Loss of flow induces leukocyte-mediated MMP/TIMP imbalance in dynamic in vitro blood-brain barrier model: role of pro-inflammatory cytokines

Ljiljana Krizanac-Bengez,1 Mohammed Hossain,1 Vince Fazio,1 Marc Mayberg,2 and Damir Janigro1,2

1Cerebrovascular Research Center, Department of Neurosurgery and 2Departments of Cell Biology and Molecular Medicine, Cleveland Clinic Foundation, Cleveland, Ohio; and 3Seattle Neuroscience Institute, Seattle, Washington

Submitted 14 October 2005; accepted in final form 9 May 2006

Krizanac-Bengez, Ljiljana, Mohammed Hossain, Vince Fazio, Marc Mayberg, and Damir Janigro. Loss of flow induces leukocyte-mediated MMP/TIMP imbalance in dynamic in vitro blood-brain barrier model: role of pro-inflammatory cytokines. Am J Physiol Cell Physiol 291: C740–C749, 2006. First published May 17, 2006; doi:10.1152/ajpcell.00516.2005.—There is substantial evidence linking blood-brain barrier (BBB) failure during cerebral ischemia to matrix metalloproteinases (MMP). BBB function may be affected by loss of shear stress under normoxia/normoglycemia, as during cardiopulmonary bypass procedures. The present study used an in vitro flow-perfused BBB model to analyze the individual contributions of flow, cytokine levels, and circulating blood leukocytes on the release/activity of MMP-9, MMP-2, and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs), TIMP-1, and TIMP-2. The presence of circulating blood leukocytes under normoxic/normoglycemic flow cessation/reperfusion significantly increased the luminal levels of MMP-9 and activity of MMP-2, accompanied by partial reduction of TIMP-1, complete reduction of TIMP-2 and increased BBB permeability. These changes were not observed during constant flow with circulating blood leukocytes, or after normoxic/normoglycemic or hypoxic/hypoglycemic flow cessation/reperfusion without circulating blood leukocytes. The addition of anti-IL-6 or anti-TNFα antibody to the lumen before reperfusion suppressed the levels of MMP-9 and activity of MMP-2, had no effect on TIMP-1, and completely restored TIMP-2 and BBB integrity. Injection of TIMP-2 in the lumen before reperfusion prevented the activation of MMP-2 and BBB permeability. These data indicate that blood leukocytes and loss of flow are major factors in the activation of MMP-2, and that cytokine-mediated differential regulation of TIMP-1 and TIMP-2 may contribute significantly to BBB failure.

shear stress; inflammation; matrix metalloproteinases

THE BLOOD-BRAIN BARRIER (BBB), a dynamic interface between the blood and the central nervous system, is composed of tight-junctioned endothelial cells (EC), astrocytic end-feet, an extracellular basal lamina, and perivascular pericytes (14, 35). Blood flow (shear stress) is required for BBB induction and maintenance (26, 41, 42, 47). High laminar shear stress promotes glycosaminoglycan synthesis (4), tight junction formation and the expression of junction related proteins (51). In vitro, either increased flow with statically adapted cells or decreased flow with flow-adapted cells can induce reactive oxygen species generation and activation of transcription factors (12, 48).

Matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs), play fundamental roles in the dynamic remodeling of the extracellular matrix (ECM) (8). Numerous studies support a contributing role for MMPs in neuroinflammatory diseases (37). The various MMPs exist as secreted or membrane-bound enzymes that require conversion from zymogen to active forms (8). The major family of enzymes controlling basement membrane ECM turnover are gelatinases MMP-2 and MMP-9, a subclass of the MMP family, which also includes collagenases, stromelysins, and membrane-type MMPs (MT-MMPs). MMP-2 and MMP-9 can degrade components of the ECM, including collagen, fibronectin, and laminin in the basal lamina and zona occludens-1 in EC tight junctions (36, 38).

A major pathological effect of MMP activity in cerebral ischemia is the disruption of the BBB through degradation of the basal lamina (39). Direct intracerebral injection of MMP-2 results in opening of the BBB with subsequent hemorrhage, and co-administration of TIMP-2 inhibits this response (40). MMP-9 knockout mice display reduced infarct size and less BBB damage after focal ischemia (5). In nonhuman primates, early opening of the BBB after stroke correlated with increased expression of MMP-2, and hemorrhagic transformation was associated with increased MMP-9 expression (11).

Cytokine-dependent regulation of TIMPs provides an important control mechanism for the activity of MMPs. At the sites of inflammation, MMPs and TIMPs contribute to cytokine and cytokine receptor turnover (2, 16). In turn, major pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, and IFN-γ) are important regulators of MMP and TIMP expression (20). In particular, TNF-α-mediated stimulation of MMP expression and synthesis is considered to be an important link between the pro-inflammatory cytokine network and the local increase of the proteolytic activity (10).

The expression of several MMPs is upregulated by IL-1β and TNF-α in EC (18), macrophages (16), and T cells (21). Human T cell activation results in the stimulation of both gelatinases MMP-9 and MMP-2 (27). Pro-inflammatory cytokines up-regulate TIMP-1 expression in both rat brain microvascular EC (BMEC) and astrocytes. The most dramatic effect was observed with the combinations of IL-1β/TNF-α or TNF-α/IFN-γ within a broad concentration range (9). TIMP-1 is considered the inducible form and is up-regulated by phorbol esters, IL-1β, TGF-β1, EGF, and IL-6 (17). TIMP-1 exerts anti-apoptotic properties and provides potent MMP-9 inhibition (7). In the central nervous system, TIMP-1 provides neuroprotective effect by inhibiting MMPs and reducing glutamate-mediated calcium influx following cytotoxic stress (45). TIMP-2 is expressed constitutively in EC and other cell
types, and its expression is not significantly influenced by cytokines and growth factors (18). Both TIMP-1 and TIMP-2 are suppressors of apoptosis (30).

Hemodynamic changes, injury, inflammation, and oxidative stress all appear to regulate MMP expression and activity (37). The regulation of MMPs and TIMPs in various cell types under conditions of oxygen-glucose deprivation, a widely used in vitro model of cerebral ischemia, has not been studied. In addition, little is known about the regulation of TIMPs and MMPs in specific cell types during flow disturbances at the BBB, as relevant for cerebral ischemia due to a cardiac arrest, or cardiopulmonary bypass; most in vitro studies are done under static conditions, lacking physiological shear stress, which is a critical modulator of EC phenotype and function (48, 51). The present study used an in vitro BBB model to analyze the individual contributions of flow, cytokine levels, and blood cells on dynamic changes in MMP-2, MMP-9, TIMP-1, and TIMP-2 under normoxia/normoglycemia or hypoxia/hypoglycemia.

MATERIALS AND METHODS

We sought to determine the interaction between loss of flow (shear stress) and leukocyte-mediated events affecting the release and activity of MMP-2, MMP-9, and their inhibitors, TIMP-1 and TIMP-2, at the BBB. The dynamic in vitro BBB model permits manipulations of shear stress with or without changing the PO2 and/or glucose levels. Flow-conditioned rat BMEC were challenged with 1 h of flow cessation under hypoxia/hypoglycemia or normoxia/normoglycemia, with or without peripheral blood leukocytes in the luminal perfusate, and followed by reperfusion for 48 h under normoxia/normoglycemia. Media samples from the lumen and abluminal compartment were analyzed for blood gases; MMP-2, MMP-9, TIMP-1, and TIMP-2 protein levels; and MMP-2 and MMP-9 activity. Tranendothelial electrical resistance (TEER) was continuously measured to assess BBB integrity (25, 42).

Cell preparation. BMEC, astrocytes, and blood leukocytes were obtained from Sprague-Dawley rats in accordance with guidelines set forth by the Institutional Animal Care and Use Committee. BMEC were obtained as described (42) from 2-wk-old male Sprague-Dawley rats (6 rats per experiment). Cells were cultured in flasks precoated with fibronectin (3 μg/cm2) (Biomedical Technologies, Stoughton, MA). BMECs were characterized by the uptake of Dil Ac-LDL (Biomedical Technologies) and by von Willebrand factor staining (Vector Labs, Burlingame, CA). On day 7, when >96% of the cells were positive for Dil Ac-LDL and von Willebrand factor, BMEC were loaded (5 × 106) into the luminal compartment of the dynamic in vitro BBB model.

Astrocytes were obtained from embryonic day 19 Sprague-Dawley fetal rat cortices (32). Brain tissue was homogenized, filtered through a 64-μm sieve, and cultured on a 60-cm2 area (100 cm2) for the abluminal space. Voltage (5 mV) was applied across the cellular layer, and resistance to alternating current flow continued to be measured via a microcomputer-driven system. TEER was continuously measured to assess BBB integrity (25, 42).

Blood mononuclear cells, obtained from the abdominal aorta of Sprague-Dawley rats anesthetized with 1% isoflurane, were prepared by density gradient centrifugation (Ficoll-Paque) (Amersham Biosciences, Uppsala, Sweden). Cell viability was 100% (determined by Trypan blue dye) before the addition to the luminal medium. One milliliter of cells (35 × 106 total/carteidge) was slowly injected into the luminal perfusate of dynamic in vitro BBB model and allowed to circulate for 24 h before flow cessation/reperfusion experiments.

Tissue culture media. BMEC were grown in low glucose DMEM (Bio-Whittaker, Walkersville, MD), enriched with: 2.5% FBS (HyClone, Logan, UT), 7.5% equine plasma-derived serum (Atlanta Biologicals, Norcross, GA), 2 mmol/l L-glutamine, 1% nonessential amino acids, 1% MEM vitamins, and 1 mmol/l sodium pyruvate (Bio-Whittaker). Endothelial mitogen (Biomedical Technologies) and heparin (Sigma) were added at a final concentration of 50 μg/ml. Astrocytes were grown in F-12-DMEM (Bio-Whittaker) with 10% FBS. The same types of media were used while the cells were loaded (BMEC and astrocytes) into the dynamic in vitro BBB model.

Dynamic model of BBB. BMEC and astrocytes were cultured in dynamic in vitro flow model, as described (42). The cartridges are connected with a media reservoir and a pulsatile pump apparatus (Cellco, Germantown, MD). The pump is capable of generating flow rates of 1–50 ml/min, corresponding to shear stress levels of 1–200 dyn/cm2. For the experiments, we used the shear stress level of 4 dyn/cm2, corresponding to the level in brain postcapillary venules (29). The pulsatile nature of flow produces a pressure waveform (15), comparable to the in vivo situation (49). BMEC are in physical contact with astrocyte end-feet through the 0.5-μm-thick pore size of the fibers. Both compartmental surfaces are coated with 3 μg/cm2 fibronectin and abluminal compartment is additionally precoated with poly-d-lysine (3 μg/cm2) according to the volume (100 ml) and surface area (70 cm2) for the lumen vs. volume (1.4 ml) and surface area (100 cm2) for the abluminal space.

BMEC, exposed to pulsatile shear stress of 4 dyn/cm2 and co-cultured with astrocytes for a 3- wk period, developed relatively high TEER (>650 Ω/cm2) and low permeability to 14C sucrose (5 × 10−6 cm/s). In experiments with blood leukocytes, blood cells were present in the luminal perfusate during the entire time of flow cessation and reperfusion. Their viability was 98% or higher (at 2-h reperfusion) and 82% (at 48-h reperfusion).

Media used in hypoxia/hypoglycemia experiments substituted aglycemic DMEM (Bio-Whittaker) for DMEM. The medium was made hypoxic by injecting nitrogen into the medium for 15–20 min. Normal medium was replaced by hypoxic/aglycemic medium in both the intra- and abluminal compartments, and cartridges were kept without flow for 1 h inside the hypoxic incubator. The oxygen tension was measured during that period both in the luminal compartment and in the hypoxic chamber using an O2 sensor (World Precision Instruments, Sarasota, FL). Analysis of O2, CO2, and electrolytes in the media were obtained using a blood gas analyzer (model ABL 77, Radiometer). After 1 h of flow cessation, hypoxic medium was replaced with normoxic, normoglycemic medium and flow was reinitiated for 48 h in normoxia.

TEER and permeability to 14C sucrose. TEER was measured by a custom device described elsewhere (42). Electodes were placed in ports allowing the access to the abluminal and luminal compartment of dynamic in vitro BBB model. Voltage (5 mV) was applied across the cellular layer, and resistance to alternating current flow continuously measured using a personal computer-driven system. TEER was confirmed by permeability to 14C sucrose (1 μCi; specific activity 565 mCi/mmol; Amersham Pharmacia), injected into the lumen, while differentiating into the abluminal space was monitored. The permeability was calculated by integration of the concentration of the radioactive compound in the lumen and abluminal space, as described (42).

ELISA for proteins levels of MMPs and TIMPs. Luminal and abluminal samples were analyzed by ELISA for MMP-2, MMP-9, TIMP-2 (EMD Biosciences, La Jolla, CA), and TIMP-1 (R&D Systems, Minneapolis, MN). The MMP-9 ELISA kit detects free and TIMP-1 bound MMP-9; the MMP-2 ELISA kit detects pro-MMP-2, active MMP-2 and the MMP-2/TIMP-2 complex. The TIMP-1 ELISA kit detects total TIMP-1, whereas the TIMP-2 ELISA kit measures the free form of TIMP-2 and the complex of TIMP-2 with active MMP-2, but does not detect the complex with pro-MMP-2. Protein levels were measured via a microplate reader system (Synergy HT, Bio-Tek Instruments, Winooski, VT) at 450–595 nm (MMP-2 and MMP-9), 450–540 nm (TIMP-1), or at 450 nm (TIMP-2).

C741 ROLE OF SHEAR STRESS, MMPs, AND TIMPs AT BBB

AJP-Cell Physiol • VOL 291 • OCTOBER 2006 • www.ajpcell.org

Downloaded from http://ajpcell.physiology.org/ by 10.221.32.246 on June 9, 2017
**Zymography.** The activity of MMP-2 and MMP-9 in the luminal and abluminal supernatants was measured by zymography, as described (3). The equal volumes (20 μl) were loaded and separated by 10% precasted Tris-glycine gel with 0.1% gelatin as substrate (In Vitrogen, Carlsbad, CA). Zymography standard (Chemicon International, Temecula, CA), containing pro- and active forms of MMP-2 and MMP-9, was used at the final concentration of 2 ng. After separation by electrophoresis, the gel was renatured with 2.5% Triton X-100 twice for 1 h at room temperature, then treated with developing buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl2, 0.2% polyoxyethylene (23) lauryl ether 35) at 37°C for 20 min, and then for 24 h with refreshed developing buffer. All reagents were purchased from Sigma. After developing, the gel was stained with 0.5% Coomassie blue R-250 (Bio-Rad, Hercules, CA) for 30 min, and destained appropriately. Gels were scanned using ScanJet model C7710A and Precision Scan Pro 3.02 software (Hewlett-Packard, Palo Alto, CA). Densitometric analysis of the grayscale image was performed using Phoretix 1D software (Nonlinear, Durham, NC).

**Blockade of IL-6 or TNF-α.** In cartridges perfused with blood cells and exposed to flow cessation/reperfusion under normoxic/normoglycemia, the activity of IL-6 or TNF-α was blocked by using an anti-IL-6 (0.5 μg/ml) or anti-TNF-α (0.3 μg/ml) mouse-anti rat monoclonal antibody (R&D Systems), added in the lumen immediately after flow cessation, i.e., before reperfusion.

**TIMP-2.** TIMP-2 protein (R&D Systems) was prepared as directed by the manufacturer to obtain a stock of 0.1 mg/ml. Stock solution was kept at −80°C. Before its use, TIMP-2 was dissolved in medium to a concentration of 25,000 pg/ml and added (1 ml) to the luminal compartment of cartridges with blood leukocytes after flow cessation.

**Statistical analysis.** Data are expressed as means ± SE. The differences between populations were analyzed by ANOVA and post hoc Mann-Whitney test was used to determine differences between samples. Experiments were performed a minimum of three times with independent cartridges prepared from independent cell cultures. A P value of <0.05 was considered to be statistically significant for all analyses.

**RESULTS**

**Effect of flow cessation on protein levels of MMP-2 and MMP-9 measured by ELISA.** The baseline level of MMP-2 in the lumen and abluminal space (Fig. 1A) was in the range of 6,000 pg/ml and was not affected by flow cessation/reperfusion under normoxic/normoglycemia or hypoxic/hypoglycemia (PO2 = 110.2 ± 4.7 mmHg before flow cessation; 45.6 ± 1.4 mmHg in the abluminal compartment and 58.5 ± 1.7 mmHg in the lumen during flow cessation/reperfusion). MMP-9 was either undetectable, or low in experiments of flow cessation/reperfusion without blood leukocytes (Fig. 1B). In experiments without blood leukocytes, the protein levels of MMP-2 and MMP-9 were reduced under hypoxic/hypoglycemic, compared with normoxic/normoglycemic flow cessation/reperfusion. In contrast to MMP-2, MMP-9 was increased (1.8×) during normoxic/normoglycemic flow cessation/reperfusion in cartridges with blood leukocytes in the luminal perfusate, compared with constant flow (Fig. 1B). Under all experimental conditions, the level of released MMP-9 protein was, on average, 30× lower than that of MMP-2 (Fig. 1, A and B), corresponding to the ratio seen in the blood of healthy people (1). In experiments with blood cells, neutralization of luminal TNF-α or IL-6 before the reperfusion period had no effect on MMP-2 levels (Fig. 2A), whereas both anti-IL-6 and anti-TNF-α reduced MMP-9 protein levels (Fig. 2B).

**Effect of flow cessation/reperfusion on zymographic levels of MMP-2 and MMP-9.** Because ELISA kits measure total MMPs (i.e., active, latent forms, as well as the complexes of MMP with TIMPs), the zymographic levels of pro- and active forms of MMP-2 and MMP-9 were measured in the luminal and abluminal media (Fig. 3). Flow cessation/reperfusion without blood leukocytes in the luminal perfusate, as well as constant...
flow in the presence of blood leukocytes, did not change the zymographic density (Fig. 3), or the ratio of pro- and active forms of MMP-2 or MMP-9 (Fig. 4A, B, and E). The MMP-2 activity in abluminal compartment was, by average, lower during hypoxic/hypoglycemic conditions compared with normoxic/normoglycemic conditions (Fig. 3). BMEC exposed to flow cessation/reperfusion under normoxia/normoglycemia in the presence of blood leukocytes induced a significant increase in MMP-2 after 24 h of reperfusion. This was due to

**Fig. 2.** Effect of neutralizing IL-6 or TNF-α on MMP-2 (A) and MMP-9 (B) ELISA levels in cartridges perfused with WBC in the luminal perfusate and exposed to flow cessation/reperfusion under normoxia/normoglycemia. Control cartridges were untreated and exposed to flow cessation/reperfusion (●), or were under constant, pulsatile flow (○). Note that neither anti-IL-6, nor anti-TNF-α had effect on the MMP-2 levels (A). Both antibodies were able to abrogate the MMP-9 levels increased in the lumen by flow cessation/reperfusion (B). Please note the difference in the scale between A and B. Results are expressed as means ± SE of 3 experiments. **P < 0.05, difference between untreated experiments vs. anti-IL-6 treated group; *P < 0.05, difference between untreated experiments vs. anti-TNF-α-treated group; *P < 0.05, difference between experiments with WBC in the luminal perfusate under constant flow vs. flow cessation/reperfusion under normoxia/normoglycemia.

**Fig. 3.** Effect of flow cessation/reperfusion on MMP-2 (A) and MMP-9 (B) zymographic (activity) levels in leukocyte-free cartridges under normoxia-normoglycemia, hypoxia/hypoglycemia, or in cartridges perfused with WBC in the luminal perfusate under normoxia/normoglycemia with either constant flow (○), or flow cessation/reperfusion (●). A: MMP-2 density levels (combined pro- and active form) were increased only in cartridges with WBC in the luminal perfusate exposed to flow cessation/reperfusion under normoxia/normoglycemia. B: unlike MMP-2, MMP-9 was not increased. Results are expressed as means ± SE of 3 (normoxia/normoglycemia) or 5 experiments (hypoxia/hypoglycemia). **P < 0.05, difference between experiments with WBC in the luminal perfusate under constant flow vs. flow cessation/reperfusion under normoxia/normoglycemia.
an increase of its pro- and active forms in both compartments (Fig. 3A and 4F). Blood leukocytes exposed to flow cessation/reperfusion without contact with BMEC did not release significant levels, and did not change the activity of MMP-2 or MMP-9 (data not shown), demonstrating that BMEC need cell-to-cell contact with blood leukocytes to release MMP-2 and MMP-9. Neutralization of IL-6 or TNF-α (D), or in cartridges perfused with WBC in the luminal perfusate under normoxia/normoglycemia with either constant flow (E), or flow cessation/reperfusion (F–H). Note that MMP-2 density was increased only in cartridges with WBC in the luminal perfusate exposed to flow cessation/reperfusion under normoxia/normoglycemia (F). This was abrogated by anti-IL-6 or anti-TNF-α (G and H). There was no change in MMP activity in cartridges without WBC, even when exposed to pro-inflammatory cytokines during flow cessation/reperfusion (C–D). ECS, extracapillary space (abluminal compartment); Std, standard for MMP-2 and MMP-9. Figure shows one representative zymogram of three (normoxia/normoglycemia) or five experiments (hypoxia/hypoglycemia).

Effect of flow cessation/reperfusion on levels of TIMP-1 and TIMP-2. Change in the levels of MMP-9 and MMP-2 under flow cessation/reperfusion may reflect the changes in their natural inhibitors, TIMP-1 and TIMP-2. Throughout the experimental time frame, in cartridges with or without blood leukocytes in the luminal perfusate, TIMP-1 protein levels were not affected by flow cessation under normoxia/normoglycemia, or hypoxia/hypoglycemia (Fig. 6A). The presence of circulating blood leukocytes during flow cessation/reperfusion induced only a 15% decrease of TIMP-1 (Fig. 6A), whereas it induced a disappearance of TIMP-2, both in the lumen and in the abluminal compartment (Fig. 6B). This corresponded to a significant increase in pro-inflammatory cytokine concentrations during the same time interval; exposure to flow cessation/reperfusion under normoxia/normoglycemia in experiments with blood leukocytes in the luminal perfusate induced a dramatic increase in the concentration of TNF-α (2 h) and IL-6.
(24-h reperfusion), accompanied by biphasic BBB opening (26). We therefore tested whether neutralization of IL-6 or TNF-α could affect the release of TIMP-1 and TIMP-2. TIMP-1 release was not affected by anti-IL-6 or anti-TNF-α antibody (Fig. 7A). In comparison, both antibodies completely restored TIMP-2 to baseline values, i.e., before the flow cessation/reperfusion period (Fig. 7B). We concluded that loss of flow decreases the levels of TIMP-2 through pro-inflammatory cytokines IL-6 and TNF-α.

Role of TIMP-2 in BBB permeability during flow cessation/reperfusion. Low concentrations of TIMP-2 facilitate MMP-2 activation, whereas higher concentrations inhibit the processing of pro-MMP-2 (43). Loss of TIMP-2 during flow cessation/reperfusion is associated with an increase in MMP-2 activity (Fig. 5). Both antibodies abrogated the MMP-2 activity increased by flow cessation/reperfusion (A). Results are expressed as mean ± SE of 3 experiments. **P < 0.05, difference between untreated experiments vs. anti-IL-6-treated group; *P < 0.05, difference between untreated experiments vs. anti-TNF-α-treated group; *P < 0.05, difference between experiments with WBC in the luminal perfusate under constant flow vs. flow cessation/reperfusion under normoxia/normoglycemia.

Role of TIMP-2 in BBB permeability during flow cessation/reperfusion. Low concentrations of TIMP-2 facilitate MMP-2 activation, whereas higher concentrations inhibit the processing of pro-MMP-2 (43). Loss of TIMP-2 during flow cessation/reperfusion is associated with an increase in MMP-2 activity (Fig. 5). Both antibodies abrogated the MMP-2 activity increased by flow cessation/reperfusion (A). Results are expressed as mean ± SE of 3 experiments. **P < 0.05, difference between untreated experiments vs. anti-IL-6-treated group; *P < 0.05, difference between untreated experiments vs. anti-TNF-α-treated group; *P < 0.05, difference between experiments with WBC in the luminal perfusate under constant flow vs. flow cessation/reperfusion under normoxia/normoglycemia.

Role of TIMP-2 in BBB permeability during flow cessation/reperfusion. Low concentrations of TIMP-2 facilitate MMP-2 activation, whereas higher concentrations inhibit the processing of pro-MMP-2 (43). Loss of TIMP-2 during flow cessation/reperfusion is associated with an increase in MMP-2 activity (Fig. 5). Both antibodies abrogated the MMP-2 activity increased by flow cessation/reperfusion (A). Results are expressed as mean ± SE of 3 experiments. **P < 0.05, difference between untreated experiments vs. anti-IL-6-treated group; *P < 0.05, difference between untreated experiments vs. anti-TNF-α-treated group; *P < 0.05, difference between experiments with WBC in the luminal perfusate under constant flow vs. flow cessation/reperfusion under normoxia/normoglycemia.
reperfusion under normoxia/normoglycemia in experiments with blood leukocytes may, therefore, result in MMP-2 activation with consequent proteolysis of microvascular ECM and BBB disruption. Decreased levels of TIMP-2 (Fig. 6B) were accompanied by an increase in MMP-2 activity (Figs. 4F and 5A), and both the MMP-2 and TIMP-2 responses were abrogated by blocking IL-6 or TNF-α (Figs. 5A and 7B). Others have shown that the administration of TIMP-2 to experimental rats results in the protection of the BBB (40). Injection of TIMP-2 protein (25,000 pg/ml; identical to controls at constant flow) into luminal perfusate of cartridges with blood leukocytes before reperfusion period, prevented the activation of MMP-2 (compare Fig. 8, A and B), and completely preserved TEER (Fig. 8C).

![Fig. 7. Effect of blocking IL-6 or TNF-α on TIMP-1 (A) and TIMP-2 (B) ELISA levels in cartridges perfused with WBC in the luminal perfusate and exposed to flow cessation/reperfusion under normoxia/normoglycemia. Control cartridges were untreated and exposed to flow cessation/reperfusion under normoxia/normoglycemia, or were under constant, pulsatile flow. Both antibodies were unable to abrogate the TIMP-1 levels (A), while they restored TIMP-2 to basal levels (B). Results are expressed as mean ± SE of 3 experiments. *P < 0.05, difference between untreated experiments vs. anti-IL-6-treated group; **P < 0.05, difference between untreated experiments vs. anti-TNF-α-treated group; *P < 0.05, difference between untreated experiments with WBC in the luminal perfusate under constant flow vs. flow cessation/reperfusion under normoxia/normoglycemia.](http://www.ajpcell.org/)

![Fig. 8. Effect of TIMP-2 protein on BBB integrity, as measured by transendothelial electrical resistance (TEER) in cartridges perfused with WBC in the luminal perfusate and exposed to flow cessation/reperfusion under normoxia/normoglycemia. A and B: one representative zymogram is shown, whereas the results for TEER (C) are expressed as means ± SE of 3 experiments. Control cartridges were untreated and exposed to flow cessation/reperfusion under normoxia/normoglycemia. TIMP-2 was added before the reperfusion in the lumen at the concentration equal to control experiments without flow cessation/reperfusion (i.e., 25,000 pg/ml). TIMP-2 was able to abrogate the increase in MMP-2 zymographic activity (compare A and B) and restored TEER (C).](http://www.ajpcell.org/)
**DISCUSSION**

Hypoxia/reoxygenation leads to disruption of BBB tight junctions (13, 31). Biochemical studies suggest that opening of the BBB may be the result of MMP expression and activation. Using a flow-perfused in vitro model of the BBB, we have demonstrated that flow cessation/reperfusion, not hypoxia/hypoglycemia, is a major factor contributing to leukocyte-mediated cytokine release and BBB disruption (26). We now report that leukocyte-mediated cytokine release during flow cessation/reperfusion, not hypoxia/hypoglycemia, differentially regulate 1) production of TIMP-1 and TIMP-2, resulting in 2) MMP/TIMP imbalance, 3) activation of MMP-2, and 4) opening of the BBB.

MMP-2 is constitutively present in its latent form in EC and in the astrocytic end feet (52). In EC cultures, stimulation with TNF-α does not affect MMP-2 mRNA expression (19). During an ischemic insult MMP-2 becomes activated primarily through a unique mechanism involving MT-MMP and TIMP-2 (52). In the flow model, the MMP-2 protein levels remained stable under all conditions of flow cessation/reperfusion, with or without treatment with anti-IL-6 or anti-TNF-α antibody. However, the activity of MMP-2 did increase during flow cessation/reperfusion in cartridges perfused with blood leukocytes; this was completely abrogated by addition of TIMP-2 protein in the luminal perfusate before the reperfusion period.

In tissue culture of human cerebral EC, TNF-α induces de novo expression of MMP-9 mRNA, but this did not lead to MMP-9 protein release into supernatant, as indicated by zymography and ELISA (19). In contrast, in flow model MMP-9 protein levels were elevated, but only in the luminal compartment of cartridges with circulating blood leukocytes exposed to flow cessation/reperfusion.

The net functional outcome of MMP and TIMP expression likely involves interactions between various cell types (38). For example, while astrocytes in culture produce pro-MMP-2 constitutively, and pro-MMP-9 in response to inflammatory stimuli, the key components required for their activation include interaction with other cells. Indeed, in the flow model, BMEC exposed to flow cessation/reperfusion without blood leukocytes did not release significant levels of MMP-2 or MMP-9, even when exposed to high concentration of IL-6 and/or TNF-α. Furthermore, blood leukocytes exposed to flow cessation/reperfusion without contact with BMEC released neither MMP-2 nor MMP-9. Therefore, BMEC need cell-to-cell contact with blood leukocytes for release of pro-form of MMP-9 and active-form of MMP-2 during flow cessation/reperfusion. In agreement with our data, MMP-9 release from human vein endothelial cells is induced by contact to lymphocytes (28).

Expression of MMP-9 within 24 h of an ischemic insult is cell specific and primarily confined to the brain endothelium (6). In vivo, MMP-9 expression after focal cerebral ischemia is primarily vascular (6, 46). Neutrophils also provide a major source of MMP-9 (22). In our experiments, neutrophils represented <5% of cells in the leukocyte perfusate, whereas the majority of blood cells were lymphocytes and monocytes. Blood lymphocytes and monocytes, however, play a major role in BBB disruption in stroke, and in patients with multiple sclerosis (23, 24).

As shown previously, flow cessation reperfusion under normoxia/normoglycemia in the presence of blood leukocytes in the luminal perfusate led to a significant increase in TNF-α and IL-6 associated with a biphasic opening of the BBB (26). Thus we assumed that neutralization of these cytokines would prevent release and/or activity of MMP-2 or MMP-9.

MMP-9 protein levels, increased by 24 h in cartridges with luminal blood leukocytes, were completely prevented by anti-IL-6 or anti-TNF-α antibody. In comparison, MMP-2 protein levels were not affected. However, neutralization of pro-inflammatory cytokines abrogated the increased activity of MMP-2. This corresponded to decreased TIMP-2 protein levels; decrease in TIMP-2 concentration could be due to its association with the active form of MMP-2.

Under constant flow, characterized by high levels of TIMP-2, and lower but stable levels of TIMP-1, the integrity of the BBB was preserved. Upon flow cessation/reperfusion, TIMP-2 was completely reduced. Neutralization of IL-6 or TNF-α induced a significant increase in TIMP-2 levels, restoring it to the baseline levels before the flow cessation. Although TIMP-1 was slightly reduced (15%) upon flow cessation/reperfusion, which corresponded to increased levels of MMP-9, TIMP-1 was not affected by anti-IL-6 or anti-TNF-α antibody. This suggests that an increase in MMP-9 levels may be mediated by the change in TIMP-2/MMP-2 pathway (50). It has been shown that induction of MMP-9 is mediated by cytokines and immediate early genes (37), whereas an early activation of MMP-2 occurs primarily through a unique mechanism involving MT-MMP and TIMP-2 (52).

TIMP/MMP imbalance is implicated in the pathogenesis of central nervous system disorders involving inflammation (44). In our hands, loss of flow decreased the levels of TIMP-1 in a cytokine-independent and TIMP-2 in a cytokine-dependent manner. These findings are in contrast with previous in vitro results obtained on static cultures, where TIMP-1 is considered to represent an inducible form (17), whereas TIMP-2 is constitutively expressed (17, 34). The sustained production of TIMP-2 by EC is refractory to modulation by pro-inflammatory cytokines (18), whereas TIMP-1 is readily induced in cultured murine BMEC by treatment with IL-1β and TNF-α or IFN-γ (9). Muir et al. (33) have shown no change in mRNA expression of TIMP-1 or TIMP-2 in astrocyte cultures following exposure to either TNF-α or IFN-γ.

The discrepancy between our current results and previous in vitro studies is due to a difference in the model system used, i.e., isolated cells in static culture vs. a system, in which cells at the BBB are exposed to flow changes. During flow cessation/reperfusion in the presence of blood leukocytes in the luminal perfusate, the highest levels for MMP-2 and TIMP-2 were found at the baseline, whereas for MMP-9 levels were higher after 24 h. A similar pattern has been described in patients with spontaneous intracerebral hemorrhage (1).

In the flow model, MMP-2 exhibited an increasing trend and TIMP-1 concentration was stable, while TIMP-2 dramatically decreased to very low levels during reperfusion, compared with the samples under constant flow. Higher concentrations of TIMP-2 inhibit the processing of pro-MMP-2, whereas low concentrations of TIMP-2 facilitate MMP-2 activation (43). Decreased levels of TIMP-2 observed during flow cessation/reperfusion may, therefore, result in MMP-2 activation with consequent proteolysis of microvascular ECM and BBB dis-
rupture. Indeed, flow cessation/reperfusion under normoxia/ normoglycemia in the presence of circulating blood cells induced an increase in the active portion of MMP-2, resulting in a complete disruption of the BBB. Others have shown that the administration of TIMP-2 results in the protection of the BBB (40). In our hands, injection of TIMP-2 protein in luminal perfuse before reperfusion reduced the portion of active MMP-2, and completely preserved TEER.

In conclusion, our results suggest that change in TIMP/MMP interplay follows the following pathway: 1) loss of flow induces release of pro-inflammatory cytokines TNF-α and IL-6 (see also Ref. 26); 2) pro-inflammatory cytokines induce release of MMP-9 from blood leukocytes and/or BMEC and decrease TIMP-2; 3) low levels of TIMP-2 induce increase in the active form of MMP-2; 4) activated MMP-2 results in opening of the BBB; and 5) co-administration of TIMP-2 inhibits this response (40). Described pattern could be achieved only in the presence of blood cells in the luminal perfusate; without them flow cessation/reperfusion under normoxia/normoglycemia or hypoxia/hypoglycemia changes neither the concentration of TIMP-2, nor the activity or concentration of MMP-2. The opposite regulation of TIMP-1 and TIMP-2 indicates that these proteins play distinct roles in the regulation of EC integrity and ECM proteolysis induced by flow cessation/reperfusion at the BBB.

ACKNOWLEDGMENTS

We thank Dr. Fiona E. Parkinson of the University of Manitoba and Danica Stanimirovic of the National Research Council, Canada, for suggestions in preparing this article. In addition, we thank Martha Tobin and Christine Moore for editorial assistance.

GRANTS

This work was supported by American Heart Association Grant 0230015N (to L. Krizanac-Bengez), and in part by National Institutes of Health Grants NS-43284, HL-51614, NS-49514, and NS-38195 (to D. Janigro). V. Fazio and D. Janigro are also supported by the Philip Morris Research Fund.

DISCLOSURES

D. Janigro holds a patent on the use of the dynamic in vitro BBB model.

REFERENCES


