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MMP2 and MMP9 in immature endothelial cells following surgical injury of rat cerebral cortex – a preliminary study

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Abstract

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of extracellular matrix remodeling. They have been implicated in various physiological and pathological phenomena involving neurovascular unit elements (neurons, astrocytes and capillary vessels). Surgical injury of the fronto-temporal region of rat cerebral cortex induces massive neurodegeneration and cell death accompanied by astrogliosis. In the area adjacent to the damage, the induction of angiogenesis is observed, in which process immature endothelial cells are important players. The aim of this study was to examine the expression of MMP2 and MMP9 in cortical blood vessels following the surgical injury of cerebral cortex in the rat. Our data demonstrated absence of immature endothelial cells and lack of or only weak MMP2 and MMP9 immunoreactivities in some cortical capillaries in intact rats. Following the surgery, we found high MMPs' immunoreactivities in walls of the vessels located in the cortex adjacent to the lesion site, and particularly in the immature endothelial cells. These results show for the first time the presence of MMPs in the immature (progenitor?) endothelial cells following surgical brain injury.

Key words: *immature endothelial cell, angiogenesis, MMP2, MMP9, Qdots.*

Introduction

Extensive data collected over many years support the existence of endothelial progenitor cells (EPC) [2-4,21,26]. Previously we investigated neovascularization in the cerebral cortex of adult rats after surgical brain injury. Using electron microscopy ultrastructural and immunocytochemical methods we have shown that the growth and development of new blood vessels are not restricted to angiogenesis, but include vasculogenesis as well [11].

Most studies on EPC differentiation concentrated on the expression of endothelial markers. Both EPC and mature endothelial cells can express similar sets of endothelium-specific markers, including vascular endothelial growth factor receptor-2 (VEGFR-2, or Flk-1), Tie-2, von Willebrand factor (vWF) and AC133 [21]. Asahara and Kawamoto [4] suggested that the

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level of AC133 expression depends on differentiation stage of endothelial progenitors, and Gill and colleagues postulated fast mobilization of Flk-1/AC133-positive EPC after vessel trauma [13].

In our previous studies, morphologically immature endothelial cells were found to resemble, by their characteristic ultrastructural features, mature endothelial cells, except that they carried fibrils in the cytoplasm [11,12]. The fibrils were being lost during the migration of immature endothelial cells from the vessel lumen to the perivascular space [9]. This phenomenon was accompanied by formation of a diversity of Flk-1- and/or AC133-expressing immature endothelial cells following brain injury [10].

Production of extracellular matrix-(ECM)-degrading proteases, and particularly matrix metalloproteinases (MMPs), by endothelial cells is a critical event during angiogenesis that occurs both in normal and pathological situations. MMPs are highly regulated at the level of synthesis and activation [29]; however, little is known about MMPs expression in endothelial cells and EPC. The importance of these enzymes in angiogenesis is supported by data showing that both endogenous and synthetic inhibitors of MMPs block this process. Moreover, angiogenesis is impaired in mice deficient for MMPs [5]. However, the functions of MMPs in angiogenesis are not completely understood. It is known that these enzymes enable migration of endothelial cells through the basement membrane and into the interstitial stroma, and participate in the final organization of the cells in tubular structures, which is an essential process in vascular remodeling and regeneration in a number of physiological and pathological states [19]. Besides their roles in the breakdown of connective tissue barriers, MMPs and their inhibitors are essential for endothelial cell attachment, proliferation and survival [29].

It has been established that MMPs play an important role in brain injury after ischemic stroke as well. In particular, it has been found that both MMP2 and MMP9 were upregulated in the infarcted tissue as compared to unaffected brain areas [7] and appeared necessary for post-ischemic EPC mobilization [15]. The goal of the present study was to test the hypothesis that immature endothelial cells (EPC?) that participate in new vessel formation after surgical injury of cerebral cortex can express these MMPs.

Material and Methods

Adult male Wistar rats of 200-250 g body weight were used for the study. The rats were randomly divided between the following groups: 1) rats subjected to surgical brain injury (N = 10), and 2) intact controls (N = 6). All animal use procedures were approved by the 4th Local Animal Experimentation Ethics Committee and were in compliance with European Union regulations on the care and use of laboratory animals. Animals were housed in an humidity- and temperature-controlled environment (20°C and 60-70% relative humidity, respectively) at 12 h/12 h light/dark cycle (lights on at 7 a.m.), and were allowed standard food for laboratory rodents (Ssniff M-Z, ssniff Spezialdiäten GmbH, Soest, Germany) and purified tap water *ad libitum*.

Surgical brain injury

The fronto-temporal region of the cerebral cortex was lesioned surgically as described earlier [11]. Briefly, the animals were anesthetized with Nembutal (20 mg/kg b.w., i.p.). After opening the skull and cutting the meninges, a 2 × 2 mm region of the cortex was removed with a scalpel, and the skin wound was sutured. After the surgery the rats were returned to their home cages in the animal facility.

Immunohistochemistry

Tissue collection and preparation

Two or four days post-lesion the operated rats and their intact counterparts were deeply anesthetized with Nembutal (80 mg/kg b.w., i.p.) and perfused through the ascending aorta, first with 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer pH 7.4 (PBS, 50ml), and then with 'regular' ice-cold fixative (4% formaldehyde in 0.1 M phosphate buffer pH 7.4, 200 ml). After the perfusion, the brains were removed from the skulls and soaked in the same fixative for 2 h. Next, the brains were sucrose-permeated by soaking in 10, 20 and 30% (w/v) sucrose solutions in PBS (for 24 h, 2-4 days, and 3-5 days, respectively). Subsequently, sister series (of 6-12 sections each) of 40 µm-thick free-floating coronal sections were cut from an 7-8 mm thick brain slice (from bregma +1.5 to bregma –5.8) encompassing the lesion area, its adjacent cortical region and fronto-temporal cortex

region distant from the lesion site, and from the corresponding brain regions in intact rats, using a model CM 1850 UV cryostat (Leica, Germany).

Immunohistochemical staining

To reduce background staining, the free-floating brain sections were placed in a 96-well microdish and preincubated for 20 min with 3% normal goat serum solution in PBS supplemented with 0.2% Triton X-100 (PBS+T). Subsequently, the sections were incubated for 1 h at 37°C with PBS+T containing 1% normal goat serum and two of the following primary antibodies (Abs): 1) murine monoclonal Ab against Flk-1 (Santa Cruz Biotechnology, CA, USA, cat. no. sc-6251, dil. 1 : 400); 2) rabbit polyclonal Ab against AC133 (Abcam, Cambridge, UK, cat. no. ab16518, dil. 1 : 200); 3) murine monoclonal Ab against MMP2 (Abcam, Cambridge, UK, cat. no. ab7032, dil. 1 : 400); and 4) rabbit polyclonal Ab against MMP9 (Abcam, Cambridge, UK, cat. no. 7299, dil. 1:400). The choice of the primary Ab pair was dictated by the choice of antigen pair of interest. The reaction was terminated by washing the sections with PBS+T (3 × 5 min). Next, the sections were incubated for 1 h at 37°C with the respective secondary Abs. The secondary Abs used were as follows: 1) goat anti-mouse Ab conjugated with Quantum dots (Qdots) 655 (Invitrogen-Molecular Probes, CA, USA, cat. no. Q11021MP, dil. 1 : 2000); and 2) goat anti-rabbit Ab conjugated with Qdots 565 (Invitrogen-Molecular Probes, CA, USA, cat. no. Q11431MP, dil. 1 : 2000). Finally, the sections were rinsed with PBS+T (3 × 5 min), mounted on silanized glass slides (Sigma) and covered with Vectashield mounting medium for fluorescence microscopy (Vector Labs Inc., Burlingame, CA, USA). Immunostaining was detected with a model Optiphot-2 Nikon fluorescent microscope (Japan) equipped with the appropriate filters, and recorded with a model DS-L1 Nikon camera (Japan).

Specifity of the immunostaining was verified by performing control ("blank") staining procedure with primary antibodies' omitted in the incubation mixture. No staining was observed in these control sections.

Electron microscopy immunocytochemistry

Tissue samples for electron microscopy studies were taken from rats anesthetized and perfused as above, except that only 20 ml PBS was used for the initial perfusion step (to shorten the pre-fixation time and prevent detrimental ultrastructural changes). The tissue was sampled from the cerebral cortex adjacent to the surgical injury in the lesioned rats and from the corresponding fronto-temporal cortex region in the control rats. The samples were treated with the regular ice-cold fixative (see above) for 20 h, postfixed in 1% (w/v) OsO_4 solution in deionized water for 30 min, dehydrated in an ethanol gradient, and finally embedded in Epon 812. Ultrathin (60 nm) sections were taken with a diamond knife and processed according to a standard postembedding immunocytochemistry procedure. Briefly, the sections were mounted on formvar-coated nickel grids, incubated for 10 min in 10% hydrogen peroxide, washed with *aqua pro injectione* and PBS (15 min each) and treated for 10 min with 1% (w/v) bovine serum albumin solution in PBS.

The primary Abs used for electron microscopy immunocytochemistry were the same as those used for fluorescence microscopy, except that their dilution was 1:20. The secondary antibodies used were as follows: 1) donkey anti mouse Ab conjugated with 12 nm gold particles (Jackson ImmunoResearch, PA, USA, cat. no. 70851, dil. 1 : 50); 2) donkey anti rabbit Ab conjugated with 18 nm gold particles (Jackson ImmunoResearch, PA, USA, cat. no. 58030, dil. 1 : 50); 3) goat anti-mouse Ab conjugated with Qdots 655 (Invitrogen-Molecular Probes, CA, USA, cat. no. Q11021MP, dil. 1 : 50); and 4) goat anti-rabbit Ab conjugated with Qdots 565 (Invitrogen-Molecular Probes, CA, USA, cat. no. Q11431MP, dil. 1 : 50). Specifity of the immunogold and Qdots' labeling was verified by performing "blank" staining procedure with primary antibodies omitted. This procedure resulted in no detectable staining.

All immunocytochemical preparations were analyzed in a model JEM-1200EX Jeol transmission electron microscope (Japan).

Results

Electron microscopy showed that the border zone of the injured cortical region comprised mostly viable parenchyma (except for a thin necrotic zone in the immediate vicinity of the lesion) showing extensive formation of new blood vessels that were partly invading the necrotic zone (not shown). The vessels were composed of cells showing immature endothelial cells features, i.e. the ultrastructural characteristics of mature endothelial cells, but with fibrils present in the cytoplasm (not shown).

In cortical samples from intact rats, immunohistochemistry revealed no or weak immunoreactivity for EPC markers (AC133 and Flk-1) and no immunostaining for either one MMP tested (not shown). However, few vessels in these samples showed weak signals for the MMP2/AC133 (Fig. 1) or MMP9/Flk-1 marker pair (Fig. 2).

In the cortical samples from lesioned rats, we found intense signals for the aforementioned marker pairs within the vessel walls adjoining the lesion, both on the 2nd (not shown) and 4th post-lesion day (Figs. 1 and 2). Isolated, i.e. showing no contact with the vessels wall, double-positive (EPC marker- and MMP-positive) cells were also present in the cortex zone adjacent to the lesion (as exemplified in Fig. 3). In an additional series of sections from the lesioned rats, MMP2 and MMP9 were found to colocalize to the same vessels (Fig. 4).

The immunohistochemistry data were confirmed and extended by electron microscopy studies. Neither newly formed blood vessels, nor cells expressing the EPC markers studied (Flk-1 and AC133) were found in control animals. Mature endothelial cells showed no specific staining or only single immunogold particles (presumably associated with MMP2 or MMP9) present in the cytoplasm (not shown).

Newly formed (as evidenced by hypertrophied endothelium) capillary blood vessels were built of cells

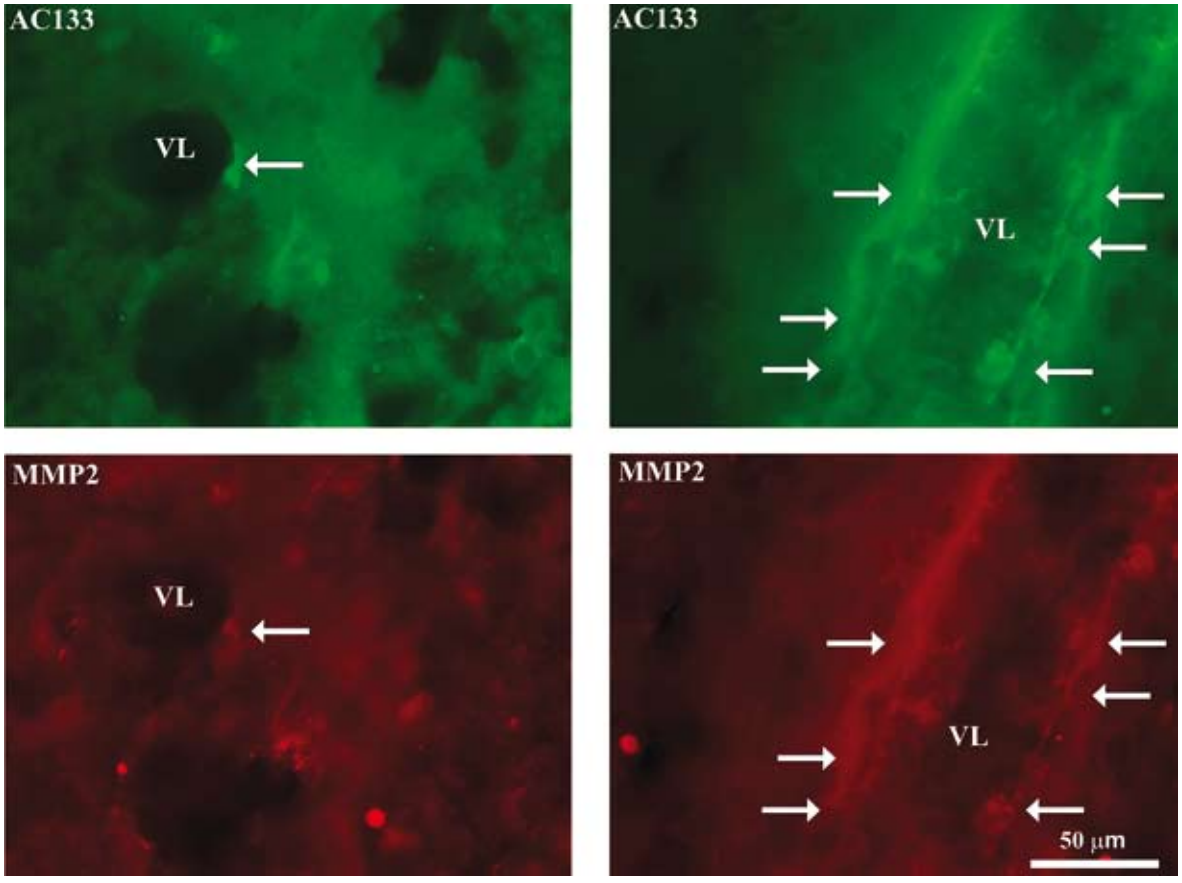

Fig. 1. Representative microphotographs showing immunofluorescent labeling for AC133 (EPC marker) and MMP2 of cerebral cortex (fronto-temporal region) from control (intact) and surgically brain-lesioned rats (post-surgery day 4). A weak staining for AC133 and a very weak staining for MMP2 (arrows) is apparent in some vessel of control rats, whereas the injured cortical tissue shows markedly higher immunostaining for both these markers. VL – vessel lumen.

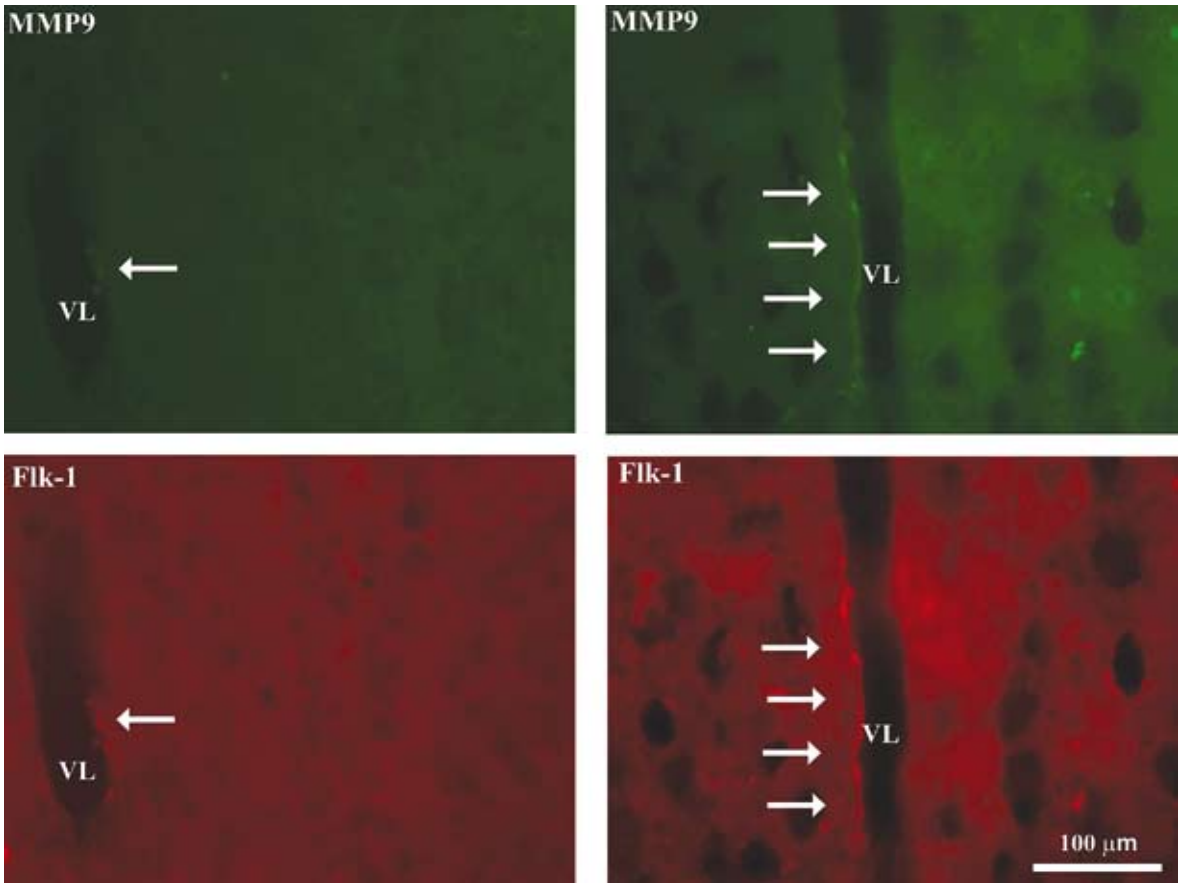

Fig. 2. Representative microphotographs showing immunofluorescent labeling for Flk-1 (EPC marker) and MMP9 of cerebral cortex (fronto-temporal region) from control and surgically brain-lesioned rats (post-surgery day 4). In control animals, some vessel walls showed the presence, at low levels, of both Flk-1 and MMP9 (arrows). Staining intensity was lower for MMP9 than for Flk-1. Vessel walls from the lesioned rats show markedly higher labeling for both these markers (arrows). VL – vessel lumen.

with immature endothelial cell appearance (intracytoplasmic fibrils). These cells (see Fig. 5) showed the expression of Flk-1 (as evidenced by the presence of 12 nm immunogold particles, single or in pairs) and MMP9 (18 nm immunogold particles). Aggregates of 18 nm immunogold particles showing the presence of MMP9 were also apparent in the basement membrane of the novel capillaries (Fig. 5). Double (AC133/MMP2) immunogold staining revealed cytoplasmic colocalization of AC133 (18 nm particles, single or in pairs) and MMP2 (12 nm particles) in the EPC-looking cells as well (Fig. 6). The MMP2-targeted immunogold particles were also apparent in the basement membrane of the new capillaries. The presence of MMP2 and MMP9 within the EPC-looking cells was confirmed by Qdots technique that showed high levels of both MMP2 (Fig. 7) and MMP9 (Fig. 8) in the cytoplasm. This technique revealed also the presence of MMP2 in the basement membrane and in the pericyte (Fig. 7), and of MMP9 in the inter-endothelial cell junctions (Fig. 8).

Discussion

Surgical lesion of the brain cortex causes massive neuronal cell death accompanied by astrogliosis [27] and angiogenesis within the injury site [11]. The first evidence for the involvement of MMPs in angiogenesis came from the finding that tissue inhibitors of metalloproteinases and synthetic MMP inhibitors can hamper significantly endothelial cell tube forma-

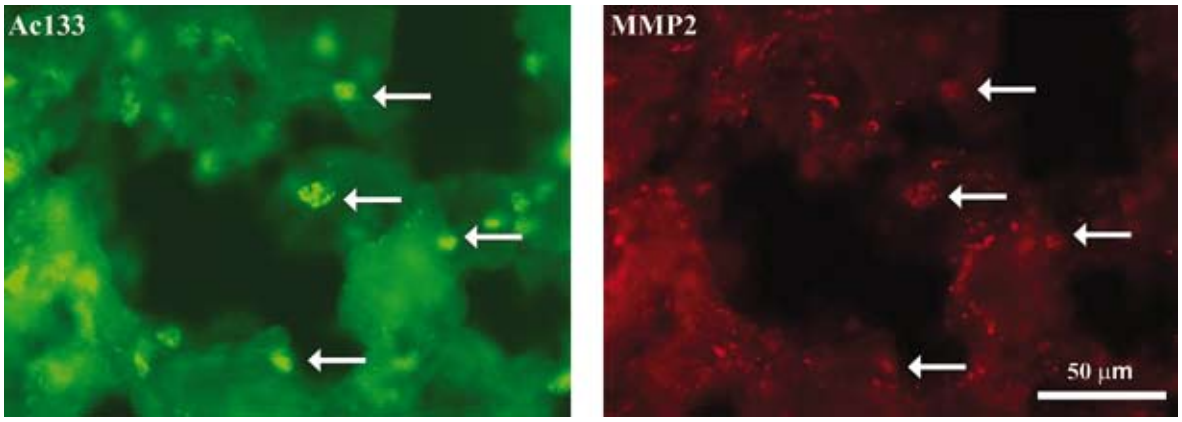

Fig. 3. Representative microphotographs showing immunofluorescent labeling for AC133 and MMP2 of cerebral cortex (fronto-temporal region) from a surgically brain-lesioned rat (post-surgery day 4). Strong immunostaining for both investigated markers is visible in many cells located within the peri-lesion cortical area (arrows).

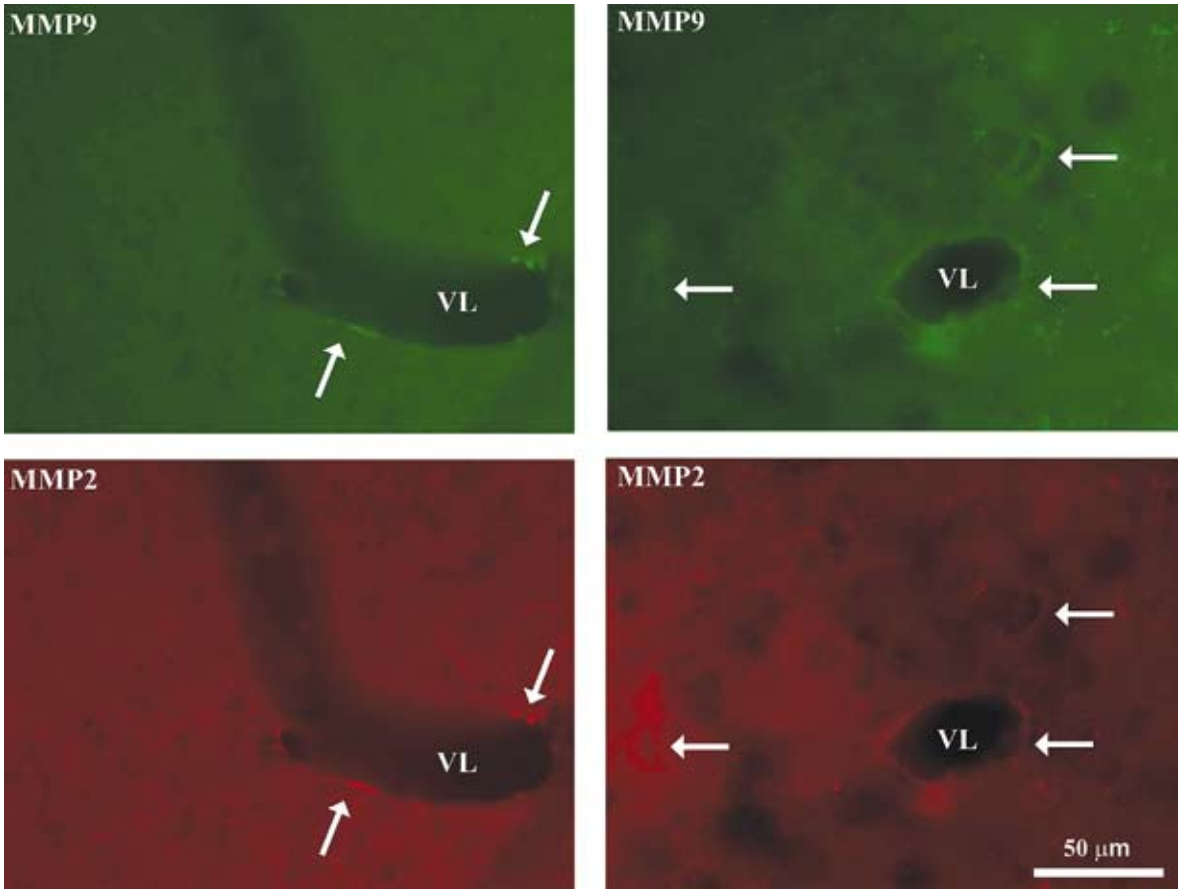

Fig. 4. Representative microphotographs showing immunofluorescent labeling for MMP9 and MMP2 of cerebral cortex (fronto-temporal region) from control and surgically brain-lesioned rats (post-surgery day 4). Please note the presence of both MMPs in the vessel walls (arrows). VL – vessel lumen.

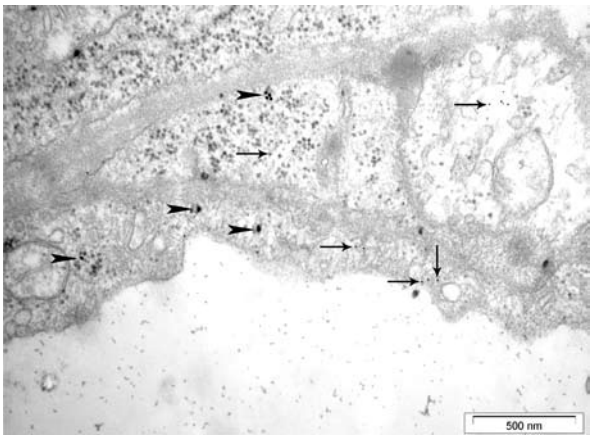

Fig. 5. Representative electron microphotograph showing Flk-1 and MMP9 immunostaining in the blood vessel wall cells with EPC characteristics (post-surgery day 2). Flk-1 immunoreactivity (arrows) is visualized by immunogold particles (12 nm diameter, single and in pairs), and MMP9 (arrowheads) is represented by 18 nm immunogold particles in the cytoplasm. Some proteinaceous plasma remnants are visible in the vessel lumen.

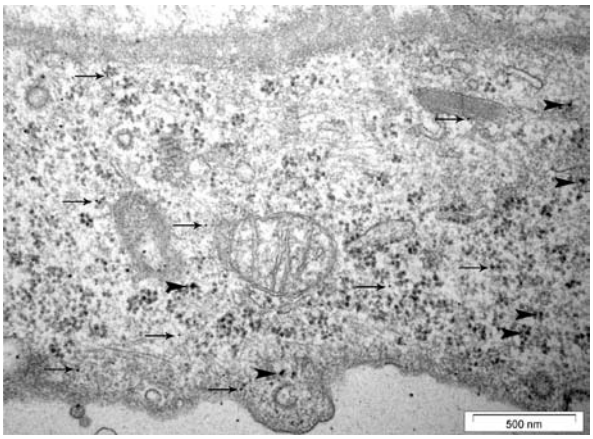

Fig. 6. Representative electron microphotograph showing AC133 and MMP2 immunostaining in the blood vessel wall cells with EPC characteristics (post-surgery day 2). High intensity of cytoplasmic AC133 (arrowheads) and MMP2 (arrows) immunostaining is apparent in the fibril-rich cell.

tion *in vitro* [25]. Identification and cloning of MMP genes enabled determining the specific MMPs involved in this process. Cultured endothelial cells have been reported to produce MMP1, MMP2, MMP3, MMP9, MMP13, and MMP14 [6,17,25].

Recently, MMPs have been implicated in a wide range of CNS pathologies (that usually involve vascular remodeling and regeneration), including Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, and stroke [24]. By degrading the ECM, MMPs damage the blood-brain barrier, resulting in edema and vascular leakage. By interrupting cell-cell and particularly cell-matrix interactions, MMPs can also trigger the peculiar type of cell death named anoikis [23,29,30]. Besides ECM proteins, MMPs can process a variety of growth factors, cytokines and other proteinaceous signaling molecules [8,20]. In this way, MMPs may contribute to the neurovascular remodeling that accompanies brain and spinal cord trauma [31].

Both MMP2 and MMP9 are constitutively expressed in the brain and spinal cord. MMP9, which is normally expressed at a much lower level than MMP2, is upregulated to a relatively bigger extent following various injuries and diseases of CNS [30]. Our finding of the presence of MMP2 and MMP9 in the vessel walls within the area adjacent to the surgical lesion of the cerebral cortex is in full agreement with those earlier studies. In addition, our study revealed that these MMPs colocalize with each other and with the EPC markers Flk-1 and AC133.

Our electron microscopy immunocytochemistry study confirmed and extended our immunofluorescence observations, and unequivocally showed the expression of both MMP2 and MMP9 in immature endothelial cells. The fact that basement membrane-associated MMPs were only seen in a close proximity to MMP-containing immature endothelial cells suggests these cells as the source of the extracellular MMPs. This observation suggests that the two MMPs may be involved not only in the process of new vessel formation, but also in rebuilding/remodeling of brain parenchyma after (mechanical) trauma.

This study revealed the presence of MMP2 in the pericyte as well. It is well known that pericytes present in capillary blood vessel walls are essential for the development of functional vascular network. Pericytes likely promote survival of endothelial cells through secretion of diffusible angiogenic factors,

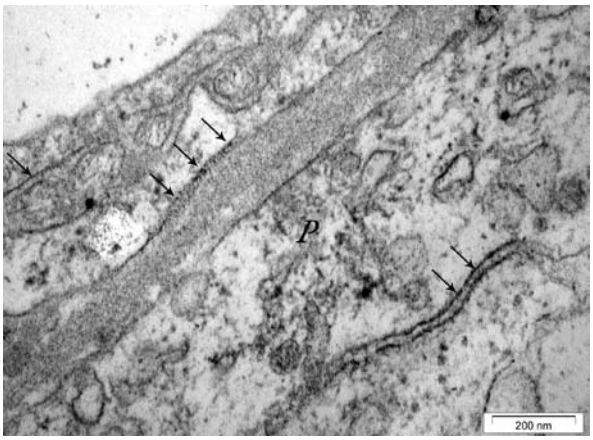

Fig. 7. Representative electron microphotograph showing MMP2 visualization with Qdots technique within a capillary (post-surgery day 4). High immunostaining intensity is apparent in the cytoplasm of the immature endothelial cell, in the basement membrane (arrows), and in the pericyte (P).

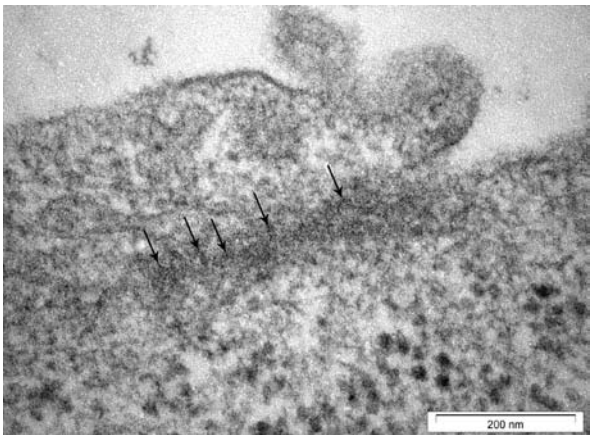

Fig. 8. Representative electron microphotograph showing MMP9 visualized by Qdots technique in a newly formed capillary (post-surgery day 4). Inter-endothelial cell junctions (arrows) show high immunostaining intensity.

e.g. vascular endothelial growth factor (VEGF) and Ang1 [22], and can also stabilize capillaries [1,14]. Hence, one may speculate that the pericyte-derived MMP2 may be involved in this process.

Some recent data indicate to yet another role of MMPs in the process of brain parenchyma rebuilding/remodeling. Namely, MMP2 and MMP9 were found to be involved in adult neurogenesis [16,18], and Wang and colleagues [28] proved that erytropoietin-activated endothelial cells secrete MMP2 and MMP9 that promote the migration of neuronal progenitors into the affected brain region after focal ischemia.

To summarize, this study is the first one to show the presence of MMPs in the immature endothelial cells (EPC?) after brain cortex surgical injury. We speculate that increased MMP2 and MMP9 levels in these cells in the vicinity of the injured tissue are directly related to post-traumatic rebuilding of brain parenchyma.

Acknowledgments

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