Glucocorticoid effects on endothelial barrier function in the murine brain endothelial cell line cEND incubated with sera from patients with multiple sclerosis

Kinga G Blecharz¹, Aiden Haghikia², Mariusz Stasiolek², Niels Kruse³, Detlev Drenckhahn⁴, Ralf Gold², Norbert Roewer¹, Andrew Chan² and Carola Y Förster¹

Multiple Sclerosis

16(3) 293–302

© The Author(s) 2010

Reprints and permissions:

sagepub.co.uk/journalsPermissions.nav

DOI: 10.1177/1352458509358189

msj.sagepub.com

Abstract

Compromised blood–brain barrier integrity is a major hallmark of active multiple sclerosis (MS). Alterations in brain endothelial tight junction protein and gene expression occur early during neuroinflammation but there is little known about the underlying mechanisms. In this study, we analysed barrier compromising effects of sera from MS patients and barrier restoring effects of glucocorticoids on blood–brain barrier integrity in vitro. cEND murine brain microvascular endothelial cell monolayers were incubated with sera from patients in active phase of disease or in relapse. Data were compared with effects of the glucocorticoid dexamethasone alone or in combination with MS sera on barrier integrity. Tight junction protein levels and gene expression were evaluated concomitant with barrier integrity. We reveal downregulation of claudin-5 and occludin protein and mRNA and an accompanying upregulation in expression of matrix metalloproteinase MMP-9 after incubation with serum from active disease and remission and also a minor reconstitution of barrier functions related to dexamethasone treatment. Moreover, we for the first time describe downregulation of claudin-5 and occludin protein after incubation of cEND cells with sera from patients in remission phase of MS. Our findings reveal direct and differential effects of MS sera on blood–brain barrier integrity.

Keywords

blood–brain barrier, endothelium, glucocorticoids, multiple sclerosis, tight junctions

Date received: 15th June 2009; accepted: 23rd November 2009

Introduction

Multiple sclerosis (MS) is a complex chronic autoimmune inflammatory disease of the central nervous system (CNS) with heterogeneous pathological and immunological phenotype and highly variable clinical course. Relapsing–remitting MS (RR-MS), the most common clinical disease course, is characterized by exacerbations,¹ initiated by breakdown of the blood–brain barrier (BBB), which allows the infiltration of activated immune effector cells, amongst them T lymphocytes, finally activating a complex cascade leading to tissue damage.^{2,3} Well-characterized mechanisms include transmigration of activated lymphocytes across the BBB mediated by ligation of the integrin VLA4,³ which has meanwhile led to the introduction of a therapeutic monoclonal antibody (natalizumab) into clinical practice. Proinflammatory cytokines (e.g. tumour necrosis factor (TNF)- α , interferon (INF)- γ or interleukin (IL)-1 β , IL-6 and IL-12)⁴ activate matrix metalloproteinases (MMPs) which

target tight junction proteins in brain endothelial cells (ECs), compromising the BBB integrity.^{5,6} High-dose glucocorticoids (GCs), which exert a variety of immunomodulatory and immunosuppressive activities, are approved as first line therapy of relapses in MS.^{7,8,9,10} In the majority of cases, this therapeutic regimen leads to a rapid improvement of the symptoms occurring during relapses. However, the

¹Department of Anaesthesiology and Critical Care, University of Würzburg, Würzburg, Germany.

²Department of Neurology, Ruhr University Bochum, St. Josef-Hospital, Bochum, Germany.

³Institute of Multiple Sclerosis Research, University of Göttingen and Gemeinnützige Hertie-Stiftung, Göttingen, Germany.

⁴Institute of Anatomy and Cell Biology II, University of Würzburg, Würzburg, Germany.

Corresponding author:

Professor Dr Carola Förster, University of Würzburg, Department of Anaesthesiology and Critical Care, University Hospital Würzburg, Oberdürrbacherstrasse 6, 97080 Würzburg, Germany.
Email: foerster_c@klinik.uni-wuerzburg.de

molecular mechanisms underlying the GC action are not yet fully understood. Pleiotropic mechanisms include induction of apoptosis in peripheral blood leukocytes,^{11,12} the repression of cytokine-dependent upregulation of adhesion molecules, such as intercellular cell adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs) or E-selectin,^{13,14} the reduction of MMP levels/activity^{5,15} and stabilization of the BBB by upregulation of tight and adherens junctions,^{16,17,18} leading to an impairment of leukocyte infiltration through BBB into the CNS.¹⁹

In this study, we used the murine immortalized brain microvascular cell line cEND,¹⁷ which is an established and validated in vitro model of the BBB, to investigate effects of MS-patient sera on the barrier restoring effects of GCs. We tested transendothelial electrical resistance (TER) in cEND cells, which showed increased values for GC treatment when cells were incubated with serum from normal donors. In contrast, there were no barrier restoring effects observed after incubation with MS-patient sera. However, we detected minor effects on occludin and claudin-5 protein related to GC administration in the presence of sera from MS patients in the cEND cell line supporting the clinical benefits of GC treatment of MS. Furthermore, we observed a downregulation of tight junction transcripts after incubation of cEND cells in sera from patients in remission state. These findings were supported by the evaluation of MMP-9 and cytokine expression measurements, since MMP-9 and proinflammatory cytokines could act as potent candidate biomarkers of various courses of MS.^{20,21}

Materials and methods

Patients and patient sera

The study was approved by local ethics committees. After gaining informed consent, sera of RR-MS patients with different time-points of last GC treatment and three normal control donors, devoid of clinical signs of infections or immunotherapies, were chosen for the following experimental set-ups.

Blood was obtained by venipuncture using CPT Vacutainer containing Na-citrate as anticoagulant (Becton Dickinson, Basel, Switzerland). Vacutainers were centrifuged for 20 min at 1650g. Serum with lymphocytes was transferred to new Falcon tubes, centrifuged again at 1200 rpm for 10 min and serum supernatant was removed and stored in aliquots at -20°C until use.

Endothelial cell culture

Cerebral microvascular ECs were isolated as described.¹⁷ Cell cultures were cultivated in Dulbecco's

Modified Eagle's Medium (DMEM) purchased from Sigma (Taufkirchen; Germany) and supplemented with 10% fetal calf serum (FCS) and 100 IU/ml penicillin and 100 mg/ml streptomycin (1% PEST). Cells were maintained in an atmosphere at 37°C containing 5.0% CO_2 .

Incubation of cells with patient sera

cEND cells were grown until confluence. Cells were then transferred into FCS-reduced medium for 24 hours. Afterwards, cells were treated with FCS-free DMEM supplemented with 2% patient serum (normal, exacerbation, remission) for 24 hours. Cells were grown for an additional 24 hours with or without 100 nM dexamethasone.

Transendothelial electrical resistance

cEND cells were plated on collagen IV (Fluka, Taufkirchen; Germany) coated transwell chambers (0.4 μm pore size) (Falcon, Heidelberg; Germany) for six-well plates (Nunc, Langensfeld; Germany). Resistance measurements were performed using an assembly containing current-passing and voltage-measuring electrodes (World Precision Instruments, Inc., New Haven, CT, USA). Resistances of blank filters were subtracted from those of filters with cells before final resistances ($\Omega \cdot \text{cm}^2$) were calculated.

Isolation of mRNA and quantitative real-time reverse transcriptase polymerase chain reaction

mRNA of cEND cells was isolated by use of RNeasy Mini Kit (Qiagen, Hilden; Germany) according to the manufacturers' instructions. Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described previously¹⁶ using the following TaqMan gene expression assays: for claudin-5 (Mm0727012_s1), occludin (Mm00516701_m1), VE-cadherin (Mm00486938_m1) and MMP-9 (Mm00486938_g1) (Applied Biosystems, Foster City, CA; USA).

Immunoblotting

Western blot analyses were performed as described previously.¹⁷ The following primary antibodies were used: claudin-5 (zy-34-1600), occludin (zy-71-1500) rat monoclonal antibody against VE-cadherin qqD4.1 (undiluted hybridoma supernatant) directed to the ectodomain of mouse VE-cadherin.^{22,23} Primary antibodies were diluted 1 : 1000. As secondary antibody the horseradish-peroxidase-labelled goat anti-rabbit IgG (for claudin-5 and occludin) and goat anti-rat IgG

(Jackson ImmunoResearch Laboratory) diluted 1 : 3000 in PBS was used.

Cytokine analysis of the sera

Levels of the human cytokines and chemokines TNF α , IL-1 β , IL-8, IL-10, IL12p70, IP-10, MCP-1, MIG and RANTES in sera were assessed by means of cytometric bead array (CBA; BD Biosciences, San Jose, CA, USA). The procedure was carried out according to the manufacturer's instructions for human sera. Fluorescence measurements were performed using FACSCanto and the CellQuest software (BD Bioscience). The results are given in picograms per millilitre.

Statistical analyses

Values of TER measurements, densitometry and gene expression were averaged to establish a single value for control cells and treated cells as indicated. Throughout the manuscript, averaged values were reported as means $\pm$ standard deviation. Mann–Whitney *U*-test was performed and $p < 0.05$ (*) was considered statistically significant and $p < 0.001$ (**) was considered highly statistically significant.

Results

Sera from MS patients decrease TER of cEND cells

Tight and adherens junction proteins as claudin-5, occludin and VE-cadherin are described to be the key mediators of endothelial barrier sealing in brain ECs. Thus, we assessed TER in order to investigate functional changes of endothelium caused by treatment with sera from MS patients. We compared this with TER of cEND cells treated with sera from normal donors (Figure 1A). First, we measured TER of brain ECs maintained in 2% FCS, which displayed values of $413 \pm 25 \Omega \cdot \text{cm}^2$. TER of cells incubated with serum from normal donors (normal) was determined as $299 \pm 21 \Omega \cdot \text{cm}^2$. This value was set as 100% and was the control for TER measurements after incubation with sera of MS patients. TER of cEND cells maintained in sera from patients in exacerbation phase showed values of $114 \pm 11 \Omega \cdot \text{cm}^2$, which was $38.1 \pm 3.2\%$ of control levels. Cells treated with sera from patients in remission phase also showed a decrease of TER which was lowered to $172 \pm 12 \Omega \cdot \text{cm}^2$ ($49.8 \pm 3.5\%$ of control). These data suggest that factors included in sera from patients either in exacerbation or remission phase of MS lead to a vascular barrier dysregulation

Figure 1. TER in cEND cells in response to patient sera. cEND cells were cultured on collagen IV-coated transwell chambers for six-well plates. The confluent cell monolayers were transferred into FCS-free medium containing 2% of patient serum and incubated for 48 hours (A) or 24 hours followed by treatment with 100 nM dexamethasone for an additional 24 hours (B). TER was assessed using an assembly containing current-passing and voltage-measuring electrodes (World Precision Instruments, Inc., New Haven, CT; USA). Resistances of blank filters were subtracted from those of filters with cells before final resistances were calculated (data are represented as an average of three independent experiments).

Dexamethasone treatment causes a minor reconstitution of TER only in cEND cells incubated with sera from patients in remission phase

cEND cells were incubated in DMEM with 2% sera from normal donors (normal untreated) or sera from patients in exacerbation (exacerbation untreated) or remission (remission untreated) phase for 24 hours as described above. Sera incubation was followed by a 100 nM dexamethasone administration for 24 hours, then TER values were assessed (Figure 1B). We were able to show that 100 nM dexamethasone treatment of cEND cells maintained in serum from normal donors (normal + dex) increased TER values from 299 ± 21 to $486 \pm 13 \Omega \cdot \text{cm}^2$, an increase of $162.5 \pm 3.8\%$ of TER values without the GC (normal untreated), which was set as 100%. In contrast, treatment with 100 nM dexamethasone did not change TER in cEND cells incubated with serum from patients in exacerbation phase (exacerbation + dex): $120 \pm 9 \Omega \cdot \text{cm}^2$ (exacerbation untreated) versus $114 \pm 11 \Omega \cdot \text{cm}^2$ ($105.3 \pm 5.3\%$ of exacerbation untreated, set as 100%). Dexamethasone effects

on cEND cells incubated with sera from patients in remission phase of MS led to an increase from $149 \pm 12 \Omega \cdot \text{cm}^2$ (remission untreated) to $218 \pm 4 \Omega \cdot \text{cm}^2$ (remission + dex) counted as $146.3 \pm 2.6\%$ of remission untreated cells. In summary, dexamethasone only led to an increase of the TER values of cEND cells, which were incubated with sera from patients in remission phase of MS (remission + dex), but these values did not achieve the TER of the control level.

Sera from MS patients cause different effects of junctional protein expression in cEND cells

Proteins of the tight and adherens junctional complex are known to constitute BBB integrity. In order to identify the mechanisms by which the TER was affected in the previous experiment in cEND cells incubated with sera from different MS patients, we examined changes in the expression levels of junctional proteins. cEND cells were incubated in DMEM with 2% of patient sera in the same experimental set-up (Figure 2A). Western blot results were analysed by densitometry and claudin-5 (23 kDa) levels measured in cEND cells incubated with sera from normal donors (normal), which were used as control and set as 100%. Incubation of cEND cells in DMEM and 2% sera of patients in exacerbation phase (exacerbation) reduced claudin-5 protein levels to $54.34 \pm 2.6\%$ of control levels. Analyses of claudin-5 content in cEND cells

incubated with sera from patients in remission phase (remission) showed a smaller decrease to $72.7 \pm 10.6\%$ of control levels. Western blot analyses of claudin-5 protein were consistent with TER measurements as shown in Figure 1A.

Furthermore, we determined protein levels of occludin (65 kDa), another tight junction protein. First, we studied occludin protein expression in cEND cells incubated with sera from normal donors (normal) which was set as 100% in densitometrical analyses. A comparison of occludin protein levels in cEND cells incubated with sera of patients in exacerbation phase of MS (exacerbation) revealed a decrease to $78.4 \pm 4.1\%$ of control level. Unexpectedly, incubation of cEND cells with sera from patients in remission phase (remission) showed an even stronger downregulation of occludin protein levels to $66.5 \pm 2.1\%$ of the control level. These findings indicate that in the case of occludin incubation of brain ECs with sera from patients in remission phase induces a stronger protein reduction of occludin than the incubation with sera from patients in the exacerbation phase of MS.

VE-cadherin was previously demonstrated to be downregulated upon incubation with MS-patient sera.²⁴ We were not able to reproduce such a prominent decrease of VE-cadherin. By contrast, we show a negligible decrease in brain ECs incubated in DMEM with 2% sera of patients in exacerbation phase of MS (exacerbation), which was $96.8 \pm 2.6\%$ of normal levels set

Figure 2. Protein levels of tight junction proteins in cEND cells incubated with sera of normal donors or MS patients. cEND cells were grown on collagen IV-coated culture flasks until confluence thereafter medium was exchanged into FCS-free DMEM containing 2% of patient sera for 48 hours (A) or 24 hours followed by treatment with 100 nM dexamethasone for additional 24 hours (B). Protein levels of claudin-5, occludin and VE-cadherin were detected by Western blot. Densitometrical analyses were performed by usage of Scion Image Beta 4.02 (Scion Corp., Frederick, MD, USA) for quantification and presented as a histogram. Bars are representing protein levels of claudin-5, occludin and VE-cadherin after incubation of cEND cells with different sera. Samples of cEND cells incubated with sera from normal donors were set as 100% (data are represented as an average of three independent experiments).

as 100% (normal). There were also no prominent changes in VE-cadherin protein levels in cEND cells incubated with sera from patients in remission phase of MS determined as $106.6 \pm 4.1\%$ as compared with the normal samples.

Dexamethasone treatment causes minor effects on junctional protein expression in cEND cells incubated with sera from MS patients

Furthermore, we were interested in the effects of dexamethasone on claudin-5, occludin and VE-cadherin protein levels in cEND cells upon incubation with patient sera (Figure 2B). As expected, a positive effect on claudin-5 protein levels in response to 100 nM dexamethasone treatment could be observed in cEND cells incubated in DMEM with 2% sera of normal donors. In detail, claudin-5 protein levels increased to $117 \pm 6.3\%$ of control (normal) in response to dexamethasone stimulation (normal + dex). A small increase was observed in cells treated with 100 nM dexamethasone incubated with sera from patients in exacerbation phase of MS (exacerbation + dex). Here, the expression of claudin-5 protein rose to $112.13 \pm 4.5\%$ from the control level (exacerbation), whereas the claudin-5 protein level in cEND cells incubated with sera from patients in exacerbation phase was still lower than claudin-5 levels in cEND cells maintained with sera from normal donors (normal). Dexamethasone treated cells incubated with sera from patients in remission phase (remission + dex) showed a minor induction of claudin-5 protein which was $105.11 \pm 2.3\%$ of the control level (remission).

To further analyse the barrier restoring effects of GC treatment in our BBB model we investigated protein levels of occludin in response to dexamethasone treatment. In agreement with our previous findings,¹⁷ dexamethasone stimulation led to an upregulation of occludin protein in cEND cells incubated with sera from normal donors (normal + dex) which was $191 \pm 10.2\%$ of the control level (normal untreated). Unexpectedly, we found that dexamethasone treatment caused even a small decrease of occludin protein levels in cEND cells incubated with sera from MS-patients in

exacerbation phase (exacerbation + dex) which was $73.9 \pm 4.4\%$ of control protein levels (exacerbation untreated). On the other hand, an increase of occludin protein content could be observed in cEND cells incubated in DMEM with 2% of sera from patients in remission phase and treated with 100 nM dexamethasone. In this case we observed an increase to $110.9 \pm 2.4\%$ (remission + dex) compared with unstimulated cells (remission untreated).

Dexamethasone treatment did not change VE-cadherin levels significantly. Western blot analyses of cEND cells incubated with sera from normal donors and treated with 100 nM dexamethasone for 24 hours showed levels of $101.2 \pm 5.9\%$ of untreated cEND cells (normal untreated) which were set as 100%. cEND cells incubated with sera from patients in the exacerbation phase of MS and treated with dexamethasone (exacerbation + dex) also revealed a minor increase of VE-cadherin protein levels which were $112.8 \pm 3.4\%$ of control (exacerbation + dex). VE-cadherin protein expression was unchanged in cEND cells incubated with sera from patients in remission phase of MS and treated with dexamethasone (remission + dex) which was $100 \pm 2.9\%$ of the untreated control (remission untreated).

Dexamethasone modulates junctional gene expression in cEND cells incubated in patient sera

In order to identify the effects on gene expression of tight and adherens junctions in cEND cells incubated with a supplement of patient sera with or without GC treatment, we performed quantitative real-time RT-PCR. Observations made on occludin genes supported our Western blot analyses described above. An induction of occludin expression in cEND cells treated with 100 nM dexamethasone and incubated in DMEM with 2% of normal control sera could be detected (Table 1). The same treatment caused only a minor claudin-5 and VE-cadherin gene upregulation as compared with untreated cEND cells incubated with normal control sera, respectively. The most prominent effects caused by incubations of cEND cells with sera from patients in the exacerbation phase of MS could be observed on

Table 1. Modulation of junctional gene expression by dexamethasone in cEND cells incubated in sera from patients

	claudin-5	occluding	VE-cadherin	
Normal	1	1	1	Untreated
	1.48 ± 0.13	1.714 ± 0.06	1.29 ± 0.04	100 nM dex
Exacerbation	0.777 ± 0.02	1.005 ± 0.16	0.994 ± 0.12	Untreated
	0.951 ± 0.06	1.025 ± 0.14	1.14 ± 0.16	100 nM dex
Remission	0.917 ± 0.02	0.876 ± 0.14	0.997 ± 0.16	Untreated
	1.57 ± 0.004	1.45 ± 0.08	1.089 ± 0.19	100 nM dex

occludin gene expression which was strongly downregulated. In contrast, claudin-5 and VE-cadherin gene expression were almost unaffected by supplementation with sera from patients in the exacerbation phase (Table 1). Incubation of cEND cells with sera of patients in remission state of MS caused a small downregulation of occludin and claudin-5 gene expression as compared with untreated cEND cells incubated in sera from normal donors. The dexamethasone effect on gene induction in cEND cells incubated with sera from MS patients in the remission state was strongest on occludin and claudin-5 levels (1.57 ± 0.004 -fold and a 1.45 ± 0.08 -fold, respectively).

Exacerbation sera induce a strong MMP-9 gene expression affecting junctional protein levels

Since patient serum effects on TER levels of cEND monolayers could only partly be attributed to gene expression modulation, we tested the induction of MMP-9 in ECs by incubation with patient sera. We previously described that treatment of cEND cells with TNF α is followed by an upregulation of MMP-9.⁵ The cytokine mediated upregulation of specific MMP proteins or an increase of MMP activity seems to be a major cause of degradation of tight junction proteins under inflammatory conditions including MS.²¹ In order to evaluate a role for MMP-9 in degradation of tight junction proteins we assessed MMP-9 by quantitative real-time RT-PCR (Figure 3). We detected the MMP transcript levels in the endothelium after incubation with sera from different patients. Incubation of cEND cells in sera from patients in exacerbation phase of MS led to a 1.5 ± 0.19 -fold increase of MMP-9 gene expression as compared with the normal control sera. In this incubation set-up dexamethasone treatment led to a 0.75 ± 0.16 -fold downregulation of MMP-9 mRNA levels as compared with the control. Incubation of cEND cells in DMEM with 2% sera from patients in the remission phase of MS caused a 1.13 ± 0.02 -fold upregulation of MMP-9 mRNA levels and dexamethasone led to a strong 0.49 ± 0.02 -fold downregulation of MMP-9 transcripts, as compared with the control of cEND cells incubated in DMEM with sera from normal donors, respectively. Dexamethasone treatment caused a 0.85 ± 0.05 -fold downregulation of MMP-9 transcript in cEND cells incubated with sera from normal donors compared with untreated cells incubated in the same sera.

In an attempt to identify serum components potentially responsible for the divergent effects of sera from active disease and remission phase, we identified cytokine levels contained in patient sera (Figure 4). While the cytokines IL-12p70, IL-10, IL-1 β and IL-8 were below the detection limit, we could detect, in tendency,

Figure 3. Effects of patient sera and dexamethasone on MMP-9 gene expression. cEND cells were seeded on collagen IV-coated 12-well plates. Then, the confluent cells monolayers were treated in the same experimental set-up. mRNA was isolated and reverse transcribed for quantitative real-time RT-PCR. The strongest effects on MMP-9 gene expression were seen in cEND cells incubated with DMEM with exacerbation serum (data are represented as an average of three independent experiments).

differences for chemokines IP-10, MCP-1, MIG and RANTES, which were differing between exacerbation and remission phase (Figure 4A and B). There was a tendency for human IP-10 to be reduced in sera from patients in exacerbation phase, while IP-10 levels appeared upregulated in sera from remission phase as compared with the control group (Figure 4A). For human MCP-1 and human MIG, a small but insignificant elevation in both active disease and sera from patients in remission phase was detected (Figure 4A). For human RANTES, a strong, but insignificant, elevation of cytokine levels was detected in sera from patients in remission phase (Figure 4B). For more conclusive results, a more thorough analysis of serum components in a wider spectrum of individuals should, however, be performed in the future.

Discussion

In the present study, we demonstrate compromised endothelial barrier function and reduced or altered junctional protein expression, resulting from incubation of the murine brain endothelial cell line cEND with sera from patients in the exacerbation or remission phase of MS. We further proved partial restoration of barrier function following administration of the GC dexamethasone. In this way, we have presented in this study for the first time, to the best of the authors' knowledge, different barrier compromising effects of

Figure 4. Levels of the proinflammatory cytokines human IP-10, MCP-1, MIG and RANTES as determined by cytometric bead array. C, normal control; E, sera from patients in the exacerbation phase; R, sera from patients in remission. (A) Cytokine levels (pg/ml) for human IP-10, MCP-1, MIG. (B) Cytokine levels (pg/ml) for human RANTES. Note the reduction in human IP-10 in sera from patients in exacerbation phase, while IP-10 levels are upregulated in remission phase as compared with the control group (A). Note the elevation of human RANTES in sera from patients in the remission phase (B).

the varying proinflammatory cytokines contained in MS patient sera during the course of RR-MS, try to identify these cytokines and evaluate the counteracting barrier restoring effects of GC.¹⁷

In detail, we have observed a reduction of monolayer tightness (TER) following incubation of cEND monolayers with patient sera. Incubation with patient sera from exacerbation or remission and simultaneous administration of dexamethasone revealed however interesting unexpected differential effects on the junctional proteins occludin, claudin-5 and VE-cadherin.

- (i) An exposure of monolayers to the exacerbation sera most significantly compromised monolayer tightness. Incubation of monolayers with sera from remission phase did compromise, as expected, monolayer tightness to a lesser extent.
- (ii) Claudin-5 protein and gene expression levels appeared to be chiefly affected (up to 50% reduction) after incubation with sera from the exacerbation phase. GC administration led to an increase in claudin-5 gene expression and restoration of its protein levels. However, stronger GC effects on

claudin-5 gene expression levels were observed after treatment with sera from MS remission patients.

- (iii) Surprisingly, occludin protein levels were more severely affected by incubation with sera from patients in the remission phase. This is a fact which was mirrored at the gene expression level. Sera from patients in an active, exacerbation phase did reduce occludin protein levels to a lesser extent. While in monolayers, which were treated with serum from healthy donors plus dexamethasone, occludin levels were strongly upregulated by dexamethasone, confirming occludin as a GR target,²⁵ unexpectedly no increase in occludin protein levels was observed for monolayers treated with patient serum of either kind plus dexamethasone. Remarkably the combination of dexamethasone and serum from exacerbation phase even led to a further reduction in occludin protein levels. At the gene expression level, dexamethasone induced occludin in monolayers treated with sera from healthy donors or patients in the remission phase only.

Increase in endothelial barrier permeability caused by compromised cell-cell adhesion is assumed to be an early event in MS.²⁶ For treatment, GCs such as dexamethasone or methylprednisolone are universally accepted as first line therapy of acute attacks of MS. These GC may thus be considered as the current gold standard for attempts to model desirable ligand-induced transcriptomic effects to treat MS.²⁷ Given the limitations and side-effects of GC therapy, there is however a profound interest in identification and sustained regulation of therapeutically beneficial GC target genes by alternative approaches. By this in vitro study we evaluated the combined effects of dexamethasone and patient sera from active and remitting disease containing different proinflammatory cytokine cocktails to provide a list of GC targets to be directed by improved GC therapy or alternative approaches in MS therapy in the future. In line with this, our observations suggest a vulnerability especially of claudin-5 protein, chiefly important for barrier sealing strand and pore formation,^{28,29} to one or more of the proinflammatory cytokines contained in sera from the exacerbation phase. In contrast, occludin, which seems to play a regulatory role in the control of vascular permeability,³⁰ appears to be the major target of proinflammatory cytokine action in the remission phase. Our data using the cEND in vitro model are partially in agreement with data from other groups,⁴ which revealed that serum from patients in the acute phase of MS downregulate tight junction proteins in cultured ECs, while the effect of sera from patients in the remission phase of the disease or barrier restoring effects of GC were not analysed in their study. One might speculate that claudin-5 and occludin

underlie differential expression and topology controlling mechanisms, a fact already observed after stimulation of endothelial monolayers derived from cerebrum (cEND) and cerebellum (cerebEND) with the proinflammatory cytokine TNF α barrier compromising effects^{5,31} were demonstrated to be composed of differential transcriptional downregulation of tight junction genes and pathological activation of the MMP-9 for this experimental set-up. Occludin and claudin-5 expression and topology in MS pathogenesis might moreover be modulated by a different set of proinflammatory cytokines contained in patient sera during the course of RR-MS.

- (iv) As for the endothelial adherens junction protein, VE-cadherin, protein levels remained unchanged in cEND cells incubated with either sera or dexamethasone, a fact we already observed for cEND monolayers treated with TNF α .⁵ One might speculate that incubation in sera from patients in exacerbation or remission phase of MS could nevertheless lead to tyrosine phosphorylation of the VE-cadherin protein resulting in a weakening of cell adhesion and thus with an increase of barrier permeability and endothelial disintegration.^{32,33,34,35} Testing this hypothesis will however remain a task for future experiments.

Several different studies have shown that chemokines and chemokine receptors are involved in the pathogenesis of MS.^{6,36} Chemokines can mediate the trafficking of immune cells across the BBB, and regulate their transfer to lesion sites. Chemokines were detected in actively demyelinating lesions and were found to be elevated in the cerebrospinal fluid of patients with MS during relapse⁶ so that the authors aimed at identifying major chemokines contained in patient sera responsible for the differential effects of sera from active disease or remission. We demonstrate a tendency for RANTES (CCL5) to be elevated in sera from patients in remission phase. In contrast, IP-10 (CXCL10) was reduced in patients sera from active disease, while IP-10 levels appeared elevated in sera from remitting patients. Different pairs of chemokine receptors and their ligands seem to play a pathogenic role in MS. For instance, significantly higher levels of CCL5 and CCL3 were found in the CSF of patients with MS in relapses,⁶ matching our finding of elevated CCL5/ RANTES levels in sera from patients in remission phase. IP-10/ CXCL10 chemokine, the ligand for CXCR3, is detected at high levels on hypertrophic astrocytes around active MS lesions.³⁷ In our study, however, in sera from active disease its levels appeared downregulated, while they appeared upregulated in sera from patients in remission phase. To clarify this discrepancy, further experiments would have to be performed. One should acknowledge

moreover that data obtained from the in vitro model might not be fully relevant for MS.

During MS exacerbation the upregulation of MMPs in endothelial cells might further contribute to the degradation of tight junction transmembrane proteins: in line with this, previous investigations³⁸ could demonstrate that the claudin-5-based tight junction structural integrity was affected at the protein level in exacerbation phase of MS. Benesova and colleagues²¹ referred to MMP-9, MMP-2 and tight junctions (claudin-5, occludin) as potent candidates for biomarkers for various courses of MS. We thus assessed gene expression of MMP-9 in cEND cells incubated in patient sera and treated or untreated with dexamethasone in this study. Incubation of cEND monolayers with sera from active disease in fact induced strong upregulation of MMP-9 gene expression. Upregulation, but to a lesser extent, was also observed after incubation with sera from remission patients. In either case, dexamethasone administration led to a reduction in mRNA levels for MMP-9. These data match previous observations in which we described that MMP-9 is strongly upregulated in TNF α -treated cEND cells.⁵ The observed downregulation of MMP-9 expression is further in arrangement with data from in vitro²¹ and in vivo^{20,21} experiments made by other groups using different MS model systems. Future studies will thus have to address the nature of the opposed effects of dexamethasone and proinflammatory cytokines on tight junction properties in brain microvascular endothelial cells exposed to the cocktail of proinflammatory cytokines contained in MS sera from active disease or remission, i.e. effects on tight junction transcription, protein synthesis, intracellular localization, phosphorylation status, on the dynamics of junctional protein interactions, and on the secretion of signalling proteins and peptidases.^{5,39,40,41,42}

Acknowledgements

This research was supported by Deutsche Forschungsgemeinschaft through the grant SFB688-TPA5 to CF and grant SFB-TPA1 RG. The authors are grateful to Dr Małgorzata Burek, Department of Anaesthesiology and Critical Care, University of Würzburg, for helpful discussions and Katharina Mattenheimer for excellent technical assistance.

References

- Steinman L. Multiple sclerosis: a two-stage disease. *Nat Immunol* 2001; 2: 762–764.
- Engelhardt B. Molecular mechanisms involved in T cell migration across the blood–brain barrier. *J Neural Transm* 2006; 113: 477–485.
- Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* 1992; 356(6364): 63–66.

4. Minagar A, Alexander JS. Blood-brain barrier disruption in multiple sclerosis. *Mult Scler* 2003; 9: 540–549.
5. Forster C, Kahles T, Kietz S, Drenckhahn D. Dexamethasone induces the expression of metalloproteinase inhibitor TIMP-1 in the murine cerebral vascular endothelial cell line cEND. *J Physiol* 2007; 580: 937–949.
6. Szczucinski A, Losy J. Chemokines and chemokine receptors in multiple sclerosis. Potential targets for new therapies. *Acta Neurol Scand* 2007; 115: 137–146.
7. Engelhardt B. Role of glucocorticoids on T cell recruitment across the blood-brain barrier. *Z Rheumatol* 2000; 59(Suppl 2): II/18–21.
8. Reichardt HM, Gold R, Luhder F. Glucocorticoids in multiple sclerosis and experimental autoimmune encephalomyelitis. *Expert Rev Neurother* 2006; 6: 1657–1670.
9. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. *N Engl J Med* 2000; 343: 938–952.
10. Reichardt HM. Immunomodulatory activities of glucocorticoids: insights from transgenesis and gene targeting. *Curr Pharm Des* 2004; 10: 2797–2805.
11. Leussink VI, Jung S, Merschedorf U, Toyka KV, Gold R. High-dose methylprednisolone therapy in multiple sclerosis induces apoptosis in peripheral blood leukocytes. *Arch Neurol* 2001; 58: 91–97.
12. Wust S, van den Brandt J, Tischner D, et al. Peripheral T cells are the therapeutic targets of glucocorticoids in experimental autoimmune encephalomyelitis. *J Immunol* 2008; 180: 8434–8443.
13. Dietrich JB. Endothelial cells of the blood-brain barrier: a target for glucocorticoids and estrogens? *Front Biosci* 2004; 9: 684–693.
14. Wang K, Wang Y, Gao L, Li X, Li M, Guo J. Dexamethasone inhibits leukocyte accumulation and vascular permeability in retina of streptozotocin-induced diabetic rats via reducing vascular endothelial growth factor and intercellular adhesion molecule-1 expression. *Biol Pharm Bull* 2008; 31: 1541–1546.
15. Rosenberg GA, Dencoff JE, Correa N Jr, Reiners M, Ford CC. Effect of steroids on CSF matrix metalloproteinases in multiple sclerosis: relation to blood-brain barrier injury. *Neurology* 1996; 46(6): 1626–1632.
16. Blecharz KG, Drenckhahn D, Forster CY. Glucocorticoids increase VE-cadherin expression and cause cytoskeletal rearrangements in murine brain endothelial cEND cells. *J Cereb Blood Flow Metab* 2008; 28: 1139–1149.
17. Forster C, Silwedel C, Golenhofen N, et al. Occludin as direct target for glucocorticoid-induced improvement of blood-brain barrier properties in a murine in vitro system. *J Physiol* 2005; 565: 475–486.
18. Forster C. Tight junctions and the modulation of barrier function in disease. *Histochem Cell Biol* 2008; 130: 55–70.
19. Pitzalis C, Pipitone N, Perretti M. Regulation of leukocyte-endothelial interactions by glucocorticoids. *Ann N Y Acad Sci* 2002; 966: 108–118.
20. Lindberg RL, De Groot CJ, Montagne L, et al. The expression profile of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis. *Brain* 2001; 124: 1743–1753.
21. Benesova Y, Vasku A, Novotna H, et al. Matrix metalloproteinase-9 and matrix metalloproteinase-2 as biomarkers of various courses in multiple sclerosis. *Mult Scler* 2009; in press.
22. Liebner S, Kniesel U, Kalbacher H, Wolburg H. Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. *Eur J Cell Biol* 2000; 79: 707–717.
23. Gotsch U, Borges E, Bosse R, et al. VE-cadherin antibody accelerates neutrophil recruitment in vivo. *J Cell Sci* 1997; 110: 583–588.
24. Minagar A, Ostanin D, Long AC, et al. Serum from patients with multiple sclerosis downregulates occludin and VE-cadherin expression in cultured endothelial cells. *Mult Scler* 2003; 9: 235–238.
25. Harke N, Leers J, Kietz S, Drenckhahn D, Förster C. Identification of a distal glucocorticoid responsive element in the occludin gene coding region. *Mol Cellular Endocrinology* 2008; 295: 39–47.
26. Simka M. Blood brain barrier compromise with endothelial inflammation may lead to autoimmune loss of myelin during multiple sclerosis. *Curr Neurovasc Res* 2009; 6: 132–139.
27. Penberthy WT. Nicotinic acid-mediated activation of both membrane and nuclear receptors towards therapeutic glucocorticoid mimetics for treating multiple sclerosis. *PPAR Res* 2009; 2009: 853707.
28. Matter K, Balda MS. Holey barrier: claudins and the regulation of brain endothelial permeability. *J Cell Biol* 2003; 161: 459–460.
29. Nitta T, Hata M, Gotoh S, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 2003; 161: 653–660.
30. Hirase T, Staddon JM, Saitou M, et al. Occludin as a possible determinant of tight junction permeability in endothelial cells. *J Cell Sci* 1997; 110: 1603–1613.
31. Silwedel C, Forster C. Differential susceptibility of cerebral and cerebellar murine brain microvascular endothelial cells to loss of barrier properties in response to inflammatory stimuli. *J Neuroimmunol* 2006; 179: 37–45.
32. Vestweber D, Winderlich M, Cagna G, Nottebaum AF. Cell adhesion dynamics at endothelial junctions: VE-cadherin as a major player. *Trends Cell Biol* 2009; 19: 8–15.
33. Yap AS, Crampton MS, Hardin J. Making and breaking contacts: the cellular biology of cadherin regulation. *Curr Opin Cell Biol* 2007; 19: 508–514.
34. Lilien J, Balsamo J. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. *Curr Opin Cell Biol* 2005; 17: 459–465.
35. Nawroth R, Poell G, Ranft A, et al. VE-PTP and VE-cadherin ectodomains interact to facilitate regulation of phosphorylation and cell contacts. *EMBO J* 2002; 21: 4885–4895.
36. Subileau EA, Rezaie P, Davies HA, et al. Expression of chemokines and their receptors by human brain endothelium: implications for multiple sclerosis. *J Neuropathol Exp Neurol* 2009; 68: 227–240.

37. Omari KM, John GR, Sealfon SC, Raine CS. CXC chemokine receptors on human oligodendrocytes: implications for multiple sclerosis. *Brain* 2005; 128: 1003–1015.
38. Mun-Bryce S, Rosenberg GA. Matrix metalloproteinases in cerebrovascular disease. *J Cereb Blood Flow Metab* 1998; 18: 1163–1172.
39. Prasad S, Mingrino R, Kaukinen K, et al. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. *Lab Invest* 2005; 85: 1139–1162.
40. Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A. Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *FASEB J* 2005; 19: 923–933.
41. Poritz LS, Garver KI, Tilberg AF, Koltun WA. Tumor necrosis factor alpha disrupts tight junction assembly. *J Surg Res* 2004; 116: 14–18.
42. Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood–brain barrier. *Nat Rev Neurosci* 2006; 7: 41–53.