaction was performed in the absence of cDNA. The PCR products obtained were analyzed by electrophoresis. For each gene (performed in duplicate for each sample), the cycle threshold (C_T) values were determined from the linear region of the amplification plot and normalized by subtraction of the C_T value for 18S (generating a ΔC_T value). The -fold change (C5-s vs. C5-d) in target gene samples, after normalization with the housekeeping gene (18S), was calculated using the $2^{-\Delta\Delta C_T}$ value, where $\Delta\Delta C_T = \Delta C_T (C5-s) - \Delta C_T (C5-d)$ and ΔC_T is the C_T value of target gene subtracted from the C_T value of the housekeeping gene. All determinations were made in the exponential phase of the reactions (Livak and Schmittgen 2001).

Western blotting

Frozen spinal cords tissue were pooled from three mice and homogenized on ice in RIPA lysis buffer (10mM Tris, pH 7.4, with 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na₄P₂O₇, 1% Triton X-100, 0.1 % SDS, 100 mM NaCl, 10% glycerol, 0.5% sodium deoxycholate, 2mM Na₃VO₄). One tablet of complete mini protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) was added just prior to use. Lysates were placed in ice for 30 min, and protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Lysates (30 µg of protein) were fractionated on 10% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA) as previously described (Cudrici et al. 2005). The membranes were blocked with 0.1% Tween-TBS containing 1% bovine serum albumin (BSA) for 1 h and incubated with primary antibody overnight at 4° C. Goat anti-rabbit or rabbit anti-goat IgG HRP-conjugated antibodies (Santa Cruz Biotech) were applied for 1 h at room temperature. After washing, reactions were developed using enhanced chemiluminescence (ECL, Pierce). The following primary antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA): goat IgG anti-IGFBP-2, rabbit IgG anti-IGFBP-3, rabbit IgG anti-IGFBP-6, goat IgG anti-CTGF, and rabbit IgG anti-TGFβ3. Membranes were stripped using Restore Western Blot Stripping Buffer (Pierce) and reprobed for the expression of β-actin (Rockland Immunochemicals, Rockville, MD). The radiographic band density was measured using UN-SCAN-IT software (Silk Scientific, Orem, UT), and the results expressed as relative density ratios to β-actin.

Immunohistochemistry

Spinal cord cryostat sections (5 µm) were air-dried and fixed in acetone for 10 min. The sections were washed in PBS, and endogenous peroxidase was quenched with 0.3% hydrogen peroxide in PBS for 10 min. Slides were incubated overnight at 4° C with the primary antibody (anti-IGFBP- 3 and 6, CTGF and TGF- β3– all from Santa Cruz Biotech.), then washed with PBS and incubated with goat anti-rabbit HRP-conjugated IgG (Jackson ImmunoResearch Labs, West Grove, PA) or donkey anti-goat HRP conjugated (Santa Cruz) for 1 h at room temperature. The reactions were developed using Nova RED (Vector Labs, Burlington, CA) as the chromogen substrate, then the sections were washed in distilled water and counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO), dehydrated, mounted, and scored by two observers in a blinded fashion.

RESULTS

Effect of complement C5 on gene expression profiling during EAE

The transcriptional profile in the spinal cord was determined by microarray analysis using the Affymetrix Mouse Expression Set 430 2.0 array chip (Affymetrix, Santa Clara, CA). The presence of C5 in mice had a profound effect on the transcriptional profile. When we restricted the profile to those genes with a ≥2-fold change in control mice without EAE, we found that 2,511 genes were differentially regulated in C5-s and C5-d mice (Table S1, Supplemental Material). During EAE, 916 and 871 genes were differentially regulated in the acute and recovery phases, respectively. In contrast, only 390 were differentially regulated in chronic EAE (Table S1, Supplemental Material). According to the microarray analysis, the most highly up-regulated genes were the gamma-aminobutyric acid A receptor subunit alpha 6 (GABRA6) (up-regulated 1928-fold), transthyretin (up-regulated 390-fold), cerebellin precursor protein 3 (up-regulated 305-fold), and carbonic anhydrase 8 (CAR8) (up-regulated 235-fold) in control C5-s mice when compared with C5-d mice (Table S1, Supplemental Material). During the acute phase, many of the down-regulated genes were involved in muscle contraction. These included triadin (down-regulated 223-fold), myosin binding protein C, fast type (down-regulated 166-fold), motylin (down-regulated 141-fold) and troponin C2 fast (down-regulated 136-fold). During the recovery phase, the most highly regulated genes were the GABRA6

(down-regulated 789-fold), glutathione peroxidase 6 (up-regulated 370-fold), and cerebellin precursor protein (down-regulated 241-fold) (Table S1, Supplemental Material). In chronic EAE, 190 genes were up-regulated and 200 were down-regulated in C5-s when compared with C5-d mice. The most highly regulated genes were immunoglobulin heavy chain 4 (up-regulated 94-fold), H19 noncoding mRNA (down-regulated 24-fold), and zinc finger protein 236 (up-regulated 22-fold) (Table S1).

Differentially regulated genes were further characterized according to their roles in biological processes (at level 3), using the Gene Ontology (GO) database (Ashburner et al. 2000). In the case of chronic EAE, more than 25% of these differentially expressed genes were involved in cell growth and maintenance, signal transduction, and cell adhesion (Fig. 1). To determine whether the identified biological processes were represented by genes that were significantly overexpressed, we used the online version of EASE (Hosack et al. 2003), which performs a statistical analysis of gene categories in the gene list to find those categories that are most overexpressed (and can therefore be described as “themes” of the gene list). This analysis revealed that in the case of chronic EAE, genes involved in insulin-like growth factor binding, extracellular space, cell growth, cell adhesion, and chemokine activity were significantly over-represented among the differentially expressed genes (Table 1). Many of the genes belonging to these categories were classified in GO database as being involved in cell growth and maintenance. Because of the significant changes in the insulin growth factor binding protein family that occur during chronic EAE, we focused our attention on this family of genes.

Real-time PCR analysis

The differential expression of several of the genes that we identified was confirmed by real-time PCR using the primers pairs and hybridization probes listed in Table 1. These included GABRA6, CAR8 and NEUROD1 genes, which showed the highest level of regulation by microarray analysis. In addition, members of the IGFBP family (IGFBP-3, -4, and -6), CTGF, WISP2, and TGF- β 3 were also tested by real-time PCR (Fig. 2). There was good agreement between the real-time PCR and the array data (data not shown). In general, the results of the RT-PCR and microarray quantification were in agreement in terms of the direction of the change; for many of the genes, the -fold changes were comparable in both studies, but the magnitude of the changes was not always identical. For example, GABRA6 showed a 1,928-fold increase in microarray analysis, whereas the real-time PCR showed a 12,288-fold increase. Of interest, members of the IGFBP family showed significant changes (over 2-fold) in the real-time PCR in all the stages of EAE examined (Fig. 2), even when no change was revealed by microarray analysis. These data indicate that microarray analysis may not identify all the genes that are differentially regulated. For instance, during acute EAE, only IGFBP-3 was found to be differentially regulated by >2-fold in microarray analysis, but when we used real-time PCR, we saw changes of >2-fold for IGFBP-3, IGFBP-4, CTGF, and TGF- β 3. Similar discrepancies were seen during the recovery phase (Fig. 2). Only IGFBP-3 was found to be differentially expressed by microarray analysis (up-regulated by 2.3-fold), but by real-time PCR, all the tested family members were significantly up-regulated (Fig. 2). These differences may reflect either a lack of sensitivity or specificity of the probe sets on the gene chip or might be related to the subsequent analysis. It is potentially significant that these genes are differentially regulated in C5-d mice, which also show increased gliosis and decreased myelination (Weerth et al. 2003). In addition IGFBP family members are known to be involved in tissue fibrosis (Holly and Perks 2006) and myelination (Chesik et al. 2007).

Western blot and immunohistochemical staining

To further validate the results of the microarray and real-time PCR analyses, we investigated whether the changes seen at the mRNA level for the insulin-like growth factor family corresponded to changes in their protein levels. For this purpose we used western blotting to

assess the expression levels of IGFBP-2, IGFBP-3, IGFBP-6, CTGF, and TGF- β 3 in chronic EAE (Fig. 3). Our data indicated that IGFBP-2 and IGFBP-3 levels were higher in C5-s than in C5-d mice. In contrast, protein levels of IGFBP-6, CTGF, and TGF- β 3 were higher in C5-d mice (Fig. 3). These data clearly show that the changes in the mRNA levels were followed by similar changes in protein levels and further implicate these proteins in the pathogenesis of gliosis seen in C5-d mice. We then used immunohistochemical staining to determine the localization of IGFBP-3, IGFBP-6, and CTGF in spinal cord sections during EAE (Table 3). Our data showed that all three proteins were localized to the infiltrative cells during the acute and recovery phases of EAE (Table 3, Fig. 4). In addition, all three proteins were found to stain spinal neurons during the acute phase and recovery and to a lesser extent in chronic EAE (Table 3). The expression of IGFBP-3 and -6 in neurons was higher in the recovery phase than in the acute phase of EAE (Table 3). We also found that IGFBP-3, IGFBP-6 and CTGF co-localized with astrocytes (Table 3). Higher levels of CTGF were found in reactive astrocytes in C5-d than in C5-s mice in chronic EAE (Table 3, Fig. 4).

DISCUSSION

To date, the factors responsible for differences seen in remyelination and gliosis during EAE in the absence of complement C5 are not clearly understood. To explore this issue, we took a genomic approach and performed gene profiling to identify genes that are differentially expressed in C5-s and C5-d mice with and without EAE. To our knowledge, this is the first transcriptome analysis performed in normal C5-s and C5-d mice and also in such mice with EAE. In the present study, we have shown that complement C5 has a major impact on transcriptional regulation in control untreated mice. In addition, C5 also significantly affects gene expression during EAE. The only biologically active complement factors that are generated in C5-s but not in C5-d mice are the C5b-9 terminal complement complex and C5a, a cleavage product of C5. Since the course of EAE is not affected by C5a (Morgan et al. 2004; Reiman et al. 2002) and C5a is not involved in OLG survival (Rus et al. 1996; Soane et al. 2001), it is likely that in addition to C5, C5b-9 might be responsible for the effect on transcription.

For all the EAE time points studied, cell cycle genes were the category of genes that was most significantly regulated by C5 when GO data mining was used for analysis (data not shown). This result is consistent with our previous studies showing that C5b-9 is able to activate the cell cycle in OLG and other types of cells (Rus et al. 1996; Niculescu et al. 1997; Halperin et al. 1993). In the case of chronic EAE, GO data mining and particularly EASE analysis identified members of the insulin-like growth factor family as being the most significantly regulated by C5. IGFBPs are secretory proteins that bind IGF I and II and modulate their bioavailability to IGF receptors. However, they also have IGF-independent functions (Holly and Perks 2006) and are involved in cell differentiation and homeostasis of the connective tissue matrix in the CNS (Carson et al. 1993). Our data showing an association of increased IGFBP-4 and -6 and reduced IGFBP-2 expression with gliosis and an absence of remyelination in C5-d mice suggest a role for these proteins in mediating efficient recovery from acute EAE in the presence of C5. Their localization in reactive astrocytes (Table 3) (Chesik et al. 2007) and their role in extracellular matrix production and induction of fibrosis in other diseases (Boers et al. 2006; Pilewski et al. 2005; Yasuoka 2006) are also consistent with the robust gliosis seen in chronic EAE in the absence of C5. The low levels of IGFBP-2 found in C5-d mice are also significant since IGFBP-2 has been implicated in promoting myelination by inhibiting OLG cell death (Chesik et al. 2006) and inducing OLG progenitor cells survival and differentiation (Kuhl et al. 2002). In addition, modulation of IGF-1 action by IGFBP-2 might represent a key mechanism that restricts neuronal cell loss following hypoxic brain injury (Beilharz et al. 1998). High concentrations of IGFBP-3 and 6 in spinal motor neurons might also lead to the reduction in free biologically available IGF-I and prevent binding of IGF-1 to IGF-1 receptors.

In addition there are growing evidences that IGFBP-3 exerts antiproliferative and pro-apoptotic effects that are IGF-1 independent (Chesik et al. 2007). In a chronic-relapsing model of murine EAE, administration of IGF-1 and IGFBP-3 during EAE was associated with an increased severity in the relapses, demyelination and axonal damage (Lovett-Racke et al. 1998). In conclusion, our data suggest that dysregulation of IGFBPs expression may be involved in the astrogliosis and decreased myelination seen in C5-d mice.

In this study, we also found increased levels of CTGF and TGF- β 3 in C5-d mice during chronic EAE. CTGF is a member of the connective tissue growth factors/cysteine-rich61/neuroblastoma family (Brigstock 1999). CTGF binds TGF- β and enhances TGF- β -mediated signaling (Abreu et al. 2002). In particular, CTGF mediates many of the profibrotic actions of TGF- β (Grotendorst 1997), which is a well-known inducer of extracellular matrix components such as collagen and fibronectin (Frazier et al. 1996). Collagen deposition and anchorage-independent growth induced by TGF- β are inhibited by neutralizing antibodies against CTGF, indicating a synergistic relationship (Kothapalli et al. 1997). This synergistic relationship between CTGF and TGF- β was implicated in the formation of glial scar in studies of spinal cord injury in rat, in which TGF- β is the most potent cytokine expressed by reactive astrocytes (Logan and Berry 1993). Induction of CTGF has also been observed in glial scarring following CNS injury, in reactive-astrocytes, fibroblasts, and endothelial cells in the peri-lesional area (Conrad et al. 2005; Hamada et al. 1996; Holley et al. 2003). Our study showing pronounced gliosis in C5-d during chronic EAE may be directly related to the up-regulation of TGF- β , and subsequently of CTGF.

In conclusion, our results suggest that in a chronic EAE environment in the absence of C5, the balance between the various growth factors involved in connective tissue matrix homeostasis is shifted toward the profibrotic state. On the other hand, the presence of C5 in chronic EAE apparently induces genes that are involved in cell growth and survival. In particular, the expression of IGFBP-2 is consistent with the enhanced OLG survival and remyelination seen in C5-s mice with EAE (Niculescu et al. 2004; Weerth et al. 2003). Insulin-like growth factor binding proteins and TGF- β /CTGF may therefore represent important therapeutic targets for preventing gliosis and promoting remyelination in diseases such as MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Gene ontology data mining

The differentially regulated genes in chronic EAE were characterized according to their biological process classification (at level 3) in the GO database (Ashburner et al. 2000). The highest number of genes belonged to the GO category of cell growth and maintenance; 109 genes did not have a GO classification.

Figure 2. Real-time quantitative RT-PCR for genes selected from the profile

Nine genes were analyzed by real-time PCR to confirm the microarray results. Down-regulated genes were arbitrarily assigned a negative value. Significant changes in the microarray results were found for GABRA6, CAR8, and NeuroD1 only in the baseline and recovery phase and were confirmed by real-time PCR. CTGF, IGFBP-3, -4, -6, TGF- β 3, and WISP2 were tested in all samples.

Figure 3. Western blotting for IGFBP-2, IGFBP-3, IGFBP-6, CTGF, and TGF-β3 in chronic EAE Spinal cords from C5-s and C5-d mice with chronic EAE were lysed in RIPA buffer, and equal amounts of protein were resuspended in Laemli buffer and fractionated on 10% PAGE gels, followed by transfer to nitrocellulose.

A. Expression of IGFBP-3, IGFBP-6, CTGF, and TGF-β3 was examined by western blotting using specific antibodies.

B. Expression of IGFBP-2 was examined by Western blotting.

C. Results are shown as density ratios to actin in the lower panel.

Figure 4. Immunohistochemical localization of IGFBP-3, IGFBP-6 and CTGF in chronic EAE
 Cryosections prepared from the spinal cords were stained for IGFBP-3, IGFBP-6 and CTGF.
IGFBP-3: Sections from C5-d mice with acute EAE revealed similar staining of inflammatory infiltrates when compared to the staining in spinal cords of C5-s mice. Original magnifications: $\times 400$.
IGFBP-6: Left panel: During the recovery phase of EAE, the staining was weaker in inflammatory infiltrates but extensive in gray matter. Right panel: IGFBP-6 positive staining of spinal neurons. Insert: higher magnification of an IGFBP-6 positive nuclear staining of neurons. Original magnifications: Left panel: $\times 100$, Right panel $\times 400$; insert: $\times 1000$.
CTGF: C5-d mice with chronic EAE. Insert: higher magnification of CTGF staining.

CTGF: Immunostaining of reactive astrocytes in C5-d mice with chronic EAE. Insert: higher magnification of a CTGF positive reactive astrocyte. Original magnifications: $\times 400$; insert: $\times 1000$.

Table 1

The primers and hybridization probes for real time RT-PCR.

Gene Symbol	Primers and hybridization probes	Product (bp)
GABRA6	For: 5'-ACTCAAGTTTGGGAGCTATGC – 3' Rev: 5'-GACTGTCATGATTACATATTCACCAG – 3' Hyb 1 GAAGTCCCAGAGAATCTTCTAGCCTCC-FL Hyb 2 LC640-CCAGTATGATTTGATTGGGCAAACAGT-PH	183
CAR8	For: 5'-AGGCTGAGGACACATGTCAA – 3' Rev: 5'-GTGGGACCACTGTCTTCTCA – 3' Hyb 1 CGGATGACTCTATCACTCAGGGGC-FL Hyb 2 LC640-GGGTAGGTCGGAAATGTCTCCCA	178
NEUROD1	For: 5'-TGAGATCGTCACTATTCAAGAACCT – 3' Rev: 5'-AGTCCTCCTCTGCATTCATGG – 3' Hyb 1 TCTCAGTTCTCAGGACGAGGAACACG-FL Hyb 2 LC640-GGCAGACAAGAAAGAGGACGAGCTT-PH	199
CTGF	For: 5'-CCTGCCCTAGCTGCCTAC – 3' Rev: 5'-GCACTTTTGGCCCTTCTTAA – 3' Hyb 1 TGTGGTCTGGACCAAGGACAGT-FL Hyb 2 LC640-GGCTCGCATCATAGTTGGGTC-PH	239
IGFBP3	For: 5'-GCAGCCTAAGCACCTACC – 3' Rev: 5'-GCTTAGACTCGGAGGAGAAGTTC – 3' Hyb 1 AAAGCCAGGTGTGCCCCAGC-FL Hyb 2 LC640-CACACCGAGTGACCGATTCCAAGTT-PH	251
IGFBP4	For: 5'-AGAGCCGTACCCACGAA – 3' Rev: 5'-CTCACTCTTGAAGCTGTAC – 3' Hyb 1 CTGTCTTCCGATCCACACACC-FL Hyb 2 LC640-CACTTGCCACGCTGTCCGT-PH	283
IGFBP6	For: 5'-GCAGCAGCTCCAGACTGA – 3' Rev: 5'-CATTGCTTCACATACAGCTCAA – 3' Hyb 1 GCCAGCCTTTGCCAGTGTC-FL Hyb 2 LC640-CCAGATGGTCAAGGAAGCACTCAGT-PH	212
TGFB3	For: 5'-CCTGGAGGAGAACTGCTGT – 3' Rev: 5'-GGTGTGTATAGTCCAAGCACC – 3' Hyb 1 CCTTAGGTTCTGTGGACCCATTGCC-FL Hyb 2 LC640-GCCTAGATCCTGCCGGAAGTCAATA-PH	171
WISP2	For: 5'-CAATCCAGCCCTCCTCAG – 3' Rev: 5'-ACTGTTCCATGAGCCGTG – 3' Hyb 1 CCATCGGCAGATGCAGGAG-FL Hyb 2 LC640-GACAAGGGCAGAAAGTTGGTGTCT-PH	235

For, forward primer; Rev, Reverse primer; Hyb 1, Hyb 2, hybridization probes; Bp, base pairs; GABRA6, Gamma-aminobutyric acid A receptor alpha 6; CA8, carbonic anhydrase VIII; NEUROD1, neurogenic differentiation 1; CTGF, connective tissue growth factor; IGFBP, insulin-like growth factor binding protein; TGFB3, transforming growth factor beta 3; WISP2, WNT1 inducible signaling pathway protein 2.

Table 2

EASE over representation analysis of the genes differentially expressed in chronic EAE.

Description	Number of genes	p-value	Fisher Exact
Insulin-like growth factor binding	7	2.9×10^{-7}	8.4×10^{-9}
Extracellular space	68	5.5×10^{-7}	3.1×10^{-7}
Extracellular	71	1.2×10^{-6}	6.7×10^{-7}
Cell growth	9	1.8×10^{-6}	1.5×10^{-7}
Regulation of cell growth	8	5.3×10^{-6}	3.9×10^{-7}
Cell adhesion	24	5.6×10^{-6}	1.8×10^{-6}
Extracellular matrix structural constituent conferring tensile strength	7	1.2×10^{-5}	7.2×10^{-7}
Collagen	7	1.4×10^{-5}	9.2×10^{-7}
Growth factor binding	7	1.7×10^{-5}	1.1×10^{-6}
Extracellular matrix structural constituent	8	4.2×10^{-5}	4.4×10^{-6}
Chemokine activity	6	0.0003	2.6×10^{-5}
Chemokine receptor binding	6	0.0003	2.6×10^{-5}
Extracellular matrix	14	0.0003	8.5×10^{-5}
G-protein-coupled receptor binding	6	0.0005	5×10^{-5}
Regulation of biological process	12	0.003	0.001
Hydrolase activity	5	0.005	0.0006
Regulation of cellular process	10	0.007	0.002

Categories with the lowest EASE score are significantly overrepresented in the list.

Table 3
Expression of CTGF, IGFBP-3 and IGFBP-6 during EAE

Protein	Expression	Acute		Recovery		Chronic	
		C5-s	C5-d	C5-s	C5-d	C5-s	C5-d
CTGF	Inflammatory infiltrate	+	++	+	+	-	-
	Neurons	-	++	-	+	-	-
	Blood vessels	-	+	-	-	-	-
IGFBP-3	Astrocytes	-	++	-	+	+	++
	Inflammatory infiltrate	+++	+++	+++	++	-	-
	Neurons	+	+	++	++	+	+
	Blood vessels	ND	+	ND	++	++	-
	Astrocytes	+	+	++	++	-	++
IGFBP-6	Inflammatory infiltrate	+++	++	+++	+	-	-
	Neurons	+	-	+++	++	+	++
	Blood vessels	+	-	+++	ND	-	-
	Astrocyte	+	-	ND	++	+	+

Data expressed as Mean±SEM (n=3). ND=not determined. -, negative; +, slightly positive, ++, positive, +++, highly positive.