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Microglial cell activation is a source of metalloproteinase generation during hemorrhagic transformation

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Hemorrhage and edema accompany evolving brain tissue injury after ischemic stroke. In patients, these events have been associated with metalloproteinase (MMP)-9 in plasma. Both the causes and cellular sources of MMP-9 generation in this setting have not been defined. MMP-2 and MMP-9 in nonhuman primate tissue in regions of plasma leakage, and primary murine microglia and astrocytes, were assayed by immunocytochemistry, zymography, and real-time RT-PCR. Ischemia-related hemorrhage was associated with microglial activation *in vivo*, and with the leakage of plasma fibronectin and vitronectin into the surrounding tissue. In strict serum-depleted primary cultures, by zymography, pro-MMP-9 was generated by primary murine microglia when exposed to vitronectin and fibronectin. Protease secretion was enhanced by experimental ischemia (oxygen-glucose deprivation, OGD). Primary astrocytes, on each matrix, generated only pro-MMP-2, which decreased during OGD. Microglia–astrocyte contact enhanced pro-MMP-9 generation in a cell density-dependent manner under normoxia and OGD. Compatible with observations in a high quality model of focal cerebral ischemia, microglia, but not astrocytes, respond to vitronectin and fibronectin, found when plasma extravasates into the injured region. Astrocytes alone do not generate pro-MMP-9. These events explain the appearance of MMP-9 antigen in association with ischemia-induced cerebral hemorrhage and edema.
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Introduction

Select metalloproteinases (MMPs), Zn^{2+} endopeptidases, are generated during experimental focal cerebral ischemia, and have been associated with hemorrhagic transformation after ischemic stroke, and with primary intracerebral hemorrhage (Clark *et al*, 1997; Rosenberg *et al*, 1998, 2001; Romanic *et al*, 1998; Heo *et al*, 1999). Heo *et al* (1999) first associated the expression of the latent precursor of MMP-9, pro-MMP-9, with hemorrhagic transformation after proximal middle cerebral artery occlusion (MCAO) in the nonhuman primate. That study indicated that hemorrhagic events in the central nervous system (CNS) could be detected by the presence of MMP-9 in brain tissue (Rosell *et al*, 2006), or in plasma from patients with hemorrhagic transformation or primary hemorrhage (Castellanos *et al*, 2003; Abilleira *et al*, 2003). However, the precise cerebral source(s) of pro-MMP-9 generated

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during focal ischemia and hemorrhage are not known.

Hemorrhagic transformation is associated with the substantial loss of extracellular matrix (ECM) components from the microvessel basal lamina, including laminin, type IV collagen, cellular fibronectin, and perlecan (Hamann *et al*, 1995, 1996; Fukuda *et al*, 2004). The basal lamina provides one of the two permeability barriers that prevent cell extravasation and leakage of plasma components in normal normoxic CNS. Degradation of microvessel ECM has been attributed to matrix protease expression and secretion, in particular by MMPs, during focal ischemia. But, members of four protease families expressed acutely could alter specific ECM proteins during MCAO (Heo *et al*, 1999; Chang *et al*, 2003; Fukuda *et al*, 2004). The association of tissue pro-MMP-9 expression with hemorrhagic transformation raises the question whether MMP-9 causes the hemorrhage, derives from the hemorrhage, or is a response to the hemorrhage.

In rodent MCAO models, MMP-9 and its zymogen appear in the ischemic tissue, independent of obvious hemorrhage (Gasche *et al*, 1999; Aoki *et al*, 2002; Gidday *et al*, 2005). In contrast, another gelatinase, pro-MMP-2, is upregulated in the ischemic core in the nonhuman primate, but has no apparent relationship to hemorrhage (Heo *et al*, 1999). pro-MMP-9 is associated with circulating polymorphonuclear (PMN) leukocytes and cells of the monocyte/macrophage lineage, which ordinarily do not enter the CNS parenchyma (del Zoppo *et al*, 1991, 2007; Delclaux *et al*, 1996). MMP-9 expression also has been reportedly associated with experimental hemorrhage, transient exposure to high-dose rt-PA (Tsuji *et al*, 2005; Tejima *et al*, 2007), and transient increased blood–brain barrier permeability (Aoki *et al*, 2002; Gidday *et al*, 2005). A small number of clinical studies have suggested that MMP-9 appears in the plasma of patients during ischemic stroke and after hemorrhagic transformation (Montaner *et al*, 2001*a*,*b*; Abilleira *et al*, 2003; Rosell *et al*, 2006). Those studies have not precisely clarified the sources and targets of the MMP-9 measured in those settings.

An interesting feature of the CNS is the compartmentalization of the ECM. For instance, ECM proteins in the cerebral vascular basal lamina affect cell adhesion receptor properties of both the endothelium and astrocytes (Milner *et al*, 2008*a*). In comparison, the ECM proteins fibronectin and vitronectin circulate in the plasma, and are not generally found beyond the cerebral microvessel wall except under conditions of tissue injury or disease (Abumiya *et al*, 1999). This compartmentalization appears relevant to cerebral tissue responses to ischemic injury.

With this background, the studies reported here tested the hypotheses that (1) pro-MMP-9 is generated by the cerebral tissue surrounding the hemorrhage, (2) this protease zymogen is generated by cells recruited to or resident in the CNS, and (3) activating factors are derived from the blood or plasma. These experiments indicate that extravasated blood is not the only source of pro-MMP-9, although it may contribute some protease content. Rather, fibronectin and vitronectin from the plasma can activate microglial cells (but not astrocytes) to generate pro-MMP-9, whose expression is further enhanced by focal ischemia. These studies indicate that pro-MMP-9 is generated exclusively by microglia in the cerebral tissue response to local hemorrhage, but do not yet prove a pathophysiologic role in microvessel matrix degradation.

Materials and methods

Animal Provisions

All experimental procedures used here were approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute and the University of Washington and performed according to the National Institute of Health guidelines.

Antibodies and Reagents

Monoclonal antibodies (MoAbs) raised against human hemoglobin (clone 8.F.126; United States Biological, Swampscott, MA, USA) and albumin (clone HSA-11; Sigma, St Louis, MO, USA) were used for localization of the two proteins in primate tissue. MoAbs to MMP-9 were obtained from British Biotech (Oxford, UK), and cross-reacted with primate MMP-9 (Heo *et al*, 1999). Polyclonal antibodies against GFAP (Abcam, Cambridge, MA, USA) and laminin (Sigma) were used for structure identification (Anderson and Davison, 1999). An MoAb against vitronectin (Millipore Bioscience Research Reagents (formerly Chemicon), Temecula, CA, USA) and a polyclonal antibody against fibronectin (Sigma) were used for their tissue localization. Microglia were identified by MoAbs to CD11b (clone M1/70) and to CD68 (KP-1) (R&D Systems, Inc., Minneapolis, MN, USA).

For flow cytometry, MoAbs to MHC-1 (clone M1/42.3.9.8; BD Pharmingen, San Diego, CA, USA), Mac-1 (integrin $\alpha_M\beta_2$, CD11b/CD18; BD Biosciences, San Jose, CA), and integrins α_4 and α_5 (BD Pharmingen) were used (Milner *et al*, 2007, 2008*a*). Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies for these studies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

For immunohistochemistry of microglial cells in culture (on coverslips), a rabbit polyclonal antibody against mouse MMP-9 (ab38898; Abcam) was used that recognizes the 92 kDa pro-MMP-9, but most probably not MMP-9 (Hadler-Olsen *et al*, 2010; Priceman *et al*, 2010; Honda *et al*, 2010).

Tissue Specimens from NonHuman Primate

Archived samples of cerebral tissues from nine adolescent male baboons (*Papio anubis/cynocephalus*) were used. The awake nonhuman primate stroke model and the

experimental procedures have been previously described (Abumiya *et al*, 1999; Heo *et al*, 1999). The cerebral tissues were removed after transcardiac perfusion with isosmotic heparinized perfusate, frozen, and archived (Heo *et al*, 1999). For the experiments here, the paraffin-embedded materials were studied shortly after removal, and again 15 years later with superb fidelity. Frozen tissues were stored at −80°C until required, with the surface 100 μm of sample discarded before sectioning. Hemorrhagic transformation occurred at 1, 2, 4, 7, 27, and 168 hours after MCAO (Heo *et al*, 1999).

Primary Cell Cultures: Astrocytes and Microglial Cells

Murine astrocytes and microglial cells were obtained as previously described with modification (Milner and Ffrench-Constant, 1994). Brains from 1- to 3-day-old postnatal C57Bl/6 mouse pups (Charles River Laboratories, Inc., Wilmington, MA, USA) were cleaned of meninges and external blood vessels, chopped finely, then dissociated for 30 minutes in a solution containing 30 U/mL papain (Worthington, Lakewood, NJ, USA) and 40 μg/mL DNAase I (Sigma) in 1 mL MEM-HEPES (Milner *et al*, 2008*a*,*b*). After trituration, the cell suspensions were centrifuged and the cell pellets resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, penicillin, and streptomycin (all from Sigma), and then plated onto poly-D-lysine (PDL) (Sigma; 5 μg/mL)-coated T75 flasks (Corning, Inc., Corning, NY, USA). Flasks were incubated at 37°C in 5% CO_2 overnight for attachment of cells and then the medium with unattached debris/cells was aspirated before adding the fresh DMEM containing 10% FBS. The established glial cultures were further incubated at 37°C with changes of the DMEM (supplemented with 10% FBS) at 3-day intervals until the cells were confluent (~10 days).

For the preparation of microglial cells, flasks of mixed glial cultures were mechanically shaken on a rotary shaker first at 130 rpm for 15 minutes to harvest floating microglia with conditioned medium. The density of microglial cells was determined by cell counting with a hemocytometer. Microglia were seeded at $\sim 4.0 \times 10^5$ cells/well onto cell culture (six well) plates that were precoated with the chosen matrix substrate. The plates were incubated at 37°C in 5% CO_2 for 20 hours until the cells were fully attached (ramified).

For the preparation of astrocytes alone, the flasks were shaken again at 37°C overnight at 300 rpm, and the medium with the remaining microglial cells and oligodendroglia was removed. The resulting basal layer of astrocytes was then washed with phosphate-buffered saline (lacking calcium and magnesium), lifted with 0.25% trypsin-EDTA. After cell detachment, equal amount of DMEM was added into the flasks and the astrocytes were harvested by centrifugation at 300 *g* for 5 minutes. In all, $\sim 2.5 \times 10^5$ astrocytes were seeded per well (six-well plates) precoated with the chosen matrix substrate. The astrocytes were grown in DMEM containing 10% FBS and the plates were incubated at 37°C in 5% CO_2 for 24 hours.

The purified matrix proteins laminin, collagen IV, perlecan (HSPG), fibronectin, and vitronectin (all from Sigma) were used as growth substrates for microglial cells and astrocytes where appropriate. Six-well plates (Nunc, Roskilde, Denmark) were prepared by coating the wells with a solution containing 10 μg/mL of each ECM protein (except perlecan, 1 μg/mL) for 2 hours at 37°C, followed by washing each well with phosphate-buffered saline. For the experiments, microglia were plated onto coated six-well plates or inserts (Greiner, Kremsmünster, Germany). Astrocytes were plated onto coated six-well plates. After plating, microglial cultures were judged to be >99% pure by Mac-1 immunohistochemistry, and astrocytes were found to be >95% pure by GFAP immunohistochemistry. In both cases, the cells were used only in first passage.

Oxygen-Glucose Deprivation

The standardized conditions for oxygen-glucose deprivation (OGD) have been described in detail (Milner *et al*, 2008*a*,*b*). Here, before induction of OGD, serum-containing media were removed from the cell cultures by simple decanting (the usual method) or by washing twice thoroughly with phosphate-buffered saline using a 'two-pipette' technique before adding serum-free high-glucose medium (4.5 g/L, DMEM containing 4 mM L-glutamine, penicillin, and streptomycin, supplemented with N1 medium) or low-glucose medium (1.0 g/L, supplemented DMEM). The cultures containing low-glucose medium were placed in a hypoxia chamber (Billups-Rothenburg, Del Mar, CA, USA), which was flushed through with a mixture of 95% N_2 and 5% CO_2 for 1 hour, and then closed for the duration of the experiment. O_2 levels decreased to 0.1% to 0.4% at 4 hours, and were maintained there throughout the experiment (18 hours total).

Tissue and Cell Homogenates

Homogenates of cerebral tissues were made by using sequential 10 μm cryostat sections placed in 100 μL of Tris-HCl lysis buffer (50 mM Tris-HCl buffer (pH 7.5), 0.1 mol/L L-arginine, 1.0% Triton X-100, 0.005% Brij 35, 150 mM NaCl, 0.05% Na^+azide). A 200-μL sample was made using enough sections to provide >50 μg tissue from each specimen. The homogenates were spun for 20 minutes at 9,000 *g* at 4°C, and aliquots of the supernatants stored frozen at −80°C until analysis.

Gelatin Zymography

Sensitive quantitative gelatin zymography detected (pro-)MMP-related activities as previously described (Heo *et al*, 1999). Zymography of cerebral tissue homogenates was performed as described by Heo *et al* (1999). For glial cultures, 40 μL aliquots from each cell culture supernatant were mixed with 10 μL 5× sample buffer (0.313 mol/L Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 50% glycerol, 0.05% bromophenol blue) and loaded onto 8% sodium dodecyl sulfate-polyacrylamide resolving gels containing 1 mg/mL gelatin (Sigma). For standardization, 250 pg recombinant human MMP-2 and MMP-9 was loaded onto

each gel. The gelatin gels were run at 4°C and 100 mA for 1.5 hours. After electrophoresis, the gels were rinsed with distilled water and washed three times with aqueous 2.5% Triton X-100, 15 minutes each time on a rotary shaker to remove the sodium dodecyl sulfate. The gels were then incubated in developing buffer (50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and 0.02% Na^+azide) at 37°C for 42 hours. After incubation, the gels were stained in 0.1% Coomassie brilliant blue R-250 solution for 1 hour, and then destained until clear zones appeared, indicating the presence of gelatinolytic activity. Quantitative densitometry of the scanned gels was performed using NIH ImageJ 1.38v software (NIH, Bethesda, MD, USA).

Flow Cytometry

Cells were mechanically removed from the plates and antigen expression was analyzed by flow cytometry (Milner *et al*, 2008*b*; Osada *et al*, 2011). Fluorescence intensity of the labeled cells was analyzed with a Becton-Dickinson FACScan (BD Biosciences), with up to 10,000 events recorded for each condition. Under conditions of OGD, with defined matrix substrates, the mean fluorescence intensity was compared with the control state (e.g., normoxia or the isotype antibody) and expressed as the percentage change relative to the control condition.

Immunohistochemistry

Immunohistochemical studies were performed on 10 μm sections from paraffin-embedded paraformaldehyde-fixed perfused primate brain tissue or on fresh frozen blocks from the same samples removed acutely (Heo *et al*, 1999). Antigens of interest on 10 μm frozen sections of cerebral tissues or isolated cells were detected as previously reported (Abumiya *et al*, 1999; Fukuda *et al*, 2004).

Pro-MMP-9 expression by microglia was developed by using the Vector ABC kit (Vector, Burlingame, CA, USA), according to the manufacturer's instructions. Briefly, cells on coverslips were washed with phosphate-buffered saline and fixed with 100% methanol for 7 minutes. After fixation, the cells were incubated with the primary anti-MMP-9 polyclonal antibody (5 μg/mL) for 1 hour at 20°C. The immunoreactive products were visualized using diaminobenzidine as the chromogen.

Video-Imaging Microscopy

For evaluation of microglial pro-MMP-9 antigen expression, five 0.14 mm^2 noncontiguous regions of interest randomly chosen were captured from each sample. Full-field photographic images were obtained and evaluated in a blinded manner. Pro-MMP-9 immunoreactivity in the nucleus and cytoplasm was judged semiquantitatively using a five-point scale as indicated in Supplementary Figure 3.

Protein and Hemoglobin Assays

Protein contents were measured using the Bradford assay or the BCA protein assay (as appropriate to the extraction buffer used) according to the manufacturer's instructions. Hemoglobin was detected by ELISA (Bethyl Laboratories, Montgomery, TX, USA).

Real-Time RT-PCR

Total RNA was extracted from microglial cells and astrocytes, subjected to normoxia or OGD, using the RNeasy Plus Mini kit (QIAGEN, Valencia, CA, USA). Eluted RNA was treated with RNase-free DNase (Ambion, Austin, TX, USA). The RT-PCR was set up according to the SuperScript III Platinum One-Step Quantitative RT-PCR System protocol (Invitrogen, Carlsbad, CA, USA). The final reaction conditions for RT-PCR were 200 nmol/L per primer, 100 nmol/L per probe, 200 μmol/L of each dNTP, 5 mmol/L $MgSO_4$, and 100 ng total RNA. The PCR cycling conditions were 45 cycles at 95°C for 30 seconds and 60°C for 30 seconds. Primers and TaqMan probes were designed with primer3 software (Supplementary Table 1). Quantitation of the amount of target in the unknown samples was accomplished using a standard curve to determine the starting concentration of the target. Expression levels were standardized using QPCR Mouse Reference Total RNA (Stratagene, La Jolla, CA, USA). The expressions reflect duplicate studies for each culture and the means and standard deviations of 4 to 8 separate cultures.

Statistical Analyses

Summary data are reported as mean ± standard deviations. For the cell-based studies, each OGD experiment was performed in triplicate for each matrix condition, against matched normoxia samples. Replicates of three or more studies were performed on separate days with different primary cultures. For flow cytometry, each experiment was performed in duplicate and repeated a minimum of three times. For real-time RT-PCR, each matrix condition was tested in duplicate with three or more studies performed on separate days. Linear regression was used to investigate pro-MMP-9 expression in the presence of hemorrhage. Analyses of variance were used to compare the effects of fibronectin and vitronectin exposure on MMP-2 and MMP-9 transcription, the levels of pro-MMP-9 expression under OGD versus normoxic conditions, and (pro-)MMP-9 in microglia alone versus microglia/astrocyte conditions. Analyses were performed in Systat 12 (Systat Software, Inc., Chicago, IL, USA, 2007). We report two-sided *P* values throughout, designated as *2P*.

Results

Hemorrhagic Transformation—Sources of Metalloproteinase-9

Gelatin zymography of striatal tissues at successive times after MCAO showed an association between pro-MMP-9 expression and the presence of hemorrhage (Heo *et al*, 1999). Tissue homogenates from the same sections and tissue blocks from the same

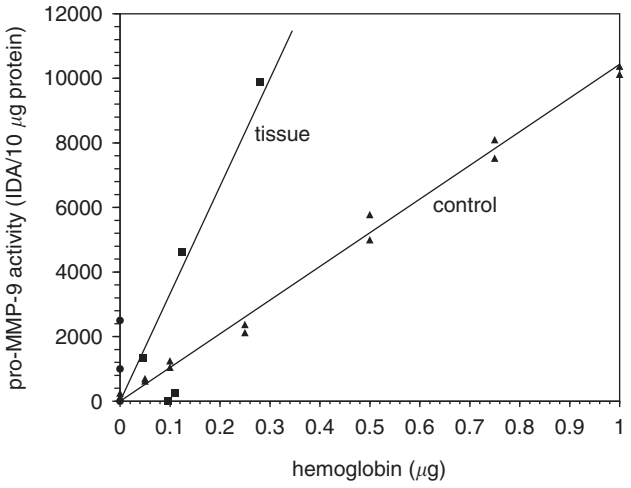

Figure 1 Relative expressions of pro-MMP-9 in striatal tissue with detectable parenchymal hemorrhage versus autologous blood mixed with homogenized normal striatal tissue *ex vivo*, by zymography (integrated density (IDA), normalized to 10 μg protein). pro-MMP-9 content of tissue with parenchymal hemorrhage (tissue, squares; $R^2 = 0.60$, $2P = 0.015$) was significantly greater than the equivalent amount of autologous blood added to homogenates of normal striatal tissue by hemoglobin content (control, triangles; $R^2 = 0.994$, $2P < 0.001$). The difference between the two relationships is significant ($F_{1,19} = 8.04$, $2P = 0.01$). See text. MMP, metalloproteinase.

subjects as in that report were assayed for immunoreactive hemoglobin content and reassayed for MMP-9 content by zymography. A significant linear relationship between pro-MMP-9 and the hemoglobin content of the tissue (at any time) was observed (Figure 1, tissue; $R^2 = 0.60$, $2P = 0.015$). To determine whether extravasated blood was responsible for the elevated MMP-9 content, homogenates of non-ischemic striatal tissue were mixed with varying amounts of autologous blood, providing hemoglobin in the range detected in the hemorrhagic specimens, processed, and then assayed (Figure 1, control; $R^2 = 0.994$, $2P < 0.001$). The observed difference between the two relationships (tissue versus control; $F_{1,19} = 8.04$, $2P = 0.01$) indicated that the hemorrhage-associated pro-MMP-9 generated during focal ischemia derived significantly from the tissue surrounding the extravasated blood.

Hemoglobin Deposition

Within the regions of hemoglobin deposition, cells displaying activated (amoeboid) microglia were frequently detected (Figure 2). Microglia or macrophages defined by both CD11b and CD68 (KP-1) antigens appeared both inside and outside the boundaries of hemoglobin deposition within ischemic striata. Those outside the boundary appeared activated (Figures 2F and 2G). These observations suggest that microglial cells could be a source of (pro-)MMP-9 in response to blood extravasation. MMP-9 immunoreactivity was not seen in the regions of hemorrhage, or in the contralateral striatum, however (Heo *et al*, 1999).

Extravasation of Plasma Extracellular Matrix Components

Within the regions of hemorrhagic transformation, hemoglobin deposition in the extravascular tissue coregistered with the presence of albumin and fibronectin and vitronectin in common areas (Figure 3; Supplementary Figure 1). No immunoreactivity was observed in the contralateral non-ischemic basal ganglia. Dual immunohistochemistry in parallel sections confirmed the deposition of all three proteins in the regions of hemoglobin deposition (data not shown). Extravasation of the three circulating proteins into the perivascular tissue is consistent with opening of the microvessel permeability barrier.

Exposure of Microglia to Plasma Extracellular Matrix Components

The effects of the circulating matrix proteins fibronectin and vitronectin on glial cell activation and gelatinase release were then determined using isolated primary murine microglia and astrocytes, and compared with the cells grown on the substrates PDL or laminin under normoxia and experimental ischemia.

Exposure of primary microglial cells to the vascular basal lamina matrix protein laminin produced little alteration in their activation status (MHC class I expression) or integrin adhesion receptor density. In contrast, fibronectin and vitronectin, proteins not likely to be encountered normally in the non-ischemic tissue, stimulated the expression of MHC class I antigens and the Mac-1 surface receptor (Supplementary Table 2), as previously described (Milner *et al*, 2007). The density of the β_1-integrin α_4 and α_5 subunits on the cell surface, responsible for microglial adhesion, significantly increased with exposure to the two plasma matrix proteins fibronectin and vitronectin (Supplementary Table 2).

Effect of Oxygen-Glucose Deprivation on Adhesion Receptor Expression

Oxygen-glucose deprivation had no effect on integrin α_4 expression, but surface integrin subunit α_5 expression modestly increased further ($6.3 \pm 7.2\%$ or $7.2 \pm 5.5\%$ over normoxia) when the cells were exposed to fibronectin or vitronectin, respectively. When the cells were grown on fibronectin, OGD enhanced Mac-1 antigen expression by $7.1 \pm 3.0\%$ over normoxia (compared with vitronectin, $< 0.08 \pm 0.05\%$ increase).

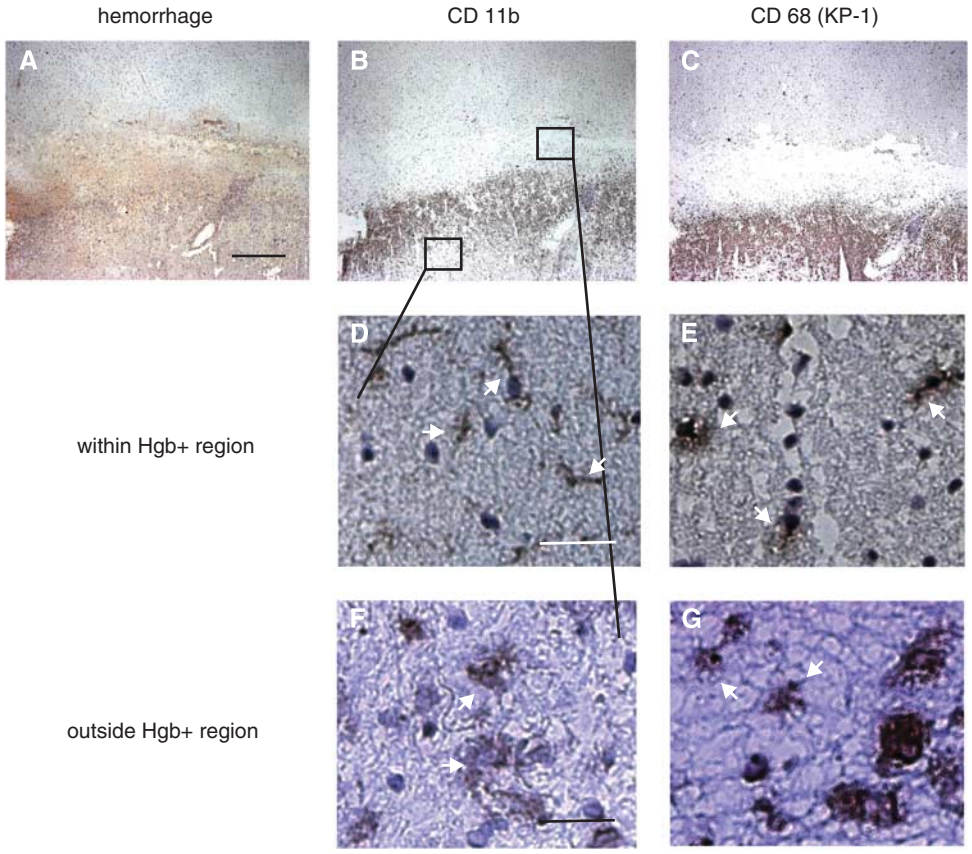

Figure 2 Microglial cells within regions of hemorrhagic transformation of the ischemic core 24 hours after middle cerebral artery occlusion (MCAO) in paraformaldehyde-fixed sections (**A**). Cells expressing the surface markers CD11b and CD68/KP-1 (**B**, **D**, and **F** and **C**, **E**, and **G**, respectively), compatible with microglial cells or macrophages (arrows), are seen inside (**D**, **E**) and outside (**F**, **G**) the areas of hemoglobin deposition. Regions of interest outside and inside the areas of hemoglobin deposition are indicated in (**B**) (that also apply to **C**). The morphologic features of the identified cells (arrows) suggest activated microglia or macrophages (but not PMN leukocytes) between the two regions of interest in (**D**) and (**E**), compared with (**F**) and (**G**). Magnification bars: (**A**–**C**) 100 μm; (**D**, **E**) 10 μm; (**F**, **G**) 10 μm.

Glial Metalloproteinase Gene Expression

To examine the effects of matrix protein exposure on MMP-2 and MMP-9 gene expression, real-time RT-PCR was performed on RNA from microglial cells or astrocytes maintained on PDL, perlecan (HSPG), or laminin under normoxia or OGD (Figure 4A). Under OGD, there was no appreciable increase in MMP-2 gene expression by microglia compared with normoxia, and no increase in MMP-9 gene expression when microglia were grown on these three substrates. By contrast, astrocytes grown on the same substrates showed a nonsignificant 1.72 ± 0.87-fold increase in MMP-2 gene expression on laminin, but no increase in MMP-9 expression compared with normoxia.

The impacts of fibronectin and vitronectin exposure on MMP-2 and MMP-9 transcription by microglia were then examined. With normoxia exposure to fibronectin, but not vitronectin, increased MMP-2 gene expression compared with PDL exposure (1.79 ± 0.54-fold ($2P = 0.029$) and 1.71 ± 1.07-fold, respectively); but, vitronectin significantly stimulated MMP-9 gene expression by 3.27 ± 1.16-fold ($2P = 0.040$) over PDL (data not shown). Under OGD (Figure 4B), vitronectin produced a 1.72 ± 1.16-fold increase in MMP-9 transcription ($2P = 0.15$), but little change in MMP-2 transcription (1.01 ± 0.30-fold difference, $2P = 0.93$), compared with normoxia. Fibronectin produced no change in MMP-9 transcription (0.87 ± 0.32-fold difference, $2P = 0.32$), but a 0.92 ± 0.078-fold decrease in MMP-2 expression ($2P = 0.03$).

Metalloproteinase Generation

Next, to carefully examine the secretion of both latent and active MMPs from isolated cells, preliminary experiments showed that FBS supplements, used routinely for culture, contain large amounts of (pro-)MMP-2 and (pro-)MMP-9. These circulating gelatinases could interfere with the read-outs of the experiments (Supplementary Figure 2), and lead to culture-to-culture variation in MMP output. Therefore, a wash technique was devised to completely remove serum from all subsequent experiments.

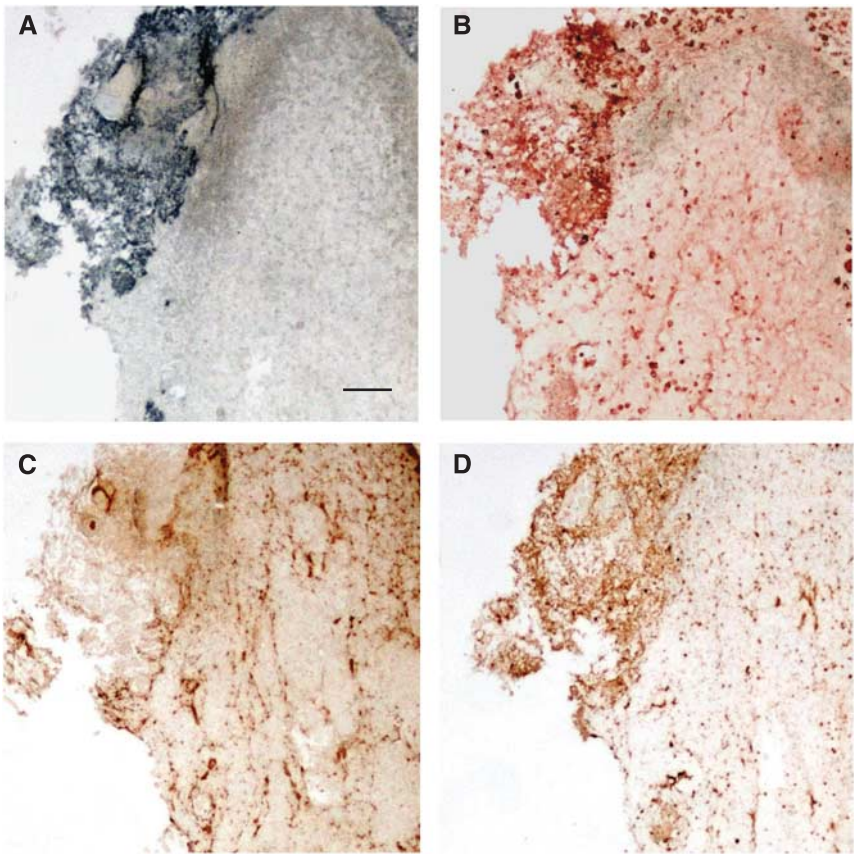

Figure 3 Plasma matrix protein extravasation in a region of hemorrhagic transformation 2 hours after middle cerebral artery occlusion (MCAO) in paraformaldehyde-fixed sections (**A**). Hemoglobin deposition identified by gray-blue coloration within the region of injury. Extravasated albumin in tissue surrounding hemorrhage (**B**), coregisters with the deposition of fibronectin (**C**) and vitronectin (**D**) at the hemorrhage boundary. Magnification bar = 200 μm (see Supplementary Figure 1).

With complete serum depletion, microglial cells were found to secrete pro-MMP-9, but not pro-MMP-2, in significantly increased quantity when the cells were exposed to vitronectin, but much less so to PDL, laminin, or fibronectin (Figure 5). Characteristic changes in microglial morphology were observed on vitronectin and fibronectin, compatible with cellular activation (Milner *et al*, 2007). Oxygen-glucose deprivation stimulated an $84.9 \pm 35.5\%$ increase in mean pro-MMP-9 generation from microglia grown on fibronectin ($2P = 0.0003$), and a $25.6 \pm 11.6\%$ increase in mean pro-MMP-9 when primary microglia were exposed to vitronectin ($2P = 0.0005$; Figure 6).

Astrocytes elaborated pro-MMP-2, but not pro-MMP-9, in response to all four substrates under normoxic conditions (Figure 5). However, OGD uniformly reduced this increased pro-MMP-2 expression by isolated astrocytes on each of the four substrates. In contrast to microglia, neither fibronectin nor vitronectin enhanced the generation of pro-MMP-2 when astrocytes were exposed to experimental ischemia. For both microglia and astrocytes, cell demise was $\leqslant 5\%$ in these studies (Figure 6).

Effects of Normoxia and Oxygen-Glucose Deprivation on Microglial Pro-MMP-9 Content

The ability of experimental ischemia to promote the release of (pro-)MMP-9 from microglia was confirmed by showing that OGD produced a significant loss of immunoreactive pro-MMP-9 from microglia cultured on PDL or fibronectin (Supplementary Figure 3). pro-MMP-9 decreased from both the nuclear and the cytoplasmic compartments, such that $>90\%$ of microglia showed detectable loss of pro-MMP-9 antigen from cytoplasm.

Interaction of Microglia and Astrocytes

To determine whether an interaction between primary astrocytes and microglial cells could further affect the generation of both gelatinases, the impact of microglia added to primary astrocytes grown on collagen IV (to mimic the vascular milieu) was examined. pro-MMP-9 secretion increased progressively with increasing microglia to astrocyte cell ratio, both under conditions of normoxia and OGD (Figure 7). At a microglia to astrocyte ratio of 1:1, pro-MMP-9 secretion significantly increased during OGD

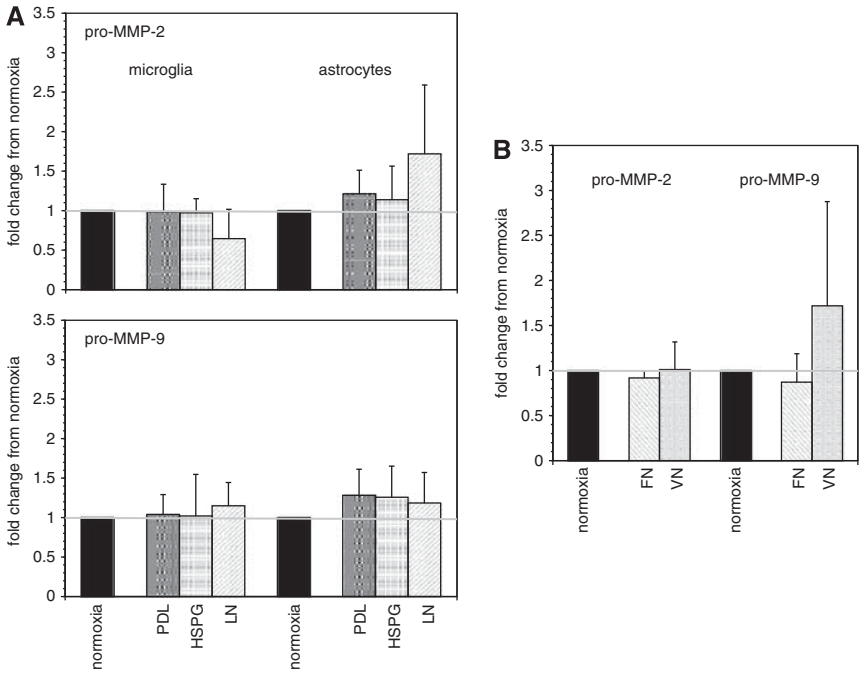

Figure 4 Gelatinase gene transcription responses of primary microglia and astrocytes to matrix exposure under oxygen-glucose deprivation (OGD) compared with normoxia. (**A**) Responses of microglia and astrocytes to poly-D-lysine (PDL), perlecan (HSPG), and laminin (LN). With OGD, microglia displayed no change in MMP-2 or MMP-9 gene expression, while astrocytes displayed increased MMP-2 transcription on LN (all changes not different from unity). $n = 5$ to 8 experiments, each in duplicate. (**B**) Gelatinase gene transcription responses of primary microglia to fibrinogen (FN) and vitronectin (VN) exposed to OGD. VN stimulates a variable increase in MMP-9 gene expression ($2P = 0.15$), and fibronectin a consistent decrease in MMP-2 gene expression ($2P = 0.03$). $n = 7$ experiments, each in duplicate. Bars = mean with standard deviation. MMP, metalloproteinase.

compared with normoxia ($F_{1,8} = 8.39$, $2P = 0.020$), and pro-MMP-9 secretion significantly increased in microglia to astrocyte cocultures compared with microglia alone ($F_{1,8} = 7.32$, $2P = 0.027$). At a microglia to astrocyte ratio of 3:1, results were similar: pro-MMP-9 secretion significantly increased during OGD compared with normoxia ($F_{1,8} = 12.34$, $2P = 0.008$), and pro-MMP-9 secretion significantly increased in microglia to astrocyte cocultures compared with microglia alone ($F_{1,8} = 6.29$, $2P = 0.036$). At the 3:1 ratio, the OGD/microglia + astrocytes response was larger than might have been expected under linearity ($F_{1,8} = 5.41$, $2P = 0.048$). In addition, the presence of microglia appeared to increase pro-MMP-2 expression by astrocytes (Figure 7).

A second experiment assessed the impact of astrocytes grown on collagen IV in proximity to microglial cells (Supplementary Figure 4). When microglia were grown on PDL in inserts under normoxia, astrocytes (on collagen, in wells) produced little effect on microglial pro-MMP-9 generation at either microglial cell density (microglia to astrocytes = 1:1 or 3:1). But, OGD stimulated a modest increase in microglial pro-MMP-9 generation in the presence of astrocytes. The small amount of pro-MMP-9 in the wells was consistent with diffusion. Exposure of microglia to fibronectin did not change the observations (data not shown). Hence, direct contact between microglia and astrocyte is required for the increased pro-MMP-9 release.

Discussion

Hemorrhagic transformation is a common feature of focal brain ischemia and is associated with the appearance of the matrix protease (pro-)MMP-9 in the ischemic territory (Heo *et al*, 1999). Clinical studies have extended those observations to the setting of intracerebral hemorrhage and focal ischemia, but have not been consistent. To explore this relationship, we have used a well-characterized nonhuman primate model of focal cerebral ischemia and hemorrhagic transformation, and isolated primary murine glial cells in culture. The glial studies are based on the observations that isolated primary murine endothelial cells and astrocytes can be cognates of their counterparts in nonhuman primate brain (Milner *et al*, 2008*a*,*b*; Osada *et al*, 2011). These experiments confirm that pro-MMP-9 appears in regions of hemorrhage within focal cerebral ischemic injury (Heo *et al*, 1999), and that a principal source of pro-MMP-9 is the tissue reaction to the extravasated plasma components. Surrogate studies indicate that the amount of pro-MMP-9 supplied by extravasated peripheral blood is insufficient to explain that

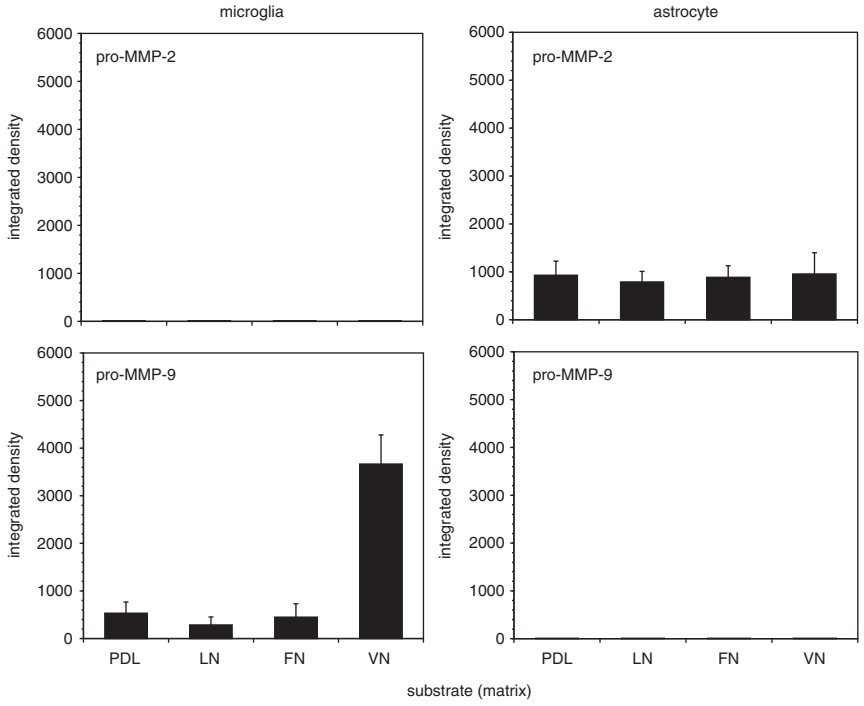

Figure 5 Microglial cell (left panel, $n = 8$) and astrocyte (right panel, $n = 5$) pro-MMP-2 and pro-MMP-9 secretion in response to matrix protein exposure, under normoxia with fetal bovine serum (FBS) depletion (by zymography). No active MMP-2 or MMP-9 was detected. Matrix substrates include poly-D-lysine (PDL), laminin (LN), fibronectin (FN), or vitronectin (VN). Microglial cells produced significant increases in pro-MMP-9 compared with baseline ($2P < 0.0005$ each substrate). VN increased pro-MMP-9 production compared with PDL ($2P = 0.0002$), whereas LN decreased pro-MMP-9 compared with PDL ($2P = 0.0148$). Astrocytes produced a uniform increase in pro-MMP-2 generation compared with baseline ($2P = 0.0075$ each substrate). Bars = mean with standard deviation. MMP, metalloproteinase.

observed in the perfused ischemic tissue suffering hemorrhagic transformation (Figure 1). These observations are compatible with the thesis that during hemorrhagic transformation leakage of the plasma-derived matrix proteins fibronectin and vitronectin can trigger the selective appearance of pro-MMP-9 derived from activated microglia (Milner *et al*, 2007). Microglia are not exposed to either matrix protein in the normal uninjured CNS. We have shown that (1) microglial cells are activated in the regions of hemoglobin deposition *in vivo*, (2) the circulating matrix proteins fibronectin and vitronectin are deposited in the regions of hemorrhagic transformation, (3) primary microglia, activated by exposure to vitronectin and fibronectin *in vitro*, significantly increase pro-MMP-9 generation and secretion only (decrease pro-MMP-9 content), and (4) experimental ischemia produces a further increase in (pro-)MMP-9 secretion by microglia, but not astrocytes, in the presence of these matrix substrates. In addition, microglia and astrocytes can interact with each other to enhance (pro-)MMP-9 release under experimental ischemia, a situation likely to occur *in vivo*. It should be pointed out that these data refer to microglial cells (and astrocytes) derived from young animals, and may not reflect the situation when cells are derived from older subjects.

Microglia are derived from cells of the monocyte/macrophage lineage (Hoogerbrugge *et al*, 1988; del Zoppo *et al*, 2007). Garcia *et al* (1994) initially described that the invasion of monocytes into the ischemic regions of a rodent model increased 24 hours after MCAO, after the incursion of PMN leukocytes. Mabuchi *et al* (2000) subsequently showed that activated macrophages appear on the inside boundary of the cortical ischemic regions, whereas activated microglia are found just outside the ischemic regions. That distribution is consistent with the appearance of activated microglia or macrophages in the face of the hemorrhagic transformation seen after ischemia in the nonhuman primate here. Activated microglial cells were found (1) at all time points after MCAO, (2) within the zones of hemoglobin deposition, and (3) associated with extravasation of the plasma proteins albumin, vitronectin, and fibronectin. In addition, the coregistration of activated microglia with regions of increased microvessel permeability suggests that activated microglia could be a source of MMP-9 activity. While it cannot be ruled out that some part of the cellular activation is due to the participation of peripheral monocytes/macrophages or PMN leukocytes, the timing, location, morphology, and association with (pro-)MMP-9 strongly suggest the local involvement

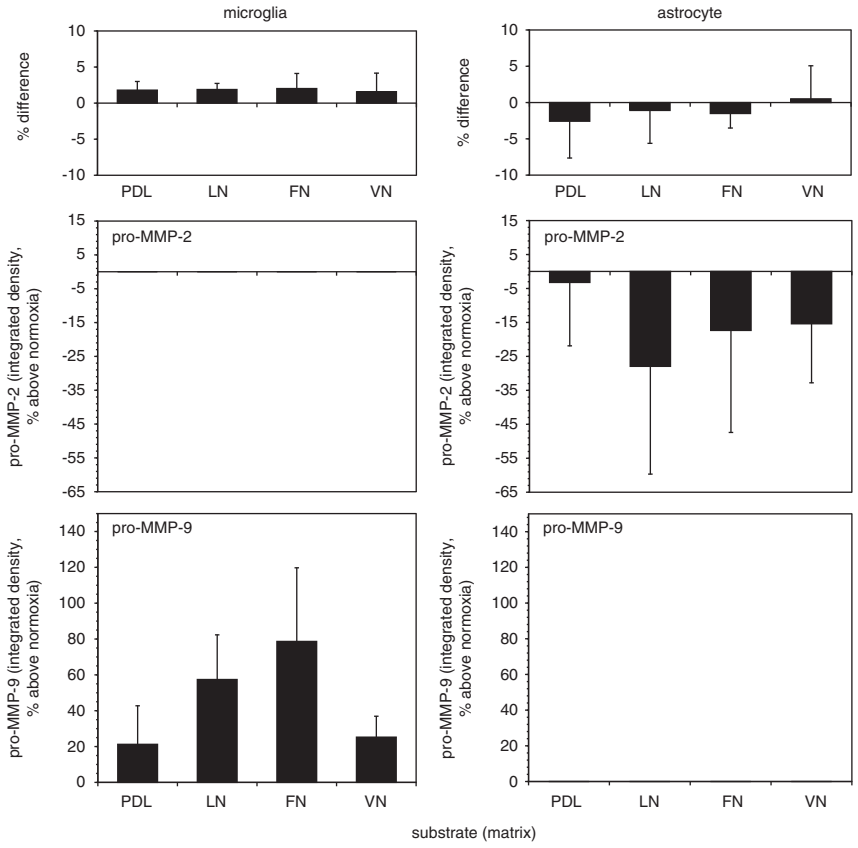

Figure 6 Relative pro-MMP-2 and pro-MMP-9 secretion by primary microglia (left panel, $n = 8$) and astrocytes (right panel, $n = 5$) to matrix protein exposure, under oxygen-glucose deprivation (OGD) with fetal bovine serum (FBS) depletion (by zymography). Matrix substrates include poly-D-lysine (PDL), laminin (LN), fibronectin (FN), or vitronectin (VN). Note the additional increase in pro-MMP-9 production by microglial cells ($2P < 0.0004$ each substrate), but no significant change in pro-MMP-2 production by astrocytes. For microglia, FN stimulated additional pro-MMP-9 production compared with PDL ($2P = 0.0006$). The conditions did not appreciably change cell viability during each experiment (upper panels). Bars = mean with standard deviation. MMP, metalloproteinase.

of resident microglia, and not astrocytes, in the regions of plasma extravasation.

Published reports indicate that all cell types from brain tissue might produce MMP-9. In samples from human ischemic cerebral tissue, MMP-9 antigen has been attributed to vascular endothelium (Rosell *et al*, 2008), astrocytes (Tejima *et al*, 2007), microglia (Clark *et al*, 1997), and PMN leukocytes (Clark *et al*, 1997; Rosell *et al*, 2008). Clark and colleagues suggested that in tissue samples from human ischemic stroke victims, MMP-9 expression (by zymography) could be explained by PMN leukocytes or microglia (Rosell *et al*, 2008). But, there are significant limitations to exact cell attributions in fixed human ischemic tissues: the timing of the initial hemorrhagic event may not coincide with the timing of tissue fixation or sampling (i.e., detectable hemorrhage can immediately follow focal ischemia; Heo *et al*, 1999) and human tissues are not perfused, so that significant protease content could derive from blood retained within the vasculature to plasma in the tissue. Rodent focal ischemia model studies have attributed MMP-9 to astrocytes (Lee *et al*, 2005), microglia (Lee *et al*, 2005), neurons (Lee *et al*, 2005), and PMN leukocytes (Gidday *et al*, 2005). A number of reports suggest that MMP-9 antigen in experimental focal ischemia is not generated by PMN leukocytes (Justicia *et al*, 2003; Maier *et al*, 2004; Tejima *et al*, 2007), although these cells participate in the early inflammatory phase of the ischemic lesion (Okada *et al*, 1994; Garcia *et al*, 1994; Gidday *et al*, 2005) and release MMP-9 activity. So, it remains open whether a single cell type or several cell populations together respond to ischemia and hemorrhage by generating MMP-9.

A few reports describe the effect of inflammatory stimuli on isolated glial cells in culture (del Zoppo *et al*, 2007). The exposure of rat microglia to lipopolysaccharide stimulated the appearance of MMP-9, and less so MMP-2 (Gottschall *et al*, 1995; Rosenberg *et al*, 2001). Zymosan, tumor necrosis factor-α, or poly (ADP-ribose) polymerase-1 stimulated MMP-9 release (Gottschall *et al*, 1995; Kauppinen and Swanson, 2005). By contrast, astrocytes appeared to generate both MMP-2 and MMP-9 on exposure to lipopolysaccharide or fragment Aβ of amyloid (Rosenberg *et al*, 2001; Deb *et al*, 2003). Inconsistencies in those results, and our previous

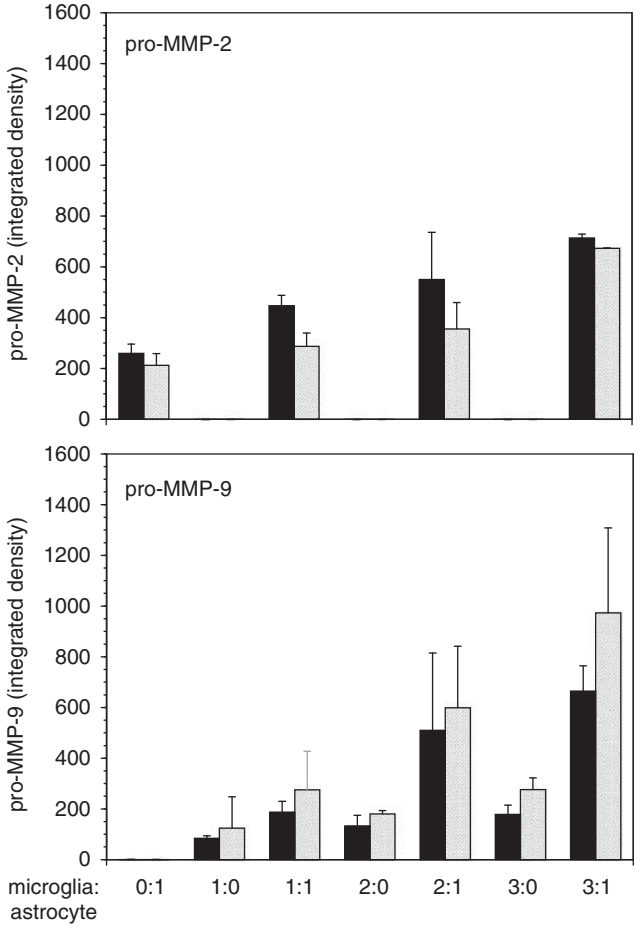

Figure 7 pro-MMP-9 generation depends on relative microglial density and astrocyte exposure. Microglia enhance pro-MMP-9 generation in the presence of astrocytes (grown on collagen IV) under normoxia and oxygen-glucose deprivation (OGD), compared with microglia alone. At a microglia-to-astrocyte ratio of 3:1 pro-MMP-9 secretion significantly increased during OGD compared with normoxia ($F_{1,8} = 12.34$, $2P = 0.008$), and pro-MMP-9 secretion significantly increased in microglia-to-astrocyte cocultures compared with microglia alone ($F_{1,8} = 6.29$, $2P = 0.036$). Bars (triplicate, mean with standard deviation): black = normoxia; hatched = OGD. MMP, metalloproteinase.

work (Heo *et al*, 1999), suggest that the impacts of ischemia, and specifically hemorrhage, on MMP-2 and MMP-9 production by both cell types would be of interest.

Metalloproteinase-9 expression has also been seen in other settings. *In vivo*, MMP-9 expression has been shown in many CNS conditions that stimulate innate inflammation, including multiple sclerosis (Detmar *et al*, 1994; Clark *et al*, 1997; Rosenberg *et al*, 2001), Parkinson's disease (Lorenzl *et al*, 2002), and amyotrophic lateral sclerosis (Dewil *et al*, 2005). There are conflicting data regarding the source of MMP-9 in the CNS and its potential roles (Crocker *et al*, 2008). It is not fully understood whether MMP-9 mediates destructive effects in the CNS, as the evidence of ECM protein degradation, including microvessel basal lamina laminin and collagen, and myelin-specific proteins is circumstantial (see Crocker *et al*, 2008). Perturbation of MMP-9 expression or activity by genetic deletion or pharmacological blockade, respectively, have yielded conflicting results. In experimental autoimmune encephalomyelitis, MMP-9$^{(-/-)}$ mice have a delay in demyelination, and consistent with this, pharmacological blockade protects mice from experimental autoimmune encephalomyelitis (see Crocker *et al*, 2008). However, MMP-9 deletion in focal cerebral ischemia can reduce the size of ischemic damage in the short term, but worsens prognosis in the long term (Asahi *et al*, 2000; Zhao *et al*, 2006). In addition, MMP-9$^{(-/-)}$ mice have been shown to have accelerated neuron injury in a model of amyotrophic lateral sclerosis (Dewil *et al*, 2005). Importantly, each of these conditions is associated with variable disruption of the microvessel permeability barrier (and extravasation of plasma). A challenge is to dissect the different roles of microglia and their gelatinases in these disorders.

Meanwhile, Tejima *et al* (2007) showed that while untreated astrocytes generated (pro-)MMP-2 when exposed to human hemoglobin the cells secreted increased amounts of both pro-MMP-2 and pro-MMP-9. Human and nonhuman primate plasmas contain variable but substantial quantities of (pro-)MMP-9 and (pro-)MMP-2 (Heo *et al*, 1999), and FBS from commercial sources contains substantial quantities of MMP-2, MMP-9 (Supplementary Figure 2), and other proteases. This is most probably due to circulating levels in normal plasma (MMP-2), and leukocyte activation (MMP-8 and MMP-9) on serum preparation. Hence, cell studies in which serum is not completely removed likely provide variable baseline MMP-2 and MMP-9 levels. Therefore, completely serum-free conditions for both normoxia and OGD were devised to clearly expose the glial responses to plasma ECM components here.

Under rigorously serum-depleted conditions, primary microglia exclusively generate pro-MMP-9 when exposed to vitronectin and fibronectin. Isolated astrocytes generate MMP-2 transcripts when grown on laminin, but secrete pro-MMP-2 in response to all matrix proteins *in vitro*. Heo *et al* (1999) had shown that in response to MCAO, pro-MMP-2 is generated in the ischemic core of the primate striatum, but not pro-MMP-9, except when hemorrhagic transformation of the ischemic regions occurred. The selective appearance of MMP-9 transcripts by microglial cells in response to vitronectin was broadly confirmed by real-time RT-PCR; but no substantial change in MMP-2 transcription by these cells was seen (Figure 4). Laminin, a matrix protein not typically associated with microglial cell adhesion, was associated with decreased MMP-9 secretion, which is compatible with the known suppression of microglial activation on this substrate. These data are consistent with the broad observation that peripheral monocytoid cells generate MMP-9, rather than MMP-2 (Gottschall *et al*,

1995; Rosenberg *et al*, 2001). Any discordance in transcription and MMP-9 release by microglia could represent the presence of subsets of microglial cells that might have different response capabilities, and the presence of small amounts of serum. In the case of astrocytes, exposure to vascular and plasma ECM substrates had no effect on pro-MMP-2 secretion compared with PDL.

Milner *et al* (2007) have shown that fibronectin- and vitronectin-induced microglial activation and pro-MMP-9 generation/secretion are mediated by the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$. The studies here confirm the ability of both matrix proteins to increase microglial cell activation under normoxia; OGD in some circumstances produces a further modest activation (Supplementary Table 2). In addition, vitronectin and OGD stimulate increased integrin α_5 expression.

Experimental ischemia further increased pro-MMP-9 secretion by microglia, in rigorously serum-depleted conditions, in a substrate-dependent manner: on fibronectin, OGD stimulated an $\sim$80% increase in pro-MMP-9 generation by microglia over baseline, whereas on vitronectin a smaller relative increase in pro-MMP-9 release was seen. The latter could be explained by either a marginal or a negative posttranscriptional regulatory effect of ischemia on vitronectin-mediated pro-MMP-9 release. *In vitro*, pro-MMP-9 antigen disappearance from microglia occurs when the cells are exposed to OGD. In contrast, OGD produced modest reduction of pro-MMP-2 output by astrocytes under serum-depleted conditions.

pro-MMP-9 generation is also modulated by the relative density of microglial cells and the presence of astrocytes with which they associate (Figure 7). pro-MMP-9 secretion increased significantly when microglia interacted directly with astrocytes under OGD. One implication is that in studies where astrocyte populations generated pro-MMP-9, the cultures contained microglia and plasma. In the normal setting of the brain, the relative density of these two glial cell types has not been recorded, but is likely to vary depending on the brain region. Of importance, clinically, it is likely that the interaction between astrocytes and microglia in cerebral tissue moderates pro-MMP-9 activity when microglia are activated.

It has been indicated that injection of autologous blood into brain in the rat produces activation of microglia (Hickenbottom *et al*, 1999). The experiments reported here show that rather than a direct cause of hemorrhage, pro-MMP-9 generation can be explained as a reaction of activated resident microglia to the presence of specific matrix components in the plasma extravasated into the injured brain that cause microglial activation. This is relevant to the possible contributions of (pro-)MMP-9 to evolving brain injury during ischemic stroke in the clinical setting. Clinical studies by Montaner *et al* have suggested that MMP-9 antigen levels in plasma correlate with ischemic cerebral injury (Montaner *et al*, 2001*a*; Rosell *et al*, 2006), hemorrhagic transformation (Montaner *et al*, 2001*b*; Rosell *et al*, 2006), intracerebral hemorrhage (Abilleira *et al*, 2003), and edema (Tejima *et al*, 2007). However, those observations have not clarified whether MMP-9 derives from the cerebral tissues directly (as here, from activated microglia), from activated PMN leukocytes or activated monocytes from the peripheral blood, or both. All are explainable by the extravasation of plasma into the brain tissue, and leakage of pro-MMP-9 into plasma. One implication is that plasma constituents trigger latent microglial matrix protease release that can affect matrix components of the neurovascular unit. Here, microglia appear to serve as 'sentinels' to the leakage of specific plasma constituents when the microvessel permeability barrier is broken.

Finally, despite known species differences in the expression of MMP-2 and MMP-9 in response to MCAO (Wang *et al*, 2000; Chang *et al*, 2003), the behavior of primary murine cells in culture again explain the observations in whole primate tissue (Milner *et al*, 2008*a*,*b*). What remains unresolved is whether the pro-MMP-9 produced by activated microglia in the setting of hemorrhage generates active MMP-9 that could participate in extravascular matrix remodeling, in injury extension, or its resolution.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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