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RESEARCH ARTICLE

Extracellular Matrix in Multiple Sclerosis Lesions: Fibrillar Collagens, Biglycan and Decorin are Upregulated and Associated with Infiltrating Immune Cells

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Abstract

Extracellular matrix (ECM) proteins can modify immune reactions, e.g. by sequestering or displaying growth factors and by interacting with immune and glial cells. Here we quantified by quantitative polymerase chain reaction (qPCR) expression of 50 ECM components and 34 ECM degrading enzymes in multiple sclerosis (MS) active and inactive white matter lesions. *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* chains were induced strongly in active lesions and even more in inactive lesions. These chains interact to form collagen types I, III and V, which are fibrillar collagens. Biglycan and decorin, which can decorate fibrillar collagens, were also induced strongly. The fibrillar collagens, biglycan and decorin were largely found between the endothelium and astrocytic glia limitans in the perivascular space where they formed a meshwork which was closely associated with infiltrating immune cells. In active lesions collagen V was also seen in the heavily infiltrated parenchyma. Fibrillar collagens I and III inhibited *in vitro* human monocyte production of CCL2 (MCP-1), an inflammatory chemokine involved in recruitment of immune cells. Together, ECM changes in lesions with different activities were quantified and proteins forming a perivascular fibrosis were identified. Induced fibrillar collagens may contribute to limiting enlargement of MS lesions by inhibiting the production of CCL2 by monocytes.

INTRODUCTION

The pathological hallmarks of active multiple sclerosis (MS) lesions are blood–brain barrier (BBB) disruption, inflammation and demyelination with axonal damage. The interactions between infiltrating immune cells and the central nervous system (CNS) environment, made up of both cellular surfaces and the extracellular matrix (ECM), contributes to control the progression of MS lesions.

ECM, the ground substance found in the interstitial spaces of all organs, provides support to cells. The ECM makes up about one-fifth of the normal brain (7, 38). Under normal conditions, the ECM, has a unique composition in the CNS as it contains relatively small amounts of fibrous proteins (collagens, laminins and fibronectin), and high amounts of linear polysaccharides [glycosaminoglycans (GAGs) such as hyaluronan, chondroitin sulfate and heparan sulfate] (32, 36, 53). Endothelial cells, astrocytes, neurons, microglia and other CNS resident cells can synthesize and secrete ECM proteins (47, 48). The ECM has traditionally been considered to play predominantly a structural role, but recently additional features of the CNS ECM have emerged. During development the ECM is involved in migration, maturation, differentiation and survival of neurons (34). In adults the ECM not only provides physical support for CNS resident cells, but also regulates ionic and nutritional homeostasis (5, 36, 57). Furthermore, the ECM binds both growth promoting and growth inhibitory factors and acts as their reservoir (11, 12, 21, 22, 40).

Recent studies showed a complex alteration in the CNS ECM during the course of MS, including altered expression of both parenchymal and basement membrane related ECM proteins (50, 52). In particular, expression of chondroitin and dermatan sulphate proteoglycans in active MS lesions is changed and foamy macrophages accumulate these proteoglycans together with myelin breakdown products (43). Loss of tenascin-C and -R immunoreactivity is seen in acute MS lesions (15). The basement membrane protein vitronectin is enhanced in the blood vessel walls of active MS lesions, at the border of chronic active lesions and on some hypertrophic astrocytes (45). Finally, altered profiles of different laminin isoforms in the basement membrane of inflamed blood vessels and increased immunoreactivity for fibronectin, agrin and collagen IV have been observed around blood vessels (14, 44, 50).

Experimental autoimmune encephalomyelitis (EAE) studies have indicated decisive roles for basement membranes and their laminins for immune cell entry into the CNS (1, 35, 54). In addition, the ECM may cause the failure of MS lesions to remyelinate as hyaluronan, a major component of the ECM in demyelinated lesions, interferes with oligodendrocyte maturation (4, 42). Further understanding of the ECM changes in MS lesions and the impact of these changes on infiltrating immune cells and CNS resident cells is of importance in understanding the dynamics of MS lesion development. Therefore, we dissected active and inactive lesions as well as unaffected white matter from control brain and determined the expression of 50 ECM molecules and 34 ECM modifying enzymes by quantitative polymerase chain reaction (qPCR). Fibrillar collagens were the ECM molecules most strikingly induced in MS lesions. They localized to the perivascular space where they were closely associated with infiltrating immune cells. *In vitro* experiments revealed decreased production of monocytic CCL2 (MCP-1) in the presence of fibrillar collagens, which may inhibit further immune cell recruitment to MS lesions.

MATERIALS AND METHODS

Tissue samples

A total of 46 tissue blocks from 25 MS patients and six controls without clinical or histological evidence of CNS disease was analyzed (Supporting Information Table S1). The tissue blocks comprised 27 frozen specimens and 19 formalin-fixed, paraffin-embedded (FFPE) samples. Autopsy samples were obtained from the BrainNet Europe, the UK MS Brain Bank, the NeuroResource tissue bank at the UCL Institute of Neurology in London and the Center for Brain Research, Vienna. Some tissue samples were provided by the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam; all material has been collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. The study was approved be the ethical committee of the Medical Faculty of Ludwig Maximilians University, Munich, Germany.

MS lesions were classified according to defined criteria: Active demyelinating lesions contained abundant macrophages with degraded myelin products [Luxol fast blue (LFB) or oil red O positive] either throughout the lesion (acute plaques) or as a broad rim around the lesion edge (chronic active plaques). Inactive demyelinated lesions were sharply demarcated from the normal appearing white matter (NAWM) and without LFB or oil red O positive macrophages and a rim of microglial activation. Slowly expanding lesions revealed mild to moderate microglia activation at the lesion edge with few macrophages containing myelin debris.

Dissection of MS lesions

Seven demyelinated inactive and four demyelinated active white matter lesions were macrodissected manually. Cryosections (20 μm) from the tissue samples were mounted on PEN slides (P.A.L.M. Microlaser, Bernried, Germany). Every sixth section (30 μm) was stained with LFB to identify demyelinated areas and the unstained sections were superimposed on stained LFB sections. The lesion area was marked and manually macrodissected. In total, 200–300 μm of each block was used. Macrodissected sections were then stained with LFB to check the dissected area. In addition, four blocks containing actively demyelinating lesions were used without macro dissection. Control tissue samples used for qPCR contained exclusively white matter.

RNA extraction, cDNA synthesis and qPCR

We used seven demyelinated inactive and eight demyelinated active lesions and six control white matter blocks. RNA was extracted twice with Trizol (TRI® Reagent, SIGMA, Munich, Germany), and cDNA was synthesized using random hexamers (High Capacity cDNA Reverse Transcription kit from Applied Biosystems (ABI; Darmstadt, Germany). qPCR was performed for 84 ECM-related genes (50 ECM components and 34 ECM modifying enzymes) using custom-made low-density arrays (LDA) (ABI). These genes are shown in Table 1 and Table 2. Data analysis was carried out using RQ Manager 1.2 software (ABI), taking *GAPDH*, *βactin* and *PPIA* as housekeeping genes.

RNA from cultured cells was isolated by lysing cells with Trizol and subsequently using RNeasy columns with DNase digestion step (Qiagen, Hilden, Germany). cDNA was synthesized using random hexamers (High Capacity cDNA Reverse Transcription kit, ABI). Genes selected for LDA for monocyte gene expression analysis included *CD80*, *PDL1-B7-H1*, *PDL2*, *CD69*, *CD200R*, *SIRPα*, *CD206*, *ADORA2A*, *IL-1R2*, *SLAM*, *IL-1*, *IL-6*, *IL-10*, *TNF*, *TGFβ*, *IL-12p40*, *CXCL10*, *CCL18*, *MPO*, *MMP9*, *CCR2*, *CX3CR1*, *CD62L*, *CD204*, *MT-2A*, *BDNF*, *NGF*, *NT-3*, *NT-4*, *NRTN*, *LIF*, *IGF-1*, *IL-4*, *IL-5*, *IFNγ*, *CD36*, *CD163*, *MFGE8*, *MERTK*, *CXCL8*, *CXCL2*, *CCL2*, *CCL5*, *CCL3*, *CCL4*, *BAFF*, *GAPDH*, *PPIA*.

Immunohistochemistry

Cryosections were fixed in 4% paraformaldehyde (PFA) and analyzed by LFB, H&E (hematoxylin and eosin), oil red O, and also CD68 immunohistochemistry (DAKO, Hamburg, Germany). Immunohistochemistry was performed using mouse peroxidase anti-peroxidase (PAP) or rabbit PAP system (DAKO). Cryosections were fixed with 4% PFA and endogenous peroxidase activity was blocked using 1.5% methanolic hydrogen peroxide. Primary antibodies were directed against collagen I (mAb, Abcam, Cambridge, UK), collagen III (mAb, Abcam), collagen V (pAb; AbD SeroTec, Düsseldorf, Germany), biglycan (rabbit serum, a kind gift from Prof Larry Fisher, NIH, USA) and decorin (mAb: R&D and rabbit

Table 1. Absolute expression and fold-change values of 50 extracellular matrix (ECM) genes. Abbreviations: NB = normal control brain; De.in = demyelinated inactive lesion; De.act = demyelinated active lesion; De.in : NB = ratio of expression level in demyelinated inactive lesion/control brain; De.act : NB = ratio of expression level in demyelinated active/control brain; ND = not detectable because of low expression level. In total six control white matter samples from four subjects, seven demyelinated inactive lesions from five subjects, and eight demyelinated active lesions from seven subjects were used for quantitative polymerase chain reaction analysis.

Absolute expression in % GAPDH				Fold change	
Genes	NB	De.in	De.act	De.in : NB	De.act : NB
Fibrillar collagens (COL)					
COL1A1	0.08	0.87	0.45	10.86	5.63
COL1A2	0.34	0.40	0.74	1.18	2.18
COL3A1	0.06	0.72	0.32	12.00	5.33
COL5A1	0.06	1.57	0.15	26.17	2.50
COL5A2	0.05	0.10	0.15	2.00	3.00
COL5A3	0.44	0.79	0.68	1.80	1.55
Basement membrane collagens (COL)					
COL4A1	0.70	2.03	1.90	2.90	2.71
COL4A2	0.12	0.16	0.28	1.33	2.33
COL4A3	0.20	0.06	0.13	0.30	0.65
COL4A4	0.10	ND	0.11	*	1.10
COL4A5	0.65	1.07	0.84	1.65	1.30
COL4A6	0.15	0.12	0.20	0.80	1.33
Anchoring collagen (COL)					
COL7A1	0.51	0.60	0.71	1.18	1.39
Nidogens (NID)					
NID1	0.60	1.11	1.32	1.86	2.20
NID2	0.40	0.31	0.60	0.76	1.50
Laminins (LAMA)					
LAMA1	0.68	2.09	0.36	3.07	0.53
LAMA2	0.64	1.30	0.57	2.03	0.89
LAMA3	0.18	0.22	0.36	1.22	2.00
LAMA4	0.71	1.25	1.24	1.76	1.75
LAMA5	0.13	0.99	0.93	7.62	7.15
LAMB1	0.11	1.06	0.40	9.64	3.64
LAMB2	2.58	5.83	7.89	2.26	3.06
LAMB3	0.14	0.11	0.13	0.79	0.93
LAMC1	0.34	0.83	0.79	2.44	2.32
LAMC2	ND	ND	ND	*	*
Lecticans					
AGC1 (Aggrecan)	0.05	0.03	0.41	0.60	8.20
BCAN (Brevican)	8.00	7.22	8.61	0.90	1.08
CSPG3 (Neurocan)	13.89	7.40	8.80	0.53	0.63
CSPG2 (Versican)	0.01	ND	0.01	*	1.00
Small leucine rich proteoglycans (SLRPs)					
BGN (Biglycan)	4.83	11.17	11.41	2.31	2.36
DCN (Decorin)	2.99	6.27	5.41	2.10	1.81
FMOD (Fibromodulin)	0.03	0.07	0.09	2.33	3.00
LUM (Lumican)	0.09	0.15	0.24	1.67	2.67
Hyaluronan and proteoglycan link proteins (HAPLNs)					
HAPLN1	0.09	ND	0.02	*	0.22
HAPLN2	29.85	5.08	25.74	0.17	0.86
HAPLN3	0.05	0.29	0.36	5.80	7.20
HAPLN4	0.03	0.17	0.03	5.67	1.00
Heparan sulphate proteoglycan (HSPG)					
HSPG2 (Perlecan)	0.63	2.75	1.51	4.37	2.40
Tenascins (TNs)					
TNC	3.68	9.46	3.49	2.57	0.95
TNR	5.05	2.78	3.98	0.55	0.79

Table 1. *Continued*

Absolute expression in % GAPDH				Fold change	
Genes	NB	De.in	De.act	De.in : NB	De.act : NB
Thrombospondins (THBSs)					
THBS1	0.12	0.98	0.41	8.17	3.42
THBS2	2.75	4.22	4.05	1.53	1.47
THBS3	0.01	0.02	0.03	2.00	3.00
THBS4	0.83	1.08	1.09	1.30	1.31
Fibrillins (FBNs)					
FBN1	2.59	3.12	1.46	1.20	0.56
FBN2	0.04	0.03	0.09	0.75	2.25
FBN3	0.11	0.10	0.13	0.91	1.18
Others					
FN1 (Fibronectin)	2.60	4.86	3.94	1.87	1.52
RELN (Reelin)	0.08	0.63	0.04	7.86	0.50
VTN (Vitronectin)	0.03	0.03	0.03	1.00	1.00

*Calculation was not possible because at least one value was under the detection limit.

serum, also a kind gift from Prof Larry Fisher). Sections were incubated O/N at 4°C followed by secondary polyclonal rabbit anti-mouse (DAKO) or polyclonal swine anti-rabbit Ig (DAKO), for 1 h at room temperature and tertiary mouse PAP (mAb, DAKO) or rabbit PAP (pAb, DAKO), for 30 minutes at room temperature. Bound antibodies were detected with diaminobenzidine and sections were counterstained with hematoxylin. For immunofluorescence primary antibodies recognizing collagen V [pAb (rabbit), AbD SeroTec], CD31 (pAb, R&D, Wiesbaden, Germany), glial fibrillary acidic protein (GFAP; mAb, Molecular Probes, Karlsruhe, Germany, directly labeled with Alexa 488) were used. As secondary antibodies donkey anti-mouse Alexa 488, donkey anti-sheep Alexa 488 and donkey anti-rabbit Alexa 594 (all from Molecular Probes) were used. Confocal images were taken from Leica SP2UV microscope. Negative controls included omission of primary antibody. For FFPE tissue, antigen retrieval was performed by treatment with citrate buffer pH6 in a steaming water bath.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by density gradient centrifugation. Monocytes were isolated by positive selection with immunomagnetic beads (CD14 MicroBeads, Miltenyi Biotech, Bergisch Gladbach, Germany) and were grown in the presence and absence of ECM proteins (coated culture plates) for 24 h.

Tissue culture plates were coated with collagen I at 2 μg/cm^2 (BD Biosciences, Heidelberg, Germany); collagen III, 2 μg/cm^2 (BD Biosciences); collagen V, 2 μg/cm^2 (BD Biosciences); biglycan, 10 μg/mL (R&D); decorin, 10 μg/mL (R&D). All ECM proteins were diluted to final concentration with Ca^{2+}, Mg^{2+} free PBS (Gibco, Karlsruhe, Germany) and cell culture plates were incubated at RT for 2 h, and then washed with distilled water (Gibco) and air dried. All the ECM proteins were tested negative for presence of LPS (Biowhittaker™ LAL kit, Walkersville, MD, USA).

The myelin basic protein (MBP) specific T cell clone ES-BP8 (30) was stimulated with HLA-DR compatible PBMC and 20 μg/mL MBP (Biogenesis, Berlin, Germany) or 10 μg/mL MBP 29-48 peptide for 2 days. On the third day cultures were pulsed with 1 μCi/well of ^{3}H-thymidine (Amersham Biosciences, Freiburg, Germany) for 24 h.

Enzyme-linked immunosorbent assays (ELISAs)

To detect CCL2, CCL4, IL10, IL1β in cell culture supernatants the Duoset ELISA system (R&D) was used. Assays were performed according to the manufacturer's instructions.

RESULTS

Altered expression of ECM components in MS lesions

In this study we quantified the expression of 50 genes coding for proteins forming the ECM (Table 1) and for 34 enzymes modifying the ECM (Table 2) in control brain, active and inactive MS lesions. Of the 50 ECM genes tested, 22 were upregulated more than two-fold in active lesions and 21 in inactive lesions (Table 1). Fifteen ECM components were induced in both active and chronic inactive lesions. Twenty-three genes in inactive and 25 in active lesions were considered unchanged, that is, they had a change between 0.51- and 1.99-fold.

Upregulated ECM components included fibrillar collagens, basement membrane collagen, laminins, SLRPs, hyaluronan link proteins, thrombospondins and perlecan (details in Table 1).

Fibrillar collagens and SLRPs form a perivascular fibrosis in MS lesions and are in close interaction with the infiltrating immune cells in the perivascular space

Our expression profiling identified a total of 22 components upregulated in active MS lesions and 21 in inactive MS lesions. Considering the relative induction in MS lesions and the absolute expression level of the ECM genes, our interest was directed to the fibrillar collagens and the SLRPs. We noted a strong induction of

Table 2. Absolute expression and fold-change values of 34 ECM modifying enzymes. Abbreviations: NB = normal control brain; De.in = demyelinated inactive lesion; De.act = demyelinated active lesion; De.in : NB = ratio of expression level in demyelinated inactive lesion/control brain; De.act : NB = ratio of expression level in demyelinated active/control brain; ND = not detectable because of low expression level. In total six control white matter samples from four subjects, seven demyelinated inactive lesions from five subjects, and eight demyelinated active lesions from seven subjects were used for quantitative polymerase chain reaction analysis.

Absolute expression in % GAPDH				Fold change	
Genes	NB	De.in	De.act	De.in : NB	De.act : NB
A disintegrin and metalloproteinase domains (ADAMs)					
ADAM8	0.11	0.05	0.31	0.45	2.82
ADAM10	16.40	13.58	13.23	0.83	0.81
ADAM12	0.30	0.29	0.40	0.97	1.33
ADAM17	1.29	0.85	1.32	0.66	1.02
A disintegrin like and metalloproteinase with thrombospondin type 1 motifs (ADAMTs)					
ADAMTS1	7.38	7.88	3.92	1.07	0.53
ADAMTS4	16.04	3.24	15.15	0.20	0.94
ADAMTS5	0.04	0.17	0.06	4.25	1.50
Matrix metalloproteinases (MMPs)					
MMP1	ND	ND	ND	*	*
MMP2	0.61	1.09	3.36	1.79	5.50
MMP3	ND	ND	ND	*	*
MMP7	ND	ND	0.03	*	*
MMP8	ND	ND	ND	*	*
MMP9	0.01	0.05	0.13	5.00	13.00
MMP10	ND	ND	ND	*	*
MMP11	0.05	0.30	0.30	6.00	6.00
MMP12	ND	ND	ND	*	*
MMP13	ND	ND	ND	*	*
MMP14	1.00	5.71	4.61	5.71	4.61
MMP15	0.71	0.83	0.36	1.17	0.51
MMP16	0.75	0.97	1.03	1.29	1.37
MMP17	0.39	5.05	0.36	12.95	0.92
MMP19	0.01	0.04	0.08	4.00	8.00
MMP20	ND	ND	ND	*	*
MMP21	0.07	ND	0.07	*	1.00
MMP23	0.02	0.01	0.01	0.50	0.50
MMP24	0.37	1.21	0.34	3.27	0.92
MMP25	ND	ND	0.01	*	*
MMP26	ND	ND	ND	*	*
MMP27	ND	ND	ND	*	*
MMP28	0.24	0.75	0.08	3.13	0.33
Tissue inhibitor of metalloproteinases (TIMPs)					
TIMP1	0.97	5.96	7.00	6.14	7.22
TIMP2	13.78	9.56	13.82	0.69	1.00
TIMP3	9.21	22.98	15.51	2.50	1.68
TIMP4	0.88	0.69	0.98	0.78	1.11

*Calculation was not possible because the values were under the detection limit.

COL1A1, *COL3A1*, *COL5A1*, *COL5A2* chains, both in active and inactive demyelinated lesions (Table 1). These collagens interact to form collagen types I, III and V, which are grouped as fibrillar collagens, known to act as structural proteins (20). Biglycan and decorin, both classified as SLRPs, were also strongly induced in active and inactive demyelinated lesions (Table 1).

The ECM molecules identified by the transcript analysis were then localized by immunostaining. In control brain tissue we saw faint staining around blood vessels with antibodies to the three fibrillar collagens, decorin and biglycan (Figure 2E, H and data not shown). Similar stainings were observed in the NAWM (Figure 1C, and data not shown). In contrast, in both active and inactive MS lesions staining of fibrillar collagens, decorin, and biglycan was more intense (Figure 2A–D, F, G) and localized around small, medium and large blood vessels (Figure 1A).

In the larger blood vessels in MS lesions the extended perivascular (Virchow Robin) space was filled by a meshwork of fibrillar collagen, biglycan and decorin (Figure 2). In chronic active and

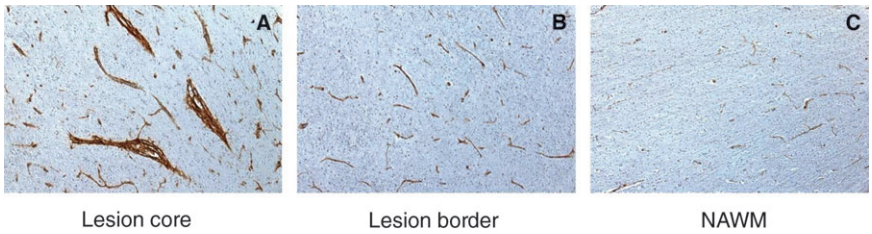

Figure 1. *Overview of the extent of induction of fibrillar collagens in multiple sclerosis (MS) lesions.* Immunostaining for collagen V is shown in **A–C**. Collagen V is deposited around small and large blood vessels in a chronic inactive MS lesion. The strong expression of collagen V in the lesion core decreases towards the lesion edge and in the normal appearing white matter (NAWM) it becomes similar to that of control brain. 3,3′-Diaminobenzidine (DAB) was used as a substrate for immunostaining and hematoxylin for counterstain. Original magnifications: A–C ×100.

also in acute lesions the infiltrating immune cells in the perivascular space were in close contact with fibrillar collagens, biglycan and decorin (Figure 2). Upregulation of these ECM molecules decreased with the distance from the lesion core and ultimately came close to the expression seen in the control brain (Figure 1A–C). The differences in the expression level of these ECM molecules in the lesion core compared to the surrounding NAWM shows that the observed induction of fibrillar collagens, biglycan and decorin is not an age-related phenomenon, but a disease-related phenomenon.

Double staining of lesions with the endothelial cell marker CD31 plus collagen V and GFAP plus collagen V located the collagens between the endothelial cells and the astrocytic glial limitans (Figure 3).

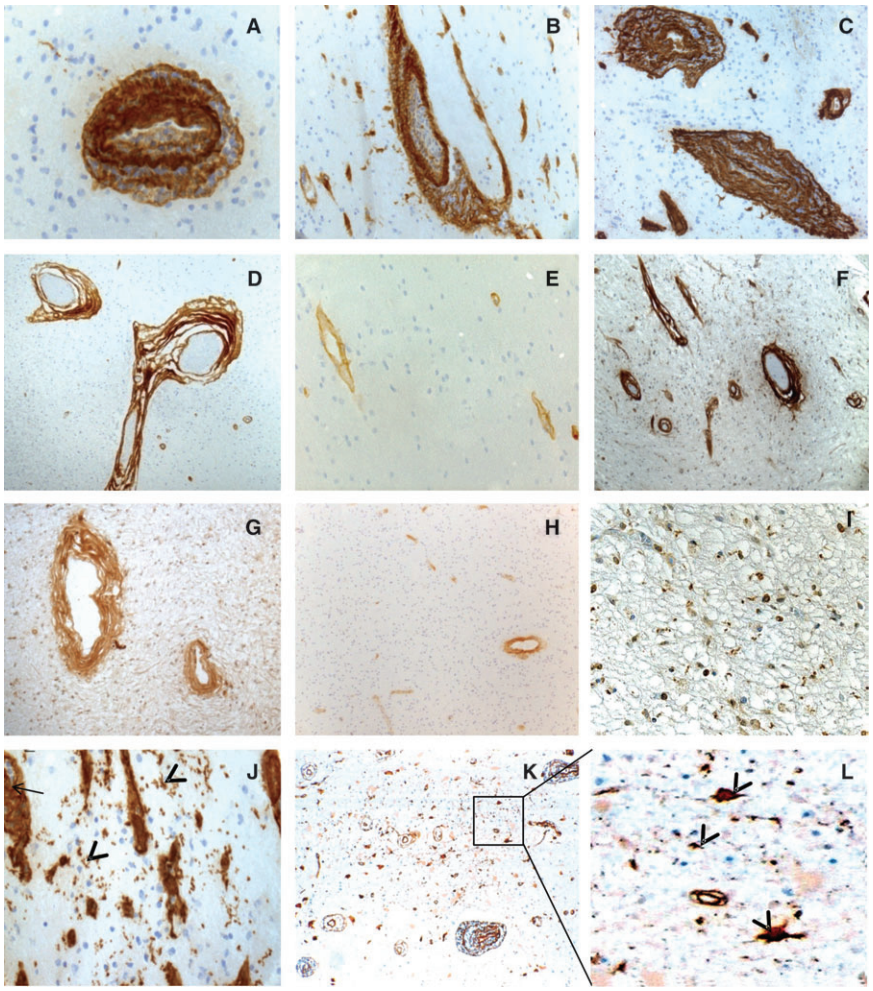

Figure 2. *Localization of upregulated extracellular matrix (ECM) components in multiple sclerosis (MS) lesions.* (**A–C**,**I–L**) Chronic active lesions; (**D**,**F**,**G**) chronic inactive lesions; (**E**,**H**) control white matter. The fibrillar collagens collagen I (**A**); collagen III (**B**,**D**); collagen V (**C**) form a meshwork in the perivascular space in MS lesions. In the control brain, fibrillar collagens are detected at lower levels around blood vessels than in chronic plaques (Collagen I in **E**). In active lesions (**J–L**) collagen V was found around blood vessels and in close interaction with the infiltrating immune cells in the PVS (arrow in **J**) and also outside the PVS (arrowhead in **J** and **L**; in **L** a higher magnification of the boxed area in **K** is shown). Biglycan is detected in infiltrating immune cells in active lesions (**I**) and in inactive lesions the fibrotic meshwork in the PVS also contains biglycan (**G**) and decorin (**F**). In control brain lower levels of biglycan (**H**) and decorin (not shown) are seen around blood vessels. DAB was used as a substrate for immunostaining and hematoxylin for counter stain. Original magnifications: **A**,**E**,**I**,**J**: ×400; **B**,**C**,**D**: ×200; **F–H**: ×100; **K**: ×250; and **L** is a sevenfold magnification of **K**.

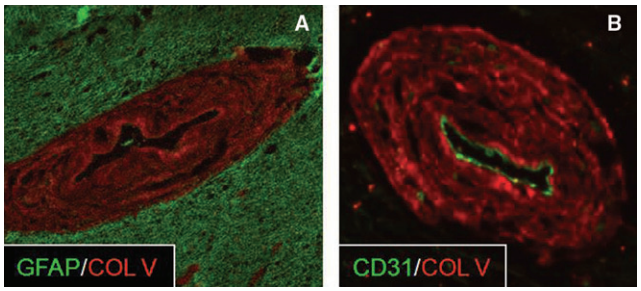

Figure 3. *Fibrillar collagens in the PVS.* The meshwork formed by fibrillar collagens is strictly limited to the PVS between endothelial cells (CD31 positive) and the astrocytic scar [glial fibrillary acidic protein (GFAP)-positive] in the parenchma. Double staining of collagen V (red) and GFAP (green) in **A** and collagen V (red) and CD31 (green) in **B**. Original magnifications: **A**,**B**: ×200.

Collagen V deposits were also observed in the parenchyma of 6/8 acute lesions, probably related to astrocytes (Figure 2J–L). Infiltrating immune cells in the parenchyma of active lesions stained positive for biglycan (Figure 2I).

ECM modifying enzymes in control brain and MS lesions

We quantified the expression of 34 enzymes that can, among other features, modulate ECM composition (Table 2). In control white matter, 7 out of 26 matrix metalloproteinases (MMPs), namely, MMP2, MMP14-17, MMP24 and MMP28 were expressed (>0.1% GAPDH). Ten of these enzymes were upregulated in active lesions and seven in inactive lesions (more than twofold; Table 2). Five of these, namely MMP9, MMP11, MMP14, MMP19 and tissue inhibitor of matrix metalloproteinase (TIMP) 1 were induced in both chronic inactive and active lesions. We also noted that additional ECM modulating enzymes were differentially regulated: MMP2, MMP9 and MMP19 were stronger induced in active lesions, whereas MMP17, MMP24 and MMP28 were stronger upregulated in inactive lesions. Among the four TIMPs analyzed, TIMP1 was most strongly enhanced. It was induced about six- to sevenfold in both active and inactive lesions, whilst TIMP3 was upregulated in inactive lesions and just slightly in active lesions (Table 2).

Fibrillar collagens upregulated in MS lesions modulate chemokine production by monocytes

Having noted a close association of infiltrating immune cells with fibrillar collagens, decorin, and biglycan in MS lesions, we tested the effect of these upregulated ECM proteins on the activation of MBP-specific T cells and cytokine/chemokine production by monocytes. We activated MBP specific T cells in the presence of fibrillar collagens (I, III, V) with MBP and PBMC as antigen-presenting cells. The stimulated antigen-specific proliferation was unchanged in the presence of either collagen I, III or V (data not shown).

Then, purified monocytes were cultured in the presence or absence of the fibrillar collagens, decorin and biglycan, and 46 immune-related genes (for gene list refer to Materials and Methods) were quantified by qPCR. Collagen I reduced the expression level of CCL2, CCL4, IL-10 and IL-1β in monocytes by about 50%, whereas collagen III reduced it by 20%–40% (Figure 4A and data not shown). In contrast, biglycan, decorin and collagen V did not modulate the production of any of the 46 genes tested.

We went on to test whether the reduced transcript levels detected in our qPCR screening were mirrored at the protein level. CCL2, CCL4, IL-10 and IL-1β were detected by ELISA in the supernatant of monocytes in three individual experiments. Although CCL4, IL-10 and IL-1β were not significantly changed in these experiments, we noted a reduction of CCL2 that mirrored the transcript

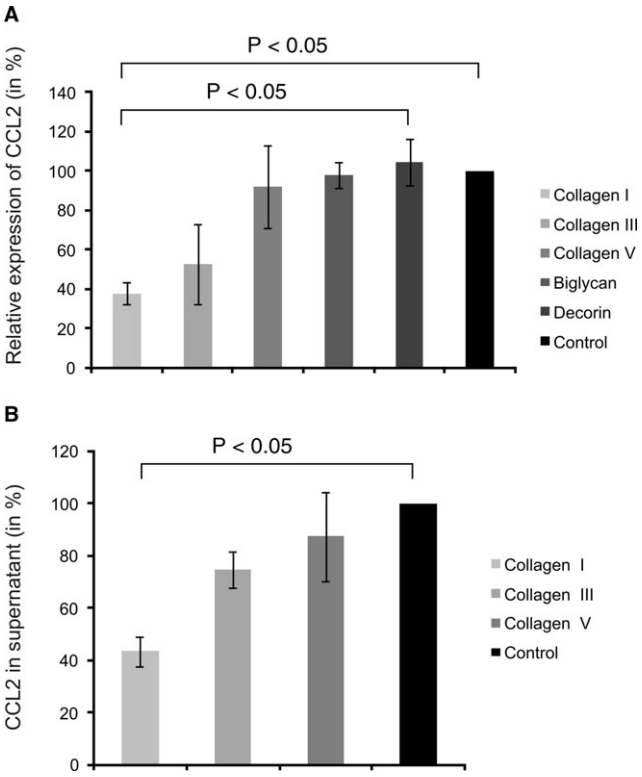

Figure 4. *Collagen I decreases CCL2 production by monocytes.* Human monocytes purified from peripheral blood mononuclear cell (PBMC) were cultured for 24 h on tissue culture plates coated with the indicated extracellular matrix (ECM) components. (**A**) The transcript level of CCL2 was determined by quantitative polymerase chain reaction using *GAPDH* as housekeeping gene. Expression of CCL2 in the control wells was set as 100% and the expression level in the presence of the indicated ECM components was calculated. The values given are mean ± standard error of the mean (SEM) of 3 (collagen I, III, biglycan and decorin) and ±SEM of 2 (collagen V) independent experiments. (**B**) The amount of CCL2 in the culture supernatant was measured by enzyme-linked immunosorbent assay and is given as percent of that in control dish without ECM component. The mean ± SEM of three experiments is given. The experiments in A and B were independently performed. Differences in diagrams derived from several independent experiments and more than two groups were analyzed by one-way repeated measures analysis of variance, followed by post hoc test (Holm–Sidak method). Normality and equal variance assumptions were fulfilled. Calculations were done using Sigma Plot.

levels. Collagen I reduced the production of CCL2 protein by 60% and collagen III by 25% (Figure 4B). This decrease in the production of CCL2 by monocytes was not explained by absorption of CCL2 on the collagens: In a parallel experiment, 1730 pg/mL of CCL2, the spontaneous level produced by unstimulated monocytes, was added to the medium in the collagen coated culture plates and >95% of CCL2 could be recovered from the supernatant after 24 h.

DISCUSSION

In this study we have quantified the transcript levels of ECM components in MS lesions, identified components of the perivascular fibrosis and studied their interaction with human immune cells *in vitro*. The most strongly altered ECM components were the chains forming the fibrillar collagens, namely *COL1A1*, *COL3A1*, *COL5A1*, *COL5A2* and the SLRPs biglycan and decorin. Decorin and biglycan can interact with and decorate fibrillar collagens (10).

Although the most prominent function of collagens is to maintain the structure of different tissues, they also have various other functions. The collagen types I, III, and V form collagen fibrils that provide many tissues such as tendon, ligament, cartilage, skin and cornea with firm but flexible characteristics (33, 49). The structure of collagens is characterized by triple helices formed by the homotrimeric or heterotrimeric interaction of three individual collagen polypeptide chains (49).

We have shown in this study that these fibrillar collagens, biglycan and decorin are molecular components of the perivascular fibrosis in MS lesions. The presence of fibrous structures around blood vessels in chronic CNS inflammation has been noted historically by neuropathologists (24, 46), and modern imaging techniques combining three MRI sequence modalities suggest that the perivascular space regulates inflammation in the brain of MS patients (56). Perivascular deposits are a feature of chronicity as fibrillar collagens were more prominent in chronic inactive lesions than in active lesions. Perivascular fibrosis is not unique to MS, it is also observed in liver cirrhosis, hypertensive heart failure and atherosclerosis (25), where collagens I and III form the perivascular deposits, which is similar to that observed in chronic MS lesions. Perivascular fibrosis is also found in HTLV-1 associated myelopathy, where the perivascular deposits consisting mainly of elastin and collagen fibers are found both in the spinal cord and in the brain (3, 55). The upregulation of some of these fibrillar collagen chains and of biglycan we observed is in harmony with previous proteomic studies on chronic active MS lesions (supporting information Table S1 in Ref. 16).

The balance of synthesis and degradation determines the final protein levels. Therefore we quantified not only the ECM components, but also the potential degrading enzymes. Among these are MMP1, -2, -3, -8, -9, -10, -13, -14 and -16 (37, 39, 58), the important collagenases degrading fibrillar collagens. MMP1, -3, -8, -10 and -13 were not detected in MS lesions. Instead, we observed strong induction of MMP2, -9, -11, -14 and -19 in the active lesions. MMP2 and -9 are involved in the BBB leakage (2, 9, 27). MMP19 may participate in the pathogenesis of MS by remodeling the ECM (51). We observed strong induction of MMP11, -14, -17, -24, and -28 in inactive lesions and this is the first study to show the involvement of these MMPs in MS. Our immunostaining showed a strong deposition of fibrillar collagens. This indicates that the synthesis of fibrillar collagens superseded its degradation by collagenases such as MMP2, -9 and -14, which were induced in the same lesions. In addition, the inhibitor TIMP1 was induced in both active and inactive lesions that might also contribute in shifting the balance towards synthesis and deposition of collagens.

The cellular source of these upregulated ECM components is not known. In our studies we saw induction of TGFβ1 in inactive and active MS lesions in parallel with the previously published data (6). TGFβ1 stimulates production of numerous ECM genes/proteins (23), the increase in TGFβ1 levels in MS lesions might trigger the expression of ECM components accumulated in perivascular fibrosis. In chronic lesions the fibrillar collagens were deposited in the PVS between endothelial cells and the astrocytic glia limitans. Candidates for the production of fibrillar collagens are, besides fibroblasts, astrocytes, which at least *in vitro* synthesize fibrillar collagens (17). In our *in vitro* studies human fibroblasts were much stronger producers of fibrillar collagens than astrocytes, in particular with regard to *COL1A1* and *COL3A1* (data not shown), which we found to be induced in MS lesions. Endothelial cells can produce decorin (31), and thus qualify as a source of decorin in the PVS. The upregulated biglycan in the perivascular space and also within infiltrating cells in the parenchyma of active lesions could also be derived from macrophages (41).

The close interaction between components of the perivascular fibrosis and the infiltrating immune cells was indicated by immunostaining and further studied *in vitro.* We found that fibrillar collagens reduced the production of CCL2 by monocytes. CCL2, a potent attractant for monocytes, dendritic cells, memory T-cells and basophils, is considered to be a major inflammatory chemokine in autoimmune CNS inflammation (8, 18, 28). The inhibition of CCL2 production by monocytes might limit lesion growth. This interpretation is supported by transfer EAE experiments (24) suggesting that reticulin fibers in the perivascular space contribute to vascular blockade and thereby limiting infiltration and lesion development.

CCL2 has been reported to be cleaved *in vitro* by MMP1, 2, 3, -8 and -9 (13, 29). The cleavage products have reduced chemotactic activity (13) or function even as an antagonist (29). In MS lesions we detected MMP2 and -9, but not MMP1, 3 and -8. Further studies are required to identify how CCL2 is processed *in vivo* in MS lesions. Additional effects of the altered ECM on immune cells could be mediated by biglycan, which has been reported to be a ligand for TLR2 and TLR4 in the mouse (41). However, in our study biglycan did not stimulate cytokine production by human monocytes. Furthermore, decorin absorbs and neutralizes TGFβ (19), a major modulator of autoimmune CNS inflammation (26). Thus the induction of decorin in MS might also contribute to lesion dynamics.

In summary, we have quantified changes in the ECM in MS lesions at different stages of development, identified the components of the perivascular fibrosis occurring in chronic MS lesions, and shown that the fibrillar collagens I and III inhibit the production of CCL2 by human monocytes. The perivascular fibrosis is a typical feature of chronic lesions and might function as both a physical and a biological barrier that limits immune cell recruitment and the expansion of MS lesions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Tissue samples of patients and controls.

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