Monocyte Chemoattractant Protein-1–Deficiency Results in Altered Blood–Brain Barrier Breakdown After Experimental Stroke

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Background and Purpose—Stroke-induced blood–brain barrier (BBB)-disruption can contribute to further progression of cerebral damage. There is rising evidence for a strong involvement of chemokines in postischemic BBB-breakdown. In a previous study, we showed that monocyte chemoattractant protein-1 (MCP-1)–deficiency results in a markedly reduced inflammatory reaction with decreased levels of interleukin-6, interleukin-1β, and granulocyte colony-stimulating factor after experimental stroke. With MCP-1 as one of the key players in stroke-induced inflammation, in this study, we investigated the influence of MCP-1 on poststroke BBB-disruption as well as transcription/translation of BBB-related genes/proteins after cerebral ischemia.

Methods—Sixteen wild-type and 16 MCP-1−/− mice were subjected to 30 minutes of middle cerebral artery occlusion. By injecting high molecular-tracer, we compared the degree of BBB-disruption after middle cerebral artery occlusion. Real-time polymerase chain reactions and Western blot technique were used to compare tight-junction gene expression, protein secretion, and BBB-leakage.

Results—Here, we report that MCP-1–deficiency results in a reduced BBB-leakage and a diminished expression of BBB-related genes occludin, zonula occludens-1, and zonula occludens-2. Real-time polymerase chain reactions and Western blot analysis revealed elevated claudin-5–levels in MCP-1−/− animals. MCP-1–deficiency resulted in reduced infarct sizes and an increased vascular accumulation of fluorescein-isothiocyanate-albumin.

Conclusions—The results of the study provide further insights into the molecular mechanisms of BBB-opening and may help to better understand the mechanisms of infarct development after cerebral ischemia. (Stroke. 2013;44:2536-2544.)

Key Words: brain-blood barrier ■ brain ischemia ■ CCL2, claudin-5 ■ inflammation ■ occludin

Ischemic stroke, which frequently results from middle cerebral artery occlusion (MCAO), leads to a sequence of multiphasic events, including inflammation, hemorrhagic transformation, and blood–brain barrier (BBB)-breakdown. The understanding of stroke pathology as a multistaged event consisting of primary tissue damage within the ischemic core and progressive cell damage in the surrounding penumbra gave rise to new therapeutic options in stroke treatment.

Monocyte chemoattractant protein-1 (MCP-1) is one of the most prominent chemokines, which is expressed in neurons, astrocytes, and endothelial cells in response to oxygen shortage. It is a dominant chemotactic factor, which attracts monocytes, neutrophil granulocytes, and macrophages into the infarcted core. After experimental cerebral ischemia, mice lacking MCP-1 develop smaller infarcts, show impaired leukocyte infiltration, and an overall reduced expression of inflammatory markers interleukin (IL)-6, IL-1β, and G-CSF, which is accompanied by a less severe neurological outcome. The BBB, a physical and metabolic barrier between blood and brain, is mainly formed by interaction of transmembrane-associated proteins claudin-5, occludin, zonula occludens (ZO)-1, and ZO-2, which connect tight-junction (TJ) proteins to the cytoskeleton. In vitro studies have shown that administration of MCP-1 leads to reduced transendothelial electric resistance as well as redistribution and phosphorylation of BBB-related proteins, resulting in altered BBB-integrity. Furthermore, it has been demonstrated that intracerebral administration of MCP-1 increases leakage of high-molecular tracer protein FITC-albumin (fluorescein isothiocyanate-albumin) into the brain, thereby providing additional evidence for a crucial role of MCP-1 in poststroke BBB-breakdown. A previous study characterized TJ-protein phosphorylation via Rho/Protein Kinase C-α (PKCα) as major mechanism leading to opening of the brain endothelial barrier. Today, it is well accepted that alterations in morphology and expression as well as post-transcriptional and post-translational modifications of TJ-proteins
are involved in regulation of BBB-specific functions particularly by preserving the low permeability of brain microvasculature.\textsuperscript{13} Whether proinflammatory chemokines like MCP-1 have a direct influence on the postischemic expression of TJ-related proteins during reperfusion injury is still unclear. Therefore, we aimed to investigate the role of MCP-1 for the expression and translation of pivotal TJ-proteins. Additionally, we compared BBBs susceptibility for high-molecular protein leakage and, using confocal immunofluorescence, possible structural and quantitative TJ-protein differences between the investigated groups. Here, we report that MCP-1\textsuperscript{−/−} mice exhibit an altered transcription and secretion of BBB-related genes claudin-5, occludin, ZO-1, and ZO-2. MCP-1\textsuperscript{−/−} deficiency resulted in an increased accumulation/retention of FITC-albumin within the cortical tissue. Furthermore, MCP-1\textsuperscript{−/−} mice developed reduced infarct size and BBB-leakage for both administered bovine FITC-albumin as well as mouse-albumin.

Material and Methods
All procedures and animal studies were performed in concordance with the local governmental authorities (Landesamt für Natur, Umwelt und Verbraucherschutz, NRW, Germany, AZ 8.87-50.10.36.09) and the European Convention for Animal Care and Ethical Use of Laboratory Animals following the current ARRIVE/STAIR guidelines. The number of animals was kept to the minimum needed.

Animal Care
Male 16- to 18-week-old C57BL/6J (Charles River, Sulzfeld, Germany; n=22) and male MCP-1\textsuperscript{−/−} mice (The Jackson Laboratory, Bar Harbor, ME; n=21) were used in this study. To ensure a pure background, Harbor, ME; n=21) were used in this study. To ensure a pure background, Animals were backcrossed for transient focal cerebral ischemia using an intraluminal technique as previously described.\textsuperscript{4}

Infarct Size and Brain Swelling Measurement
Comparison of infarct sizes between MCP-1\textsuperscript{−/−} and wild-type mice was performed using 2 toluidine-stained coronal brain sections of each animal (bregma \pm 1 mm) to 0 mm were cut with a cryostat (Leica CM 3050, Nussloch, Germany), mounted on glass slides (SuperFrost, Langenbrinck, Germany) and stored at \textdegree C until further use. To avoid degradation of mRNA, mice used for subsequent gene expression analyses were perfused with 0.9% NaCl terminal xylazine/ketamine anesthesia. Brains were quickly removed, embedded in TissueTek, frozen on dry ice, and stored at \textdegree C. Coronal sections for immunohistochemical staining (10 \mu m) covering bregma \pm 1 mm to 0 mm were cut with a cryostat (Leica CM 3050, Nussloch, Germany), mounted on glass slides (SuperFrost, Langenbrinck, Germany) and stored at \textdegree C until further use. Specimen for subsequent laser capture microdissection and gene expression analysis were cut (12 \mu m) and collected on polyethylene naphtalate (PEN) membrane coated slides (PALM MicroLaser Systems, Germany).

Transient Focal Cerebral Ischemia
Focal cerebral ischemia was induced in mice (25–30 g) for 45 minutes via MCAO using an intraluminal technique as previously described.\textsuperscript{4} MCAO was performed by 1 of the authors without knowledge of the genetic background. Briefly, animals were anesthetized with 1.5% isoflurane in 30% O\textsubscript{2}/70% N\textsubscript{2}O. A thermostat-controlled heated blanket served to maintain a constant body temperature (37°C\pm0.5°C). MCAO was performed by introduction of a 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicone resin (Xantopren; Haerueas, Dormagen, Germany) into a small incision of the left common carotid artery. Advancing of the monofilament to the distal carotid bifurcation led to a temporary occlusion of the MCA. Regional cerebral blood flow was continuously monitored using a laser Doppler probe (Periflux 5001; Perimed, Stockholm, Sweden) to confirm ischemia and reperfusion. The occlusion was considered successful when the cerebral blood flow dropped \textless 20% of the preoperative flow-value and \textgreater 60% recovery after reperfusion. Sham-operated mice were subjected to the same procedure without advancing the filament to the MCA. Only mice showing appropriate signs of successful operation (impaired locomotion and/or flexion of the affected limb) were subjected to the subsequent experimental procedures. Two wild-type mice and 1 MCP-1\textsuperscript{−/−} deficient mouse died within 12 hours after surgery and were, therefore, excluded from the investigation. All surviving mice subjected to MCAO showed appropriate deficits and were included in the survey.

FITC-Albumin Administration and Tissue Preparation
To determine a possible influence of MCP-1 on ischemia-induced BBB-integrity loss, a bolus of 10 mg bovine FITC-conjugated albumin (dissolved in 100 \mu L phosphate buffered saline, pH 7.4) was injected into the venous cavae (20 minutes before perfusion. Animals used for immunohistochemical fluorescence investigations were perfused with 6% hydroxyethyl-starch solution (HAES steril; Fresenius, Bad Homburg, Germany) through the left ventricle for 1 minute, followed by 4% buffered paraformaldehyde (pH 7.4) for 10 minutes under deep xylazine/ketamine anesthesia. Brains were removed, postfixed in 4% buffered paraformaldehyde (3 hours, 4\degree C), immersed in 10% sucrose overnight, embedded in TissueTek compound (Sakura Finetek, Netherlands), frozen and stored at \textdegree C until further use. To avoid degradation of mRNA, mice used for subsequent gene expression analyses were perfused with 0.9% NaCl under terminal xylazine/ketamine anesthesia. Brains were quickly removed, embedded in TissueTek, frozen on dry ice, and stored at \textdegree C. Coronal sections for immunohistochemical staining (10 \mu m) covering bregma \pm 1 mm to 0 mm were cut with a cryostat (Leica CM 3050, Nussloch, Germany), mounted on glass slides (SuperFrost, Langenbrinck, Germany) and stored at \textdegree C until further use. Specimen for subsequent laser capture microdissection and gene expression analysis were cut (12 \mu m) and collected on polyethylene naphtalate (PEN) membrane coated slides (PALM MicroLaser Systems, Germany).

Laser Capture Microdissection and RNA Extraction
Immediately after sectioning, PEN-foil–mounted brain sections were fixed in 96% alcohol (\textdegree C, 5 minutes) and air-dried for 1 minute. Toluidine blue staining was used for identification of blood-vessel structures (0.1% Toluidine blue [Sigma-Aldrich, Munich, Germany]/0.1 mol/L sodium phosphate buffered solution, pH 5.5 for 1 minute). Dehydration of brain slices was performed in graded ethanol dilutions (70%, 96%, 100%, 30 seconds each). Afterward, specimens were air-dried and stored at \textdegree C until further use. Capillary blood-vessel structures were identified and isolated using a Zeiss/PAVM LMD microscope (Carl Zeiss MicroImaging GmbH, Germany; Figure 1A–1C). Blood vessels were collected in microfuge caps containing 45 \mu L of RNA extraction buffer RLT (Qiagen, Hilden, Germany) supplemented with 2-mercaptoethanol (143 mmol/L; Sigma-Aldrich, Munich, Germany). Blood vessels of 20 brain slices per animal were cut and collected for subsequent gene expression analyses. RNA purification was performed following the manufacturer protocol provided for RNA extraction from microdissected tissue (RNaseasy micro kit, Qiagen, Hilden, Germany). RNA integrity and quantity of each sample were analyzed using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA; Figure 1D).
Real-Time Polymerase Chain Reaction

Primers used for expression studies were purchased from Qiagen (QuantiTect Assays, Hilden, Germany): glyceraldehyde-3-phosphate dehydrogenase (QT01658692); claudin-5 (QT00254905); occludin (QT0011055); ZO-1 (QT00493899); and ZO-2 (QT00134687). cDNA concentrations were measured by semiquantitative real-time polymerase chain reactions (RT-PCR) using the ABI PRISM 7700 RT-PCR-System (Applied Biosystems, Foster City, CA) and SYBR-green fluorescence. Standard LightCycler conditions were used for the expression arrays, and all measurements were done in duplicates.

Expression Analysis

Gene expression was related to the individual expression of glyceraldehyde-3-phosphate dehydrogenase as endogenous control. Expression analysis was performed using expression software tool REST-MCS 2.1.6 (for a detailed description of used algorithms see REST homepage: http://www.gene-quantification.de/rest-mcs.html). Comparison between wild-type and MCP-1–deficient mice was inhibited by incubation in Blocking Reagent (15 minutes, Roche, Basel, Switzerland). Protein lysates were collected after centrifugation (12,000 g, 20 minutes, 4°C), protein concentration was determined using BCA Protein Assay (Pierce Chemical Co, Rockford, IL), and all probes were adjusted to a concentration of 1 mg/mL. Equal amounts of protein (12 µg) were separated on 12.5% sodium dodecyl sulfate–polyacrylamide gels and transferred onto 4% to 20% gradient membranes. Membranes were incubated (overnight, 4°C, in TBS-T) with primary antibodies rabbit-anti-albumin (Serotec, Duesseldorf, Germany, 1:1000), rabbit-anti-claudin-5 (Abcam, Invitrogen, 1:100), rabbit-anti-occludin (Invitrogen, 1:200), rabbit-anti-ZO-1 (Invitrogen, 1:100), and rabbit-anti-ZO-2 (Invitrogen, 1:200). For detection of protein bands, a horseradish conjugated secondary antirabbit antibody was applied at a dilution of 1:5000 for 45 minutes. Visualization was performed using horseradish peroxidase substrate (Lumi-LightPLUS, Roche). Signal intensity normalization was performed by stripping membranes and reprobing with glyceraldehyde-3-phosphate dehydrogenase antibodies and β-Tubulin antibodies (in case of claudin-5). For analysis of relative protein changes, normalized concentrations of mouse-albumin, claudin-5, occludin, ZO-1, and ZO-2 of sham-operated animals were set to 100% as baseline. Concentrations of respective post–MCAO-proteins are shown as percentage in relation to their controls. FITC-albumin leakage was determined by measuring FITC-fluorescence intensity within the respective brain tissue samples prepared for Western blot analysis. FITC-albumin concentration was measured using a TECAN Infinite-200 (Tecan Group Ltd, Maennedorf, Switzerland; emission 488 nm, excitation 518 nm) fluorescence reader. Final intracerebral FITC-albumin amounts were determined using a standard curve.

Measurement of FITC-Albumin Extravasation

Images of the whole hemisphere were taken with a Nikon Eclipse 80i microscope (Nikon GmbH, Duesseldorf, Germany) using the Stereo Investigator Software (MicroBrightField Inc., Williston, VT) and proper filter sets for fluorescein isothiocyanate signal. Measurements of FITC-albumin positive areas were performed using a standard computer-assisted image analysis technique.

Western Blot and FITC-Albumin Uptake

Western blot and FITC-albumin uptake/leakage analyses were performed using protein lysates of ipsilateral brain tissue samples of the saline perfused animals (covering bregma –1 to 2.5 mm). Brain tissue specimens without cerebral ischemia (baseline), 12 and 36 hours after MCAO were prepared by ultrasonic homogenization (in lysis buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP-40 and a protease inhibitor mixture (Roche, Basel, Switzerland). Protein lysates were collected after centrifugation (12,000 g, 20 minutes, 4°C), protein concentration was determined using BCA Protein Assay (Pierce Chemical Co, Rockford, IL), and all probes were adjusted to a concentration of 1 mg/mL. Equal amounts of protein (12 µg) were separated on 12.5% sodium dodecyl sulfate–polyacrylamide gels and transferred onto 4% to 20% gradient gels (MiniProtean TGX, BioRad, Munich, Germany). After blocking (5% wt/vol milk powder in TBS-T) for 1 hour at room temperature, membranes were incubated (overnight, 4°C, in TBS-T) with primary antibodies rabbit-anti-albumin (Serotec, Duesseldorf, Germany, 1:1000), rabbit-anti-claudin-5 (Abcam, Invitrogen, 1:100), rabbit-anti-occludin (Invitrogen, 1:200), rabbit-anti-ZO-1 (Invitrogen, 1:100), and rabbit-anti-ZO-2 (Invitrogen, 1:200). For detection of protein bands, a horseradish conjugated secondary antirabbit antibody was applied at a dilution of 1:5000 for 45 minutes. Visualization was performed using horseradish peroxidase substrate (Lumi-LightPLUS, Roche). Signal intensity normalization was performed by stripping membranes and reprobing with glyceraldehyde-3-phosphate dehydrogenase antibodies and β-Tubulin antibodies (in case of claudin-5). For analysis of relative protein changes, normalized concentrations of mouse-albumin, claudin-5, occludin, ZO-1, and ZO-2 of sham-operated animals were set to 100% as baseline. Concentrations of respective post–MCAO-proteins are shown as percentage in relation to their controls. FITC-albumin leakage was determined by measuring FITC-fluorescence intensity within the respective brain tissue samples prepared for Western blot analysis. FITC-albumin concentration was measured using a TECAN Infinite-200 (Tecan Group Ltd, Maennedorf, Switzerland; emission 488 nm, excitation 518 nm) fluorescence reader. Final intracerebral FITC-albumin amounts were determined using a standard curve.

Immunofluorescence Microscopy

Brain slices cut for immunohistochemical staining were postfixed with 4% paraformaldehyde (15 minutes), and endogenous peroxidases were blocked (3% H₂O₂/Methanol, 10 minutes). Unspecific protein binding was inhibited by incubation in Blocking Reagent (15 minutes, Roche...
Primary antibodies used for immunohistochemistry were the following: rabbit-anti-mouse-albumin (1:100; Serotec, Duesseldorf, Germany), rabbit-anti-clathrin (1:100; Abcam, Cambridge, United Kingdom) rabbit-anti-claudin-5 (1:100; Abcam), rabbit-anti-ZO-1 (1:100; Invitrogen, Darmstadt, Germany), rabbit-anti-ZO-2 (1:100; Invitrogen, Darmstadt, Germany), rabbit-anti-occludin (1:100; Invitrogen), and rabbit-anti-von Willebrand Factor (1:250; DukOcytomaion, Hamburg, Germany). For detection of rabbit antibodies, we used a biotinylated goat-anti-rabbit antibody (1:200; Jackson Labs, Bar Harbor, ME). A streptavidin/fluorescent dye (AlexaFluor594, Molecular Probes, Leiden, The Netherlands) was used as chromogen. Nuclear counterstain was performed with fluorescent-preserving mounting medium containing 4',6-diamidino-2-phenylindole (Vector, Burlingame, CA). Confocal images were taken with a fluorescence microscope (Axiovert, Zeiss, Oberkochen, Germany) with appropriate filter sets for Alexa Fluor594, FITC, and 4',6-diamidino-2-phenylindole. Digitizing was done with AxioVision software.

Statistical Analysis

Differences between infarct sizes, mouse-albumin, and bovine FITC-albumin-leakage; RT-PCR data; and Western blot analysis were evaluated by 2-way ANOVA followed by Bonferroni post hoc test using GraphPad Prism software version 5.01. Results are presented as mean±SD. P≤0.05 was considered as significant.

Results

FITC-Distribution and Infarct Size Development

Fluorescence guided investigation revealed an increased accumulation of FITC-conjugated albumin within vessel related structures of the ipsilateral hemisphere. Immunofluorescence analyses showed FITC-albumin accumulation particularly within the perilesional cortex and to a lesser extent within the infarct core (Figure 2A and 2B). FITC-distribution points toward a widespread cortical vessel-associated accumulation. In MCP-1−/− mice, FITC-positive areas were extended compared with wild-type mice 12 hours (P<0.001) and 36 hours after MCAO (Figure 2C).

MCAO led to consistent brain tissue damage within the striatum expanding into the cortical structures. Twelve hours after MCAO, lesion size of wild-type animals was significantly increased (54.3%±9.74%) compared with MCP-1−/− mice (29.36%±8.02%; P<0.001). Thirty-six hours after experimental stroke lesion size remained in wild-type animals (53.66%±8.02%) and slightly expanded in MCP-1−/− animals (37.17%±10.81%; not significant; Figure 2D). Determination of hemisphere swelling showed a pronounced edema formation in all investigated MCAO groups, but no significant differences could be observed (Figure 2D).

FITC-Albumin and Mouse-Albumin Leakage

Confocal microscopy confirmed the close vicinity of FITC-positive signals to blood-vessel-related structures overlapping with vessel marker von Willebrand factor (Figure 3A). FITC-albumin was mainly located surface-bound within the vessel lumen and the perivascular space. Costaining of FITC-albumin and endocytotic vesicle marker clathrin did not show evidence for active endothelial ingestion of FITC-albumin

![Figure 2](http://stroke.ahajournals.org/)

Cerebral ischemia results in reduced infarct size and increased vascular retention of Fluorescein-isothiocyanate (FITC)-albumin in monocyte chemoattractant protein-1 (MCP-1)−-deficient mice. A, Whole section image depicting FITC-albumin distribution mainly within the ipsilateral hemisphere. B, Administered FITC-albumin accumulates within vascular structures of the ipsilateral hemisphere. Comparison of FITC-albumin distribution pattern and toluidine staining revealed FITC-positive blood vessels particularly within the perilesioned cortex and, to a lesser extent, the infarcted core (arrows). C, Fluorescence positive areas showed an extended and more pronounced accumulation of FITC-albumin within the brains of MCP-1−/− mice at 12 hours and 36 hours (not significant) after middle cerebral artery occlusion (MCAO; exemplary image shown). D, Infarct size and hemisphere swelling assessed 12 and 36 hours after MCAO. Lesion volume is expressed as percentage of the ipsilateral hemisphere. Hemisphere swelling as percentage of ipsilateral vs contralateral hemisphere. Values show mean±SD. Asterisks denote significant difference between the investigated groups (**P<0.01; ***P<0.001).
Figure 3. Von Willebrand factor (vWF) immunostaining (A) shows concomitantly fluorescence signal with FITC, scale bar=20 µm. B, Double-fluorescent analyses of Fluorescein-isothiocyanate (FITC)-albumin and vesicle marker clathrin showed no colocalization excluding a possible endocytic uptake of FITC-albumin by endothelial cells. Scale bar=25 µm. C, Immunofluorescence staining showed a widespread leakage of mouse-albumin within the infarcted core (arrows) and surrounding cortex. Compared with mouse-albumin, FITC-albumin showed a similar distribution pattern, albeit to a lesser extent, within the infarcted core. Double-fluorescence analysis of FITC-albumin and mouse-albumin showed a widespread colocalization (inlay). Band intensity analyses showed an increase of mouse-albumin in both groups after middle cerebral artery occlusion (MCAO). On day 1, mouse-albumin concentration was lower in monocyte chemoattractant protein-1 (MCP-1) −/−–deficient mice (P<0.01). Exemplary bands are shown (digitally merged). MCAO also led to an increase of intracerebral FITC-albumin in wild-type (WT) and MCP-1–deficient mice. Compared with wild-type animals, FITC-albumin leakage was reduced in MCP-1–deficient mice on day 1 and day 7 (P<0.05) after MCAO. Values show mean±SD. Nuclear counterstain with 4′,6-diamidino-2-phenylindole (DAPI). KO indicates knockout.

(Figure 3B). Fluorescence analysis about mouse-albumin and bovine FITC-albumin showed widespread colocalization of both proteins leaked into the ischemic tissue covering the infarcted core and parts of the ipsilateral cortex (Figure 3C). Protein analyses showed a noticeably post-MCAO increase of high-molecular proteins in all investigated groups. Western blot analysis revealed a decreased mouse-albumin concentration within the brains of MCP-1–deficient mice compared...
with wild-type animals 12 hours \( P<0.01 \) and 36 hours (not significant) after MCAO. Fluorescence intensity measurements of FITC-albumin within brain tissue lysates showed similar results as the mouse-albumin Western blot data. FITC-albumin leakage was slightly elevated in wild-type animals compared with MCP-1–deficient mice 12 hours (not significant) and 36 hours \( P<0.05 \) after MCAO.

**Gene Expression in Wild-Type and MCP-1–Deficient Animals**

To rule out differences within the constitutional expression of respective genes claudin-5, occludin, ZO-1, and ZO-2, we directly compared the mRNA ratios between sham-operated baseline animals. Data showed no significant differences between wild-type and MCP-1–deficient animals in the healthy brain (Figure 4A–4D).

**Claudin-5 Expression Is Upregulated in MCP-1–Deficient Mice**

Analysis of wild-type mRNA claudin-5–expression showed no relevant differences after MCAO compared with sham-operated animals (Figure 4A). In contrast, claudin-5–expression was upregulated in MCP-1–deficient mice at both investigated time points \( P<0.01 \) compared with sham-operated MCP-1−/− mice and wild-type animals. Western blot analysis confirmed this trend on the protein level, admittedly not significant. Immunofluorescence analysis showed no structural differences of claudin-5 staining between MCP-1–deficient and wild-type animals, but confirmed claudin-5 signals in close vicinity to vascular-accumulated FITC-albumin in all investigated groups.

**MCP-1–Deficiency Leads to a Reduced Occludin Expression**

RT-PCR analyses showed that MCAO results in a reduced occludin-expression in both investigated groups (Figure 4B). Moreover, in MCP-1–deficient mice, transcript expression of occludin was significantly reduced compared with baseline animals and wild-type mice \( P<0.001 \), which was also reflected on the protein level, although less pronounced (12 hours after MCAO; \( P<0.05 \)). Double-fluorescent studies showed occludin and FITC-albumin in close vicinity to vascular structures. Because occludin is known to be an extracellular protein interconnecting endothelial cells, these results could indicate that FITC-albumin is, to a certain extent, also accumulated within the luminal and perivascular space. However, confocal imaging was not sufficient to depict any quantitative/qualitative differences between wild-type and MCP-1−/− immunofluorescence signal.

**Cerebral Ischemia Leads to a Breakdown of ZO-1 Expression**

After MCAO, transcript expression of ZO-1 was clearly reduced in all investigated groups (Figure 4C). MCP-1–deficiency resulted in a significantly reduced expression of the ZO-1 transcript 12 hours \( P<0.01 \) and 36 hours \( P<0.01 \) compared with wild-type animals. Analysis of ZO-1 protein levels confirmed mRNA data showing a significantly reduced protein concentration in both groups 12 hours after onset of ischemia. Thirty-six hours post-MCAO, ZO-1–protein levels remained almost absent in MCP-1–deficient mice in contrast to wild-type animals, which showed re-elevated ZO-1 protein concentration \( P<0.001 \). Confocal immunofluorescence analyses showed FITC-albumin signaling in close vicinity to vascular ZO-1–staining although slightly shifted to the luminal side of blood vessels.

**Experimental Stroke Results in a Reduced Expression of ZO-2**

In wild-type mice, ZO-2 expression was only marginally increased after MCAO, whereas MCP-1–deficiency led to a significant decrease of ZO-2 expression 12 hours \( P<0.001 \) and 36 hours \( P<0.001 \); Figure 4D) compared with baseline animals. The downregulation of ZO-2 in MCP-1–deficient mice was also confirmed by Western blot showing a reduced ZO-2 protein concentration 12 hours \( P<0.001 \) and 36 hours \( P<0.001 \) after MCAO.

Z-stack visualization showed FITC-albumin in close vicinity to ZO-2 within vessels of the ipsilateral hemisphere. According to the double-staining studies of claudin-5, occluding, and ZO-1, no relevant structural differences in ZO-2 fluorescence signals could be detected visually between MCP-1−/− and wild-type mice.

**Discussion**

Loss of BBB-integrity is a pivotal feature in the progress of the ischemia-reperfusion injury cascade after cerebral ischemia and may contribute to further tissue damage. Therefore, in-depth understanding of the pathophysiological mechanisms resulting in BBB-dysfunction is crucial to develop new therapeutic strategies in stroke treatment. Because MCP-1−/− mice show milder inflammation and smaller infarcts, we investigated the influence of MCP-1–deficiency on stroke-induced BBB-dysfunction and expression of BBB-related genes. In the present study, we show that cerebral infarction leads to remarkable alterations in BBB-related gene expression along with an increased vascular accumulation/retention of FITC-albumin in MCP-1–deficient mice. Immunofluorescence analysis revealed that administered FITC-albumin tends to accumulate within cortical areas surrounding the infarcted core mainly in vicinity to endothelial cells and the perivascular space. About the increased accumulation of FITC-albumin within the infarct core surrounding areas, a recent study describes pinocytic vesicle formation and enhanced pinocytosis by endothelial cells after cerebral ischemia in rats, which could suggest that MCP-1 could be involved in regulating vesicular formation and transport through the BBB. However, our double-immunofluorescence analyses about a possible FITC-albumin uptake via clathrin-positive endocytotic vesicles showed no colocalization, ruling out an active uptake by at least clathrin-positive vesicles. In our opinion, insufficient perfusion as explanation for vascular-accumulated tracer protein can be ruled out because FITC-albumin was only rarely seen within the contralateral hemisphere of all investigated animals. However, the increased accumulation of FITC-albumin points toward an alteration of endothelial
surface structures, vessel constriction/obstruction, or milder cortical BBB-impairment attributable to smaller lesions in MCP-1-deficient mice, which could be an interesting topic for further studies.

In several reports, cerebral ischemia has been shown to result in severe BBB-integrity loss leading to hemorrhagic transformation and massive leakage of high-molecular molecules into the damaged tissue.\(^{2,18,19}\) Our findings of a reduced FITC-signal intensity within the infarcted core could be simply explained by the high vascular fluorescence leading to an underestimation of lower signal intensities by extravasated and diffused intraparenchymal FITC. For this reason, we decided to measure the amount of intracerebral diffused mouse-albumin and FITC-albumin. It is well known that both molecules do not cross the

Figure 4. Real-time polymerase chain reactions (RT-PCR) and Western blot analysis were performed for claudin-5, occludin, zonula occludens (ZO)-1, and ZO-2 in wild-type (WT) and monocyte chemoattractant protein-1 (MCP-1)-deficient mice 12 hours and 36 hours after cerebral ischemia. Immunohistochemical staining of BBB proteins showed similar distribution patterns with fluorescein-isothiocyanate (FITC)-albumin confirmed by z-stack depiction (exemplary confocal images shown). A, RT-PCR analysis showed a marked upregulation of claudin-5 gene in MCP-1-deficient mice. In wild-type animals, no altered claudin-5 expression could be detected. Western blot analyses showed no significant changes of claudin-5 protein amount in both groups. B, RT-PCR and Western blot analyses revealed a reduced expression and protein concentration of occludin in MCP-1−/− mice. Wild-type animals showed no altered occludin-expression and concentration compared with baseline. C, Analyses of ZO-1-transcription and protein concentration showed a marked downregulation in MCP-1−/− mice compared with baseline and wild-type animals. D, ZO-2-expression was not affected by middle cerebral artery occlusion (MCAO) in wild-type mice. In contrast, MCP-1-deficiency led to a significant reduced amount of ZO-2-mRNA and protein compared with wild-type mice and baseline animals. RT-PCR data shown represent mean levels ± SD of n-fold changed gene expression compared with the respective sham-operated group. RT-PCR was performed twice for each sample. Western blot data show semiquantitative protein concentration changes compared with the respective baseline protein amount (set to 100%). Exemplary band for each group and time point shown (digitally merged). For ZO-2 (ko; 36 hours) and occludin (ko; 12+36 hours), no cDNA-sample did exceed the set threshold after 40 cycles of RT-PCR. In these cases, ΔC\(_T\) was equated with 40 for further analyses, therefore generating no error-bar (denoted with #). Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) between wild-type vs MCP-1-deficient (ANOVA followed by Bonferroni post hoc test). Scale bar=10 µm. KO indicates knockout.
BBB within the healthy brain and, indeed, the analyses show a massive extravasation of both endogenous mouse-albumin and administered FITC-albumin in all groups. MCP-1–deficient mice showed a reduced intraparenchymal protein concentration despite elevated vascular FITC-accumulation. This indicates a less severe BBB-breakdown and maybe enhanced vascular detaining of tracer proteins compared with wild-type animals. These findings are in concordance with the results of a previous study, which shows that MCP-1 administration leads to a significant increase in the BBB-permeability for FITC-albumin. One of our main findings, the decreased expression of occludin, ZO-1, and ZO-2 in wild-type animals is also supported by results of several other groups. Recent studies show decreased intracerebral occludin and ZO-1 trancription as well as decreased ZO-1 and occludin protein concentration up to 120 hours after experimental MCAO in rats. In our study, both wild-type and MCP-1–deficient mice showed reduced expression of TJ-related genes. Interestingly, the expression of occludin, ZO-1, and ZO-2 was even lower in MCP-1−/− mice. Because secretion or administration of MCP-1 worsens BBB-leakage, reduces transendothelial electric resistance in vitro, and triggers redistribution, phosphorylation, and attenuation of TJ-protein secretion, the decreased occludin, ZO-1, and ZO-2 gene expression/protein concentration in MCP-1–deficient mice was surprising. Previous studies suggest that MCP-1 increases the activity of PKCα as well as PKCδ via the MCP-1–specific CC chemokine receptor-2. MCP-1–triggered induction of PKCα has been reported to alter BBB-integrity via phosphorylation of TJ-proteins occludin, ZO-1, ZO-2, and claudin-5 in vitro, whereas PKCδ activity opens the BBB by loosening the association of occludin and ZO-1 with the cytoskeleton. This suggests that MCP-1 modulates the BBB-integrity indirectly considering TJ-protein phosphorylation state as a transcriptional modulating feedback loop. Our findings of a decreased occludin and slightly elevated claudin-5 protein concentration are supported by the findings of a recent study in which rats were subjecting to MCAO, showing that ischemia triggers matrix metalloproteinase-2–mediated occludin degradation. Furthermore it has been shown, by using an in vitro BBB model, that oxygen deprivation does not alter claudin-5 protein concentration, but leads to a caveolin-1–mediated dissociation from the endothelial cytoskeleton. These findings are also supported by the intriguing results of a recent study reporting that MCP-1–exposure to brain endothelial cells results in a rapid internalization of claudin-5 and occludin within caveolin-1–positive endosomes. Stamatovic et al conclude that internalized claudin-5 and occludin are stored away within recycling endosomes to be available for recycling back to the cell surface to re-establish the BBB-integrity. No evidence for degradation of both internalized TJ-proteins could be found. Taken together, these findings suggest that claudin-5 and occludin despite their altered transcription are possibly, in contrast to ZO-1 and ZO-2, less susceptible for proteinase degradation, which could be an explanation for the minor changes in protein concentration in our study. However, MCP-1–deficient mice showed an increased claudin-5 mRNA and minor increased claudin-5 protein concentration, but a reduced ZO-1 and ZO-2 mRNA/protein concentration in comparison with wild-type mice. This was surprising as MCP-1–deficient animals showed a reduced infarct size and BBB-leakage. These findings could highlight the importance of claudin-5 maintaining the structural integrity of the BBB and could give further evidence that BBB-integrity is not just linear correlated with the plain concentration of TJ-proteins.

Concerning leukocyte infiltration, it is well accepted that MCP-1, which is widely expressed after cerebral ischemia, attracts hematogenous cells into the inflamed tissue. There is strong evidence that immigrated leukocytes contribute to vascular permeability regulation by secreting cytokines, matrix-modeling proteins, and influencing adhesion molecules. In a previous study, we showed that MCP-1–deficiency results in a diminished influx of monocytes, macrophages, and neutrophil granulocytes. Other studies report that various other proinflammatory cytokines like IL-1β or IL-6 are major contributors to BBB-integrity loss after oxygen deprivation in vitro and in vivo. Because MCP-1–deficiency leads to reduced IL-1β, IL-6, and G-CSF expression after experimental stroke, diminished secretion of proinflammatory proteins could prevent further disruption of the BBB in MCP-1−/− mice. A limitation of this study could be the fact that we used laser-captured microvessels for the expression analyses and brain lysates for the protein studies, although it is well known that BBB-related TJ-proteins are solely secreted by vascular-associated cells. Besides, no littermates but backcrossed animals were used in this study. Double-immunofluorescence studies clearly showed a direct correlation between vascular FITC-albumin and respective TJ-protein signal. However, image analyses of structural BBB-alterations are limited because of the given resolution of fluorescence microscopy. In this in vivo study, no significant differences in BBB-protein structure could be observed.

Conclusions

Taken together, our findings show that MCP-1 is involved in the transcriptional and translational regulation of pivotal TJ-related genes claudin-5, occludin, ZO-1, and ZO-2. MCP-1–deficient mice develop smaller infarcts and show reduced BBB-breakdown. The present study provides new insight into the mechanisms of BBB-damage, concluding that MCP-1 could be a major player in the regulation of the delayed BBB-breakdown and subsequent secondary brain damage. Clearly, further studies are needed to clarify the in vivo phosphorylation states of the respective proteins and whether blocking or inhibition of the MCP-1 pathway could be a beneficial and promising strategy in the treatments of inflammation related diseases, including stroke.

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Disclosures

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References


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