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The asparaginyl endopeptidase legumain after experimental stroke

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Various proteases in the brain contribute to ischemic brain injury. We investigated the involvement of the asparaginyl endopeptidase legumain after experimental stroke. On the basis of gene array studies and *in situ* hybridizations, we observed an increase of legumain expression in the peri-infarct area of rats after transient occlusion of the middle cerebral artery (MCAO) for 120 mins with a maximum expression at 24 and 48 h. Immunohistochemical analyses revealed the expression of legumain in Iba1$^+$ microglial cells and glial fibrillary acidic protein-positive astrocytes of the peri-infarct area in mice after MCAO. Post-stroke recovery was also studied in aged legumain-deficient mice (45 to 58 weeks old). Legumain-deficient mice did not show any differences in physiologic parameters compared with respective littermates before, during MCAO (45 mins), and the subsequent recovery period of 8 days. Moreover, legumain deficiency had no effect on mortality, infarct volume, and the neurologic deficit determined by the rotating pole test, a standardized grip strength test, and the pole test. However, a reduced number of invading CD74$^+$ cells in the ischemic hemisphere indicates an involvement in post-stroke inflammation. We conclude that legumain is not essential for the functional deficit after MCAO but may be involved in mechanisms of immune cell invasion.
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Introduction

After experimental ischemic stroke, activated intra- and extracellular proteases including calpains (Bevers and Neumar, 2008), cathepsins (Wen *et al*, 2008), and matrix metalloproteinases (Rosell and Lo, 2008) contribute to acute and delayed brain damage. Multiple actions of these enzymes attribute to cleavage of extracellular matrix proteins (Fukuda *et al*, 2004) and to other cell death mechanisms (Jourquin *et al*, 2003). Lysosomal cystein proteases and matrix metalloproteinases may also regulate post-stroke inflammation by processing proinflammatory cytokines (Amantea *et al*, 2007) and modulation of leukocyte infiltration into the brain parenchyma (Campbell *et al*, 2004). However, delayed upregulation of proteases may be involved in tissue re-organization and re-modeling, suggesting a dual function dependent on the spatial and temporal activation patterns (Zhao *et al*, 2006).

The asparaginyl endopeptidase legumain is a unique late endosomal cysteine protease expressed in many mammalian tissues, including the brain (Chen *et al*, 1998, Shirahama-Noda *et al*, 2003). Legumain is activated under acidic conditions in an autocatalytic process (Li *et al*, 2003) and it specifically hydrolyzes the carbonyl side of a surface asparagine residue of the substrate sequence (Chen *et al*, 1997). Several legumain substrates have been identified, including the extracellular matrix protein fibronectin (Morita *et al*, 2007), progelatinase (Chen *et al*, 2001), cathepsins H, B, L (Shirahama-Noda *et al*, 2003), and α-thymosin (Sarandeses *et al*, 2003). The intracellular localization at cell membranes (Liu *et al*, 2003), the involvement in secretion from

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monocytes (Clerin *et al*, 2008), and an increased migratory and invasive activity of legumain-overexpressing tumor cells (Liu *et al*, 2003) indicate that legumain may be important for cell migration. Legumain has been proposed to be involved in processing antigens for class II major histocompatibility complex (MHC) presentation (Manoury *et al*, 1998) though it has not been confirmed (Maehr *et al*, 2005). Hence, legumain-deficient mice show no differences in processing of the invariant chain or maturation of class II MHC products. Moreover, the presentation of ovalbumin and myelin oligodendrocyte glycoprotein, two antigens that contain asparagine residues, to primary T cells was not affected in legumain-deficient mice (Maehr *et al*, 2005).

We earlier showed an increased expression of various proteases in the peri-infarct region after experimental stroke (Rickhag *et al*, 2006). Upregulation in late-expression clusters suggests an involvement in mechanisms secondary to acute neurotoxic cascades. The aim of this investigation was to specify the expression of legumain in different areas of the lesioned hemisphere after middle cerebral artery occlusion (MCAO). We further tested the hypothesis if legumain deficiency affects brain damage and post-stroke recovery after MCAO. Moreover, brain-derived molecular cues have not been identified to regulate the invasion of peripheral immune cells after stroke. On the basis of previous studies (Manoury *et al*, 1998; Maehr *et al*, 2005), we hypothesize that legumain is involved in attraction of those cells into the ischemic hemisphere.

Materials and methods

Transient Middle Cerebral Artery Occlusion in Rat

All animal experiments were carried out with the approval of the Malmö-Lund ethical committee. Transient MCAO was induced as described previously (Rickhag *et al*, 2008). In brief, male Wistar rats (325 to 350 g, HsdBrlHan; Harlan, Scandinavia, Denmark) were housed under diurnal light conditions and were fasted for 12 h before surgery. During surgery, physiologic parameters (arterial blood pressure and gases after tail artery cannulation and insertion of a catheter (SIMS Portex, Hythe, UK) for rectal body temperature) were measured and controlled within physiologic limits. Moreover, body temperature was measured after 1 and 2 h of occlusion and after 1 and 2 h of recirculation. Rats were anesthetized (initial 4% fluothane in N_2O/O_2 (70:30), during surgery 2% fluothane in N_2O/O_2; AstraZeneca, Lund, Sweden) and the right common carotid artery and external carotid artery were occluded permanently, the internal carotid artery was exposed and ligated. A nylon filament (top diameter 0.3 to 0.4 mm) was introduced into the internal carotid artery through a small incision into the distal end of the common carotid artery and pleaded up to occlude the origin of the MCA for 2 h. The filament was withdrawn and awake rats were placed in a cooling box. At 2 h after occlusion, a neurologic score was assessed and only rats showing rotational asymmetry and dysfunctional limb placement were included into the study. Rats were killed at different time points after tMCAO (3, 6, 12, 24, and 48 h) and fixative perfused brains were processed for immunohistochemical analyses. The same procedure was performed in sham-operated animals but no filament was introduced into the internal carotid artery.

Transient Middle Cerebral Artery Occlusion in Mouse

Studies were performed on age-matched male legumain wild-type and knockout mice with a C57BL/6J background (44 to 45 and 55 to 66 weeks old) (Shirahama-Noda *et al*, 2003). Transient MCAO occlusion was induced as essentially described by Nygren and Wieloch (2005). Briefly, anesthesia was induced by inhalation of 3% isoflurane in N_2O/O_2 (70:30) and maintained by inhalation of 2% isoflurane in N_2O/O_2 (70:30) through a face mask during the initial phase of surgery. During surgery, body temperature was maintained at 37°C using a heating pad and controlled by a temperature probe inserted into the rectum. Regional cerebral blood flow in the MCA was controlled by a flexible optical fiber connected to a laser Doppler (PeriFlux System 5000; Perimed, Jarfalla, Sweden) mounted on the skull of the right hemisphere 4 mm lateral from midline and 2 mm posterior from bregma. A silicon-coated filament (Prolene 6-0; Johnson & Johnson, New Brunswick, NJ, USA) was introduced into the internal carotid artery through an incision in the external carotid artery. The filament was advanced until it blocked the origin of the MCA. Placement was confirmed by a reduction in laser Doppler flow and isoflurane concentration was decreased to 1.5% during MCAO. We have only included mice with adequate occlusion, which was considered by (1) regional cerebral blood flow reduction by at least 70% immediately after filament placement, (2) sustained reduction of regional cerebral blood flow during occlusion time of 45 mins, and (3) completely recovered regional cerebral blood flow within 5 mins after the filament was removed.

Carbon Black Perfusion

Legumain-deficient mice and respective wild-type littermates were perfused with carbon black as described previously (Berry *et al*, 1975). Briefly, animals were anesthetized with 2% isoflurane and perfused with NaCl, followed by 4% formaldehyde. Finally, animals were perfused with a carbon black solution containing 40 mL encre de Chine India ink (Panduro Hobby AB, Malmö, Sweden), 20 mL of a 25% mannitol solution, and 7 g gelatin dissolved in 120 mL water. Brains were dissected immediately and kept at 4°C, overnight, in a fixative. Pictures were acquired with a MicroPublisher 3.3 RTV CCD camera (QImaging, Surrey, BC, Canada) under standard conditions.

Post-Surgical Management of Mice

After surgery, mice were transferred to heating cages and checked regularly during the first 48 h after surgery. The cage temperature was regulated to maintain the body

temperature for 2 days. Body temperature was measured every hour for the first 5 postoperative hours, at 24 h, and further every 24 h for 5 days. Body weight was controlled every 24 h after MCAO for 5 consecutive days. Glucose solution (5%, intraperitoneally) was injected after a standardized protocol to prevent weight loss after MCAO.

Rotating Pole Test

Rotating pole test was performed in mice as described previously (Ruscher *et al*, 2009). An elevated wooden pole (750 mm above ground, diameter 15 mm, length 1,500 mm) was rotating at 0 or 3 or 10 r.p.m. to the right or left, respectively. Mice were trained to traverse the pole to reach a platform on the opposite side at any rotation, 12 h before surgery. After MCAO, mice were tested at days 2 and 8 using a score from 0 to 6: 0, the mouse falls off the pole; 1, the mouse tries to walk forward but falls off; 2, the front paws embrace the pole while crossing and the mouse manages to reach the platform; 3, the mouse is jumping with its hind legs or slipping with its feet while crossing; 4, the mouse traverses the pole with more than eight slips; 5, the mouse crosses the pole with three to seven slips; 6, the mouse crosses the pole fast with zero to two slips. All tests were recorded and evaluated by an experienced person masked to the study. A healthy mouse performs with a score of 5 to 6.

Pole Test

Mice were placed head upward on the top of a vertical wooden pole with superficial circular notches every 5 mm (diameter: 8 mm, length: 500 mm). The time to turn to the head-down position (t_{turn}) and the total time to turn and reach the floor with all four paws (t_{total}) was taken in five trials. If the mouse did not turn but descended in the head-up or lateral position, the value of the t_{total} was attributed to the t_{turn}. The maximum time to perform the test was set to 120 secs. The best performance was used for the statistical analyses. The pole test was performed 1 day before and at 2 and 8 days after MCAO.

Grip Strength Test

Forelimb strength was measured using the Grip Strength Test Meter GS3 (Bioseb, Chaville, France). Mice voluntarily gripped a grid with either the healthy or the paralyzed forelimb and pulled it backward. The maximum strength out of three trials was taken for analysis.

Infarct Size Measurement

Coronal brain sections (thickness 30 μm) were stained for the neuronal specific antigen NeuN (dilution 1:1,000; Millipore, Hampshire, UK). Infarcted areas, the nonlesioned area of the infarcted hemisphere, and the nonlesioned contralateral hemisphere were outlined and the infarct volume was calculated as described previously (Ruscher *et al*, 2009).

In Situ Hybridization Histochemistry

Wistar rats were subjected to MCAO and killed at various time points of recovery (3, 6, 12, 24, 48 h, $n=5$ for each time point). Isolated brains were cut on a cryostat in coronal sections and mounted on Superslide glasses (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). After fixation for 15 mins with 4% paraformaldehyde, sections were rinsed in phosphate-buffered saline and dehydrated in ethanol. An oligonucleotide specific for legumain mRNA (Rickhag *et al*, 2006) was 3′-labeled with α-[^{35}S]dATP using terminal deoxynucleotidyl transferase (Amersham Biosciences, Uppsala, Sweden). Hybridization was performed in a humidified chamber for 18 h at 42°C using a hybridization cocktail containing 50% formamide and 10^7 c.p.m./mL of labeled oligonucleotide. After hybridization, the sections were washed at 55°C in 1 × SSC for 3 × 15 mins followed by 1 × SSC at room temperature for 15 mins. Further, the sections were rinsed in water and dehydrated in ethanol. The radioactive slides were then exposed to BioMax MR films (Kodak, Kista, Sweden) and the intensity from the autoradiograms was photographed.

Immunohistochemistry

Brain sections (thickness 30 μm) from 4% paraformaldehyde-perfused animals were washed in phosphate-buffered saline and quenched (3% H_2O_2, 10% methanol) for 15 mins. After blocking with 2% normal horse serum in phosphate-buffered saline supplemented with 0.25% Triton X-100 for 60 mins, the sections were incubated with the following primary antibodies: polyclonal rabbit anti-legumain (dilution 1:400; Shirahama-Noda *et al*, 2003), polyclonal sheep anti-legumain (dilution 1:400; R&D Systems, Minneapolis, MN, USA), monoclonal anti-NeuN (dilution 1:2,000; Millipore), and CD74 (dilution 1:600; BD Biosciences Pharmingen, San Jose, CA, USA) at 4°C overnight followed by a secondary biotinylated horse anti-goat antibody (dilution 1:400; Vector Laboratories, CA, Burlingame, USA). Visualization was achieved through the Vectorstain ABC Elite kit (Vector Laboratories) using 3,3-diaminobenzidine/H_2O_2. Omission of the primary antibody served as a negative control.

Immunofluorescence

Sections were processed as described for immunohistochemistry. For colocalization of proteins, the following antibodies were used: polyclonal sheep anti-legumain (dilution 1:200; R&D Systems Europe, Abingdon, UK), polyclonal rabbit anti-legumain (dilution 1:200; Shirahama-Noda *et al*, 2003), monoclonal mouse anti-NeuN (dilution 1:1,000; Millipore, Solna, Sweden), monoclonal directly Cy3-conjugated anti-GFAP (dilution 1:5,000; Sigma-Aldrich, MO, USA), and biotinylated polyclonal rabbit anti-Iba1 (dilution 1:500; Millipore). After overnight incubation at 4°C, cells were incubated with appropriate secondary antibodies (Cy3, Cy5; conjugated donkey anti-mouse antibody and biotinylated horse anti-sheep antibody, both diluted at 1:200; Jackson ImmunoResearch Laboratories, Suffolk, UK). Sections

exposed to the secondary biotinylated donkey anti-sheep antibody were further incubated with an Alexa 488 streptavidin conjugate (dilution 1:300) at room temperature for 60 mins. Fluorescent signals were visualized using a confocal microscopy system (LSM510; Zeiss, Jena, Germany).

Western Blotting

Protein (10 μg) was separated on a 10% SDS polyacrylamide gel. Blocking was performed onto polyvinyldifluoride membranes using blocking buffer (20 mmol/L Tris, 136 mM NaCl (pH 7.6), 0.1% Tween 20, 5% nonfat dry milk), and detected using primary polyclonal antibody against the legumain (dilution 1:2,000; R&D Systems). After incubation at 4°C overnight, the membrane was incubated with a secondary anti-sheep biotin-linked antibody (Dako A/S, Glostrup, Denmark) and a horseradish-peroxidase-linked anti-biotin antibody (dilution 1:3,000; Cell Signaling, Danvers, MA, USA). Signals were visualized by exposing the membrane to a CCD camera (LAS1000; Fujifilm, Tokyo, Japan) using a chemiluminescence kit (Millipore).

Statistical Analysis

Unless otherwise stated, all data are presented as medians with the 25th and 75th percentiles. Data from the rotating pole test were analyzed by the Mann–Whitney *U*-test and $P<0.05$ was considered statistically significant. All other statistical analyses were performed as stated in the figure legends.

Results

We have used *in situ* hybridizations to study the spatiotemporal expression of legumain in the ischemic hemisphere after experimental stroke. As shown in Figure 1, a basal expression was detected in sham-operated animals and in the noninfarcted brain areas immediately after MCAO for 120 mins. Signal intensity was markedly decreased in the infarct core compared with corresponding regions of the nonischemic hemisphere. At 3 h after occlusion, an increase of legumain RNA was observed in the parietal peri-infarct area, whereas the signal further decreased in the infarct core, particularly in the striatum. During the following hours, legumain mRNA was further elevated in layers of the peri-infarcted motor cortex (Figure 1D) with a maximum expression at 24 and 48 h after MCAO (Figure 1E and 1F). At 24 h, legumain expression was spread throughout the noninfarcted areas of the ischemic hemisphere. An accumulation was observed toward the infarct core in the striatum, in the parietal peri-infarct cortex, and a legumain-expressing zone was found at the brain surface. In these regions maximum expression was found at 48 h after MCAO. Accordingly, we found a delayed increase in legumain immunoreactivity in the ischemic hemisphere (Figure 2). Although legumain immunoreactivity was present in round cells at 2 days after tMCAO, it appeared in cytoplasmic globules of ramified cells of the peri-infarct area at 4 and 7 days (Figure 2). No specific immunoreactivity was detectable in sham-operated rats. Collectively, the data show a time-dependent upregulation of legumain within peri-infarct areas after transient MCAO.

At 3 days after MCAO, legumain immunoreactivity was also increased in glia-like cells of the peri-infarct area in legumain wild-type mice (Figure 3). Immunoreactivity in the peri-infarct area was abolished by serial dilution of the primary antibody (data not shown). No immunoreactivity was found in legumain-deficient mice in this region. In addition, legumain immunoreactivity accumulated in

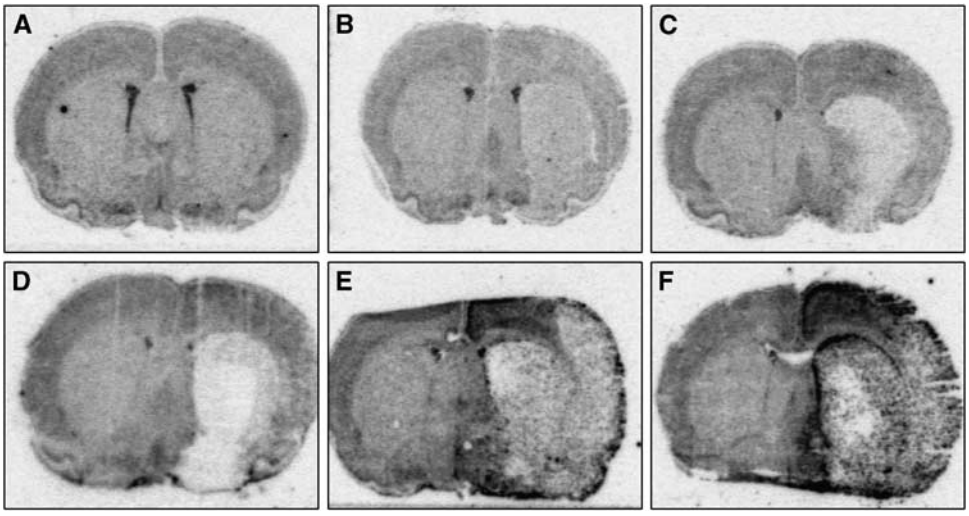

Figure 1 Transcriptional regulation of legumain after middle cerebral artery occlusion (MCAO). Representative coronal sections (bregma −1.8 to −1.6) showing legumain mRNA expression in a sham-operated rat (**A**) and at 3 h (**B**), 6 h (**C**), 12 h (**D**), 24 h (**E**), and 48 h (**F**) after transient MCAO for 120 mins, respectively ($n = 5$ each time point).

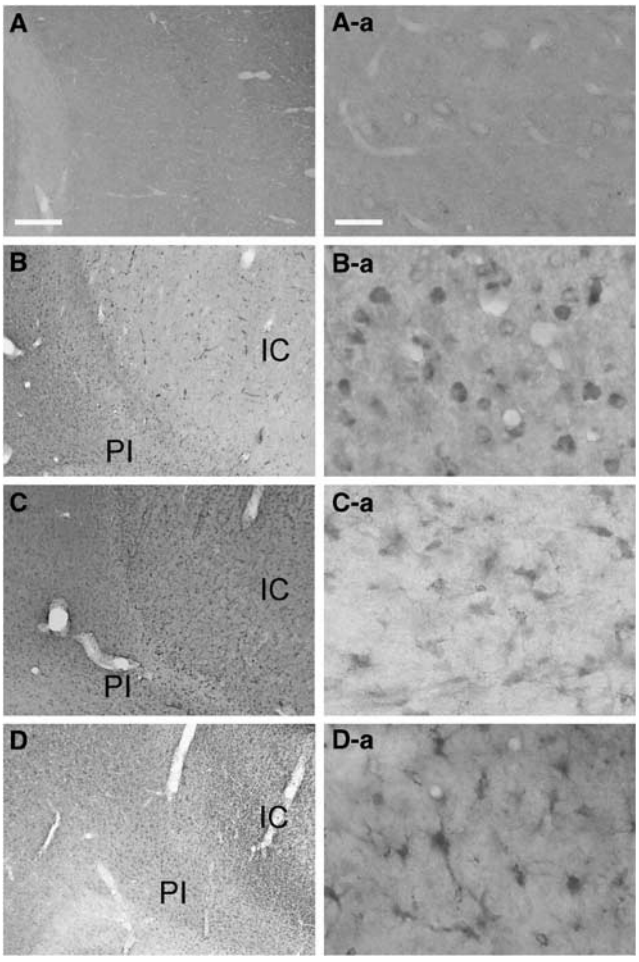

Figure 2 Legumain expression in rat after transient middle cerebral artery occlusion (MCAO). Phase contrast micrograph (**A**) and higher magnification (**A-a**) showing weak immunoreactivity in neuron-like cells and microvessels in the neocortex of a sham-operated rat. (**B**) Legumain immunoreactivity in the ischemic hemisphere 2 days after transient MCAO (120 mins) with (**B-a**) an increased immunoreactivity in microglia like cells inside and around the ischemic core. (**C**) Legumain immunoreactivity 4 days after occlusion with higher number of ramified cells in the peri-infarct area in **C-a**. (**D**) Legumain immunoreactivity accumulates in astrocyte like cells in the peri-infarct region (**D-a**). IC, ischemic core, PI, peri-infarct area, scale bars: 100 μm, magnifications 12.5 μm.

microglia-like cells of the infarct core in wild-type mice but also in legumain-deficient mice. Although legumain immunoreactivity was more pronounced in wild-type animals, it could not be abolished by serial dilution of the primary antibody (data not shown) in both genotypes. The infarct core was devoid of legumain-positive cells by omission of the primary antibody. In addition, recombinant legumain added to the primary antibody solution clearly decreased legumain immunoreactivity in the infarct core (data not shown). In addition, further testing of legumain antibodies revealed no legumain-specific band in western blots from legumain-deficient mice (data not shown). Further immunofluorescence analyses from the peri-infarct area confirmed an expression of legumain in fibrillary acidic protein-positive ($GFAP^+$) astrocytes and $Iba1^+$ microglial cells (Figure 3).

We also performed western blot analyses to evaluate whether legumain is processed into its 36 kDa active form. As shown in Figure 4, an increase of pro-legumain (57 kDa band) was found in the ischemic hemisphere compared with the contralateral cortex. However, no difference was observed between the ischemic core and the peri-infarct area. More importantly and compared with the other regions, active legumain (the 36-kDa band in Figure 4) was significantly increased in the peri-infarct area. In addition, we found a legumain-specific band of around 50 kDa in samples from the peri-infarct area and the contralateral cortex, but not in the ischemic core region, indicating that active legumain might have been processed through a 46/47-kDa intermediate as described before (Li *et al*, 2003). Results corroborate that active legumain is increased in glial cell populations in the peri-infarct area of the ischemic hemisphere after MCAO.

We further investigated the brain damage in legumain knockout mice after MCAO. In total, we studied 16 wild-type and 14 knockout mice. Out of these, seven wild-type and five knockout animals were excluded because of death or lack of recirculation after MCAO. The average age was 55.8 ± 10.4 weeks (mean $\pm$ s.d.) in wild-type and 53.3 ± 9.3 weeks in the knockout group. The body weight did not differ significantly between the study groups (wild-type mice: 36.5 ± 3.7 g, knockout mice 32.0 ± 4.0 g). Postoperative temperature was maintained around 37°C during MCAO and around 36°C for the first 48 h after MCAO to prevent hypothermia that significantly affects the stroke outcome (Auer, 2001). During and within the first 24 h after MCAO, we could not detect any temperature differences between knockout and wild-type mice (Figure 5).

Legumain-deficient mice did not show abnormalities of the larger brain arteries and their branches (Figure 6A). In particular, we found a normal configuration of the circle of Willis with normal branching of the anterior and posterior cerebral arteries. Despite being elongated, the middle cerebral arteries and ramifying branches showed a normal anatomic configuration (Figure 6A). At 8 days after tMCAO, animals were perfused in standard procedure and infarct sizes were evaluated from serial coronal sections and stained for neuronal nuclear protein (NeuN). The infarct size did not differ between the groups with 23.9 mm^3 (25th percentile: 20.8 mm^3; 75th percentile: 25.7 mm^3; $n = 6$) found in legumain wild-type mice and 26.8 mm^3 (25th percentile: 22.5 mm^3; 75th percentile: 28.8 mm^3; $n = 7$) found in legumain-deficient mice (Figure 6B). Moreover, we could not detect significant differences in behavioral tests after tMCAO. Using the rotating pole test, a sensitive approach to test sensorimotor and balance function, we found a similar spontaneous

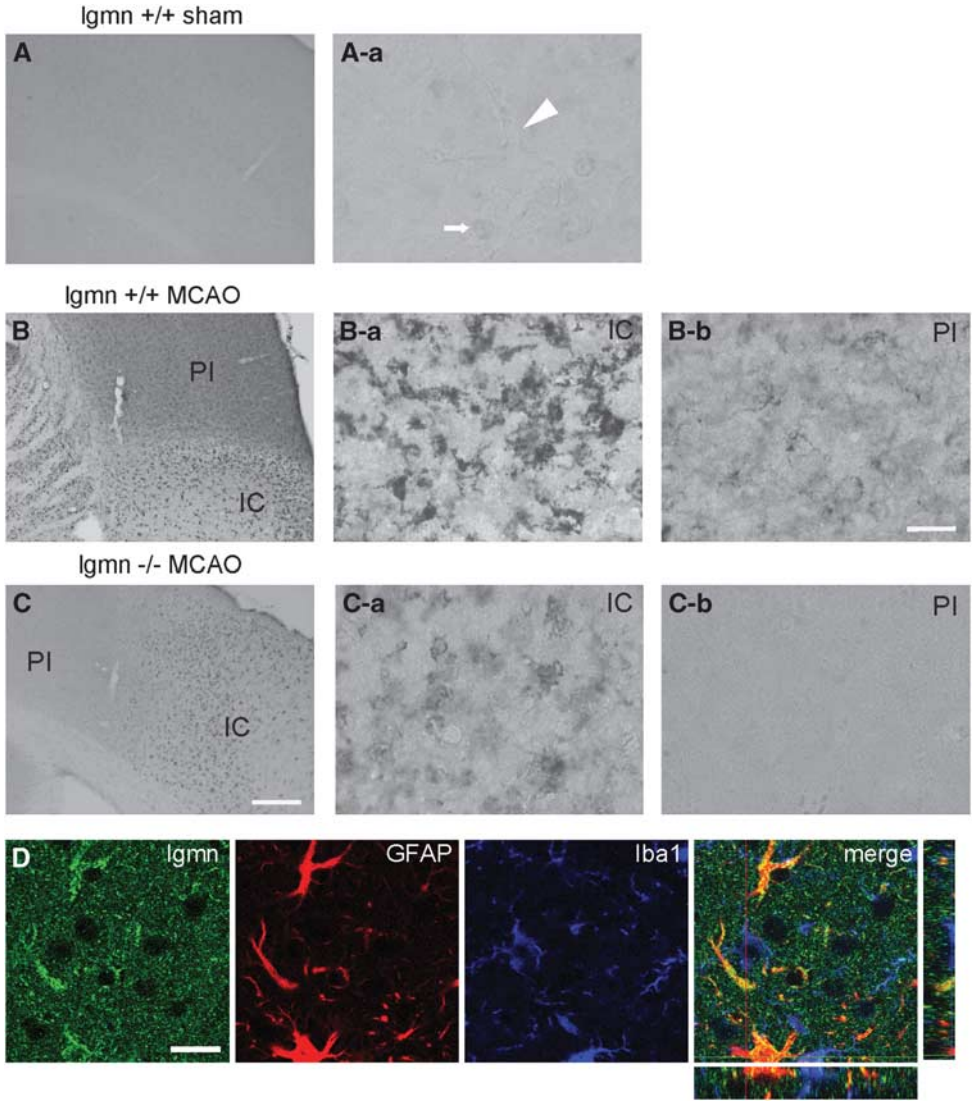

Figure 3 Legumain (lgmn) expression in mouse after transient middle cerebral artery occlusion (tMCAO). Phase-contrast micrograph (**A**) and higher magnification (**A-a**) showing weak lgmn immunoreactivity in microvessels (arrowhead) and associated cells (arrow) in the neocortex of a sham-operated mouse. (**B**) lgmn immunoreactivity in the hemisphere ipsilateral to the ischemic lesion 72 h after tMCAO with (**B-a**) an increased unspecific immunoreactivity in microglia-like cells inside the ischemic core (IC) and in (**B-b**) astrocyte-like and microglial-like cells of the peri-infarct (PI) area. (**C**) lgmn immunoreactivity in the ischemic hemisphere of an lgmn-deficient mouse with (**C-a**) an unspecific immunoreactivity of microglial-like cells of the IC and (**C-b**) no immunoreactivity in the PI area. (**D**) lgmn (green, AF488)/glial fibrillary acidic protein (GFAP) (red, Cy3)/Iba1 (blue, Cy5) costaining in the PI area 72 h after MCAO for 45 mins. Scale bars: (**A–C**) 100 μm, magnifications: 12.5 μm, (**D**) 20 μm.

recovery in legumain-deficient and wild-type mice (Figure 7A). No test score differences were also found in the pole test (Figure 7B). We also used a standardized grip test to measure the maximum strength of the paralyzed (left) and nonparalyzed (right) front paw before and after tMCAO. No differences were observed between wild-type (left: 75.9 ± 16.3 g, right: 84.9 ± 21.8 g) and knockout mice (left: 86.6 ± 3.8 g, right: 85.4 ± 4.5 g) before tMCAO. At 8 days of recovery, grip strength was generally decreased (wild-type: left 61.9 ± 15.1 g, right 65.7 ± 17.3 g; knockout: left 52.2 ± 20.9 g, right 59.2 ± 18.8 g), but also this test did not reveal a difference between wild-type and knockout mice. The results of our study support the notion that legumain deficiency has no significant impact on functional outcome after tMCAO.

However, legumain may contribute to an invasion of inflammatory cells toward the ischemic core. We determined the number of antigen-presenting $CD74^+$ cells in the ischemic hemisphere of legumain wild-type and deficient mice 8 days after MCAO. As shown in Figure 8, $CD74^+$ cells accumulate in the ischemic core but also in the peri-infarct area of legumain wild-type mice (448 ± 81 cells, $n = 6$). Reduced numbers of those cells were found in the

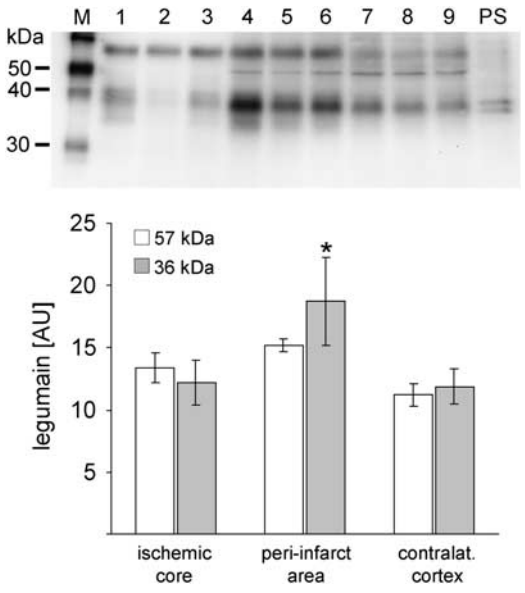

Figure 4 Legumain processing in the peri-infarct area after transient middle cerebral artery occlusion (MCAO). Western blot of samples from the infarct core (lane 1 to 3), the peri-infarct area (lane 4 to 6), and the contralateral neocortex (lane 7 to 9) from ischemic legumain wild-type mice 72 h after transient MCAO (45 mins) and densitometric analysis (mean ± s.d., $*P < 0.05$). Each lane represents an individual animal. Abbreviations: M, marker lane, PS, legumain positive control (kidney extract).

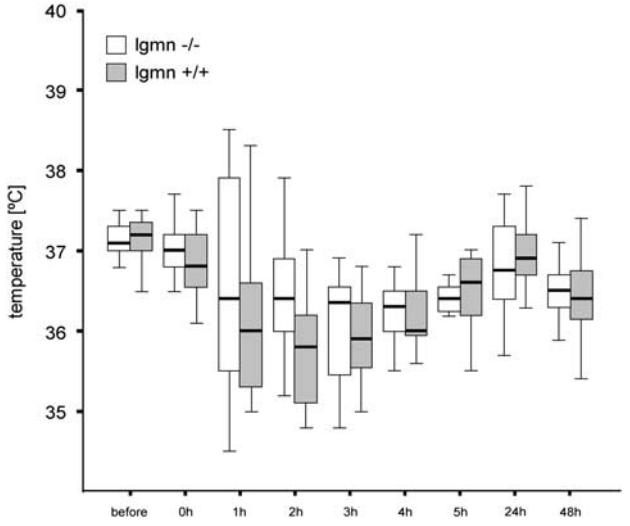

Figure 5 Poststroke temperature in legumain (lgmn)-deficient mice. Rectal body temperature was measured immediately before and at the indicated time points after transient middle cerebral artery occlusion (45 mins) by fine lubricated probe (−/− $n = 11$; +/+ $n = 17$). Note that no difference in body temperature was observed between legumain-deficient and wild-type littermates at all time points studied.

ischemic hemisphere of legumain-deficient mice (301 ± 100 cells, $n = 7$). Interestingly, a decreased number of $CD74^+$ cells in legumain-deficient mice

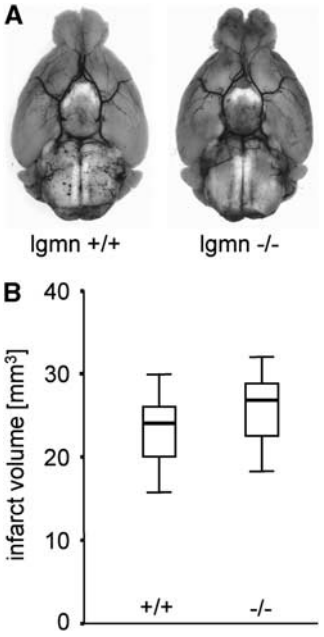

Figure 6 Brain arteries and infarct volumes in legumain-deficient mice. (**A**) Circle of Willis and major arteries of an aged (58 weeks) legumain-deficient mouse and a wild-type mouse. (**B**) Infarct volume from legumain-deficient mice (−/−; $n = 7$) and respective wild-type littermates (+/+; $n = 6$).

was mainly detected in the ischemic core. This suggests that legumain may be involved in mechanisms of post-stroke tissue inflammation and reorganization in the ischemic core and the peri-infarct area after MCAO.

Discussion

The aim of this study was to investigate the expression of the late endosomal asparaginyl endopeptidase legumain in the ischemic rodent brain and to evaluate the functional outcome of legumain-deficient mice after experimental stroke. We present three important findings: (1) legumain expression is increased in the peri-infarct area of rats after transient MCAO and is upregulated in microglial cells and reactive astrocytes; (2) legumain deficiency does not affect infarct volume or neurologic outcome in aged mice after MCAO; and (3) the number of $CD74^+$ cells in the ischemic hemisphere is significantly reduced in legumain-deficient mice, indicating an altered inflammatory response in the ischemic hemisphere.

Legumain Expression in the Brain after Middle Cerebral Artery Occlusion

Legumain is differentially expressed in various mammalian tissues (Chen *et al*, 1998; Shirahama-Noda *et al*, 2003). In line with results from a previous

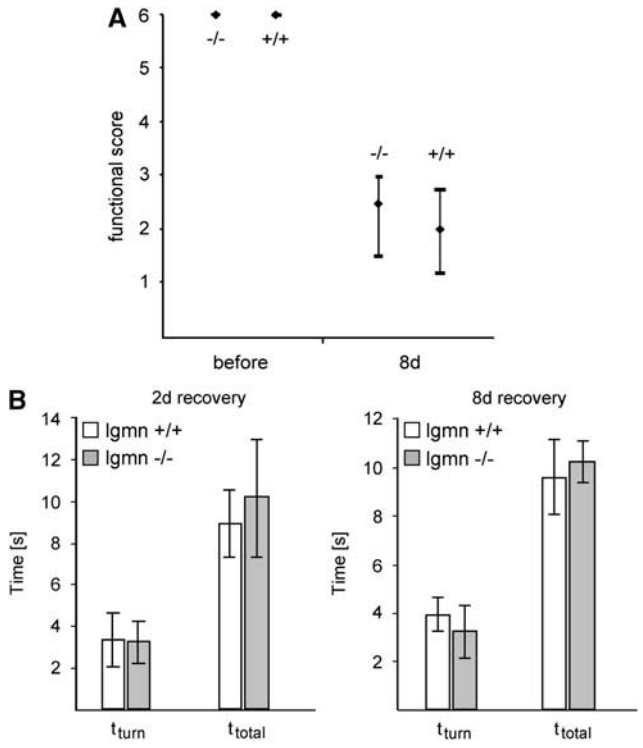

Figure 7 Functional recovery in legumain (lgmn)-deficient mice after transient middle cerebral artery occlusion. Evaluation of sensorimotor function in lgmn-deficient (−/−, $n = 8$) and wild-type littermate (+/+, $n = 8$) mice 8 days after brain ischemia. (**A**) Animals were tested on the rotating pole at 10 turns per minute to the left. Similar results were obtained with 10 turns to the right (data not shown). (**B**) The pole test shows the time to turn to the head-down position (t_{turn}) and the total time to turn and reach the floor with all four paws (t_{total}) at 2 and 8 days of recovery. Behavioral assessment did not show significant differences between deficient and wild-type mice.

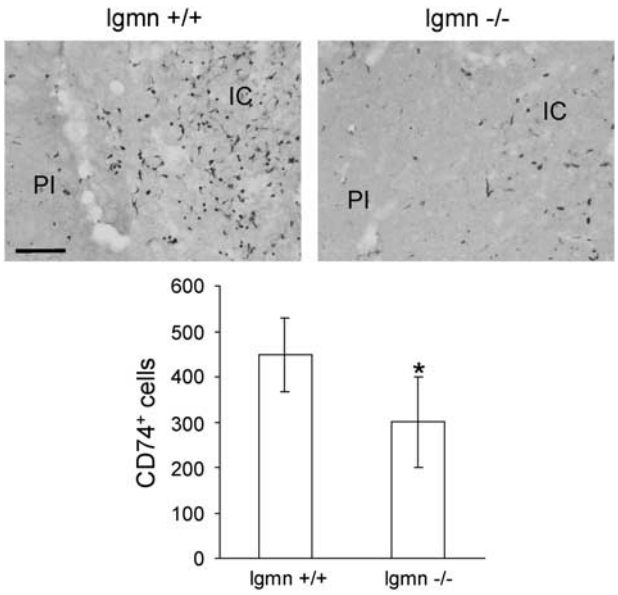

Figure 8 Analysis of CD74 positive cells in the ischemic hemisphere of legumain (lgmn) wild-type and deficient mice after transient middle cerebral artery occlusion (tMCAO). Quantification of $CD74^+$ cells from the ischemic hemisphere of lgmn wild-type ($n = 6$) and deficient ($n = 7$) mice 8 days after tMCAO for 45 mins (mean ± s.d., $P < 0.05$, Student's t-test). Abbreviations: IC, infarct core, PI, peri-infarct area. Scale bar: 100 μm.

gene array study, we show here an upregulation of legumain expression and elevated legumain protein levels in the rat brain. Legumain mRNA increased in the peri-infarct region from 3 h and onwards and followed a delayed time pattern similar to that for other proteins, including proteases such as cathepsin B (Rickhag *et al*, 2006). Although legumain was weakly expressed in microvessels and adjacent cells in sham-operated mice, microglial cells and astrocytes strongly expressed legumain in the peri-infarct area after MCAO.

Macrophage-specific legumain was proposed to be released in atherosclerotic plaques and promote inflammatory cell invasion, contributing to the progression of atherosclerosis (Clerin *et al*, 2008). Our *in situ* hybridization and immunohistochemistry study findings that legumain is upregulated after MCAO were confirmed by western blot, and in addition we show that legumain is processed into the active 36 kDa form (Li *et al*, 2003) particularly in the peri-infarct area. This result and the delayed dot-like accumulation of immunoreactivity in branches of astroglial cells but also in microglia of the peri-infarct area suggest that brain-derived legumain may be secreted (Clerin *et al*, 2008) and may function as a chemoattractant for invading inflammatory cells. However, we cannot exclude that legumain is secreted by invading peripheral monocytes but also by resident microglia and may be taken up by astroglial cells.

Glial cells produce both pro- and anti-inflammatory cytokines that may modulate the entry and migration of inflammatory cells in the CNS parenchyma (John *et al*, 2003). In addition, it was shown that legumain increases the *in vitro* migratory and invasive activity of tumor cells (Liu *et al*, 2003). Little is known about secreted glial molecular guidance cues for cell migration toward the peri-infarct tissue and the ischemic core region (Sofroniew, 2005). However, our observation of a reduced number of legumain antigen-presenting $CD74^+$ cells in the ischemic core and adjacent peri-infarct area of legumain-deficient mice supports the idea that legumain is involved in cell migration toward the lesion side after MCAO. Further studies will clarify the effect of different legumain levels on cell migration and expression of proinflammatory cytokines in the ischemic hemisphere.

Potential Function of Legumain in Tissue Re-organization

This study was also conducted to evaluate a putative function of legumain after MCAO. However,

legumain deficiency did not affect the infarct volume compared with the respective littermates. In addition, behavioral tests showed no difference between the two genotypes. Low legumain levels in the early phase after MCAO when damage is developing and delayed upregulation may explain why legumain is not directly involved in the mechanisms of neurotoxicity or neuroprotection. More likely, the spatial and temporal distribution of legumain-positive microglial and astroglial cells in the peri-infarct area suggests an involvement in the mechanisms of wound healing and tissue reorganization. Recently, a number of studies have brought to attention the importance of the extracellular matrix turnover in these mechanisms (Rosell and Lo, 2008). In particular, late inhibition of matrix metalloproteinase-9, colocalized with markers of neurovascular remodeling, increases ischemic brain injury and impairs functional recovery 2 weeks after experimental stroke onset (Zhao *et al*, 2006). Legumain has been shown to regulate extracellular matrix turnover through the degradation of fibronectin (Morita *et al*, 2007). Extracellular fibronectin may be taken up by caveolin-mediated endocytosis and degraded intracellularly in legumain-enriched lysosomes (Sottile and Chandler, 2005). Hence, legumain-positive astrocytes of the peri-infarct area that are enriched in submembraneous globular structures indicate such a mechanism for extracellular matrix proteins.

The type II transmembrane glycoprotein CD74 (invariant chain, Ii) is associated with the MHC class II α- and β-chains and functions as a chaperone in transport of $\alpha\beta$Ii (invariant chain) complexes from the endoplasmic reticulum to endocytic compartments (Wolf and Ploegh, 1995). CD74 further prevents peptide binding in the endoplasmic reticulum, and contributes to peptide editing in the MHC class II compartment (Stumptner-Cuvelette and Benaroch, 2002). In addition to its function as a chaperone molecule, CD74 has a role in cell signaling. Recently, CD74 was reported to be a high-affinity binding protein for the proinflammatory cytokine, macrophage migration-inhibitory factor (Leng *et al*, 2003). Except for an accumulation in neurofibrillary tangles (Bryan *et al*, 2008), CD74-positive cells have not been reported in the normal brain and under pathologic conditions.

A reduced number of $CD74^+$ cells indicate an altered inflammatory response in the ischemic core and in the adjacent peri-infarct area of legumain-deficient mice. However, we also observed $CD74^+$ cells in the ischemic hemisphere of deficient mice, indicating that legumain (together with other molecules) indirectly contributes to mechanisms of $CD74^+$ cell invasion after MCAO. It has to be determined whether low levels of legumain are associated with MHC class II processing as previously shown in endotoxin-tolerant monocytes (Wolk *et al*, 2005). However, it has to be determined whether brain-derived glia can express and process MHC class II proteins on an ischemic insult. Our study results indicate that brain-derived legumain contributes to $CD74^+$ cell infiltration in the ischemic hemisphere after experimental stroke indirectly by modification of molecules involved in mechanisms of immune cell attraction.

We used aged (approximately 1-year-old) mice in this study. All animals showed severe impairment and, in contrast to young animals, recovery was markedly delayed in all mice independent from genotype. We also observed a slight increase in mortality compared with previous studies using young mice (Nygren and Wieloch, 2005), which can be most likely attributed to age as similar observations have been made in aged rats subjected to transient MCAO (Badan *et al*, 2003). Neuronal degeneration, glial scar formation, and post-stroke inflammation contributing to stroke recovery are significantly altered in the aged rodent brain (for a review, see Petcu *et al*, 2008). Delayed activation may explain that no differences were detected in behavioral tests in legumain-deficient and wild-type mice 8 days after MCAO.

Previous studies have shown an increased body temperature in legumain-deficient mice (Chan *et al*, 2009). High body temperature is associated with poorer outcome after experimental and clinical stroke (Dietrich *et al*, 1990; Zaremba, 2004). In this study, we did not detect any temperature differences between legumain wild-type and deficient mice. This discrepancy could be due to the small number of animals included in the study reported by Chan *et al*. Further, temperature differences obtained in younger animals may be ameliorated in aged mice similar to body weights as shown previously (Manoury *et al*, 1998; Shirahama-Noda *et al*, 2003). It has also been shown that legumain-deficient mice develop symptoms resembling a hemophagocytic syndrome (Chan *et al*, 2009) accompanied by increased serum levels for the proteohormone erythropoietin. Apart from a possible relevance for an increased reactive hematopoiesis (Chan *et al*, 2009), it remains speculative whether elevated erythropoietin levels exert any function in the brain of legumain-deficient mice after MCAO in particular in view of its neuroprotective actions in other models of experimental stroke (Ruscher *et al*, 2002).

Conclusion

In summary, legumain expression level and activity are increased in the peri-infarct area of rats after transient MCAO and are upregulated in microglial cells and reactive astrocytes. Legumain does not appear to be involved in the acute stage of neurodegeneration but may influence the immune response during stroke.

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Conflict of interest

The authors declare no conflict of interests.

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