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Involvement of MMPs in delayed neuronal death after global ischemia

Teresa Zalewska, Małgorzata Ziemka-Nałęcz, Anna Sarnowska and **Krystyna Domańska-Janik**

Laboratory of Molecular Neuropathology, Department of Neurochemistry, Medical Research Centre, Polish Academy of Sciences, 5 Pawiński St., 02-106 Warsaw, Poland, Email: terezal@cmdik.pan.pl

Abstract. Spatial and temporal relations between metalloproteinase (MMP-2 and MMP-9) activation and laminin degradation in gerbil hippocampus after transient cerebral ischemia has been studied. Activity of MMPs was determined by gelatin zymography in homogenates from dorsal (DP, an equivalent of CA1 sector) and abdominal (AbP, containing CA2-4 and *gyrus dentatus*) parts of hippocampus. A significant activation of both investigated metalloproteinases was found at 72 h of recovery. Whereas MMP-2 up-regulation did not show any spatial preferences, the increase of MMP-9 activity was observed exclusively in DP. Activation of MMP-9 at this time point correlated spatially with degradation of laminin - protein of extracellular matrix. These results show that MMP pathway may function as a component of delayed neuronal death cascade in the apoptogenic CA1 sector after transient global ischemia.

Key words: transient cerebral ischemia, matrix metalloproteinases, MMP-2, MMP-9, laminin

INTRODUCTION

Brief periods of transient global brain ischemia in rodents cause delayed cell death in hippocampal CA1 pyramidal neurons. Other hippocampal neurons in CA2-4 and dentate gyrus are much less vulnerable to a mild ischemic insult (Kirino 1982, Pulsinelli et al. 1982). In gerbils, cell death visualized by the terminal transferase dUTP nick-end labelling (TUNEL) reaction appeared selectively in the CA1 region of the pyramidal cell layer between the third and fourth days of reperfusion, suggesting an apoptotic type reaction (Domańska-Janik et al. 1999). Global, moderate ischemia has been shown to promote apoptosis-like events in vulnerable brain regions, including chromatin condensation, cell shrinkage, caspase and calpain substrates cleavage and neuroprotection offered by protein synthesis, endonuclease or caspase inhibitors (Goto et al. 1990, Loddick et al. 1996, Chan and Mattson 1999). On the other hand, an exact mechanism of ischemic neuronal death induction, the interplay between different signaling pathways as well as the mode of final neuronal death is still not clear. In effect this ischemia - induced cellular reaction recently is classified as an apoptosis-like PCD (programmed cell death) rather than caspase- and chromatin fragmentation-dependent apoptosis.

It was emphasized in several *in vitro* investigations conducted during the last ten years that extracellular signals, mediated by integrin receptors, are critical for survival of cells including neurons, and in their absence the cells are proned to die by apoptosis (Raff 1992, Frish and Ruoslahti 1997, Gary and Mattson 2001, Lesay et al. 2001). The disruption of cell-extracellular matrix (ECM) interaction and in consequence the suppression of a survival promoting pathway may be due to excessive proteolytic modification of ECM components. In fact, it was found that in several models of brain injury extracellular proteases are up-regulated and cleave the extracellular matrix (Rosenberg 1995, Fujimura et al. 1999, Heo et al. 1999).

Degradation of ECM was primarily linked to the activation of metalloproteinase -2 (MMP-2, gelatinase A) and metalloproteinase -9 (MMP-9, gelatinase B), belonging to the family of at least 16 zinc-dependent proteolytic enzymes which are capable of degrading almost all proteinaceous components of extracellular matrix such as collagen, fibronectin and laminin in a variety of physiological as well as in pathological conditions (Nagase and Woessner 1999). Considering their central involvement in general biology such as embryonic development, wound healing, and angiogenesis, it is not surprising that deregulation of MMPs could be engaged in neurodegenerative disorders e.g. multiple sclerosis and Alzheimer's disease (Yong et al. 1998) as well as in acute brain injuries. Accumulating evidence points to a deleterious role of metalloproteinases, particularly MMP-9, in the development of brain injury after focal ischemia (Rosenberg et al. 1996, 1998, Romanic et al. 1998, Gasche et al. 1999, Planas et al. 2001). The marked enhancement of their activity with simultaneous degradation of ECM and in consequence the altering of adhesive contact of neuronal cells with the extracellular matrix was postulated as a factor contributing to neuronal degeneration in ischemic penumbra.

This prompted us to investigate if global brain ischemia induces degradation of matrix proteins which would temporarily and spatially coincide with brain regions that exhibit neuronal apoptosis. To test this hypothesis we investigated the MMPs expression at the level of enzymatic activity in brain hippocampus, employing a well defined model of transient global cerebral ischemia in gerbils, which leads to neuronal death exclusively in pyramidal cells layer of the CA1 region. To understand further the role of MMPs in induction of postischemic apoptosis, in our experiment the hippocampus was divided into two parts: the dorsal part containing vulnerable CA1 neurons and the abdominal part considered as an ischemia-resistant area. In these two regions we have measured the activity of MMPs in two arbitrarily chosen time points: 3 h reperfusion, a time well in advance of any cellular sign of induced postischemic degeneration, and 72 h, when typical neuronal death is observed.

Furthermore, we determined the expression of laminin, as a putative substrate for MMP-9, in the similar course of postischemic reaction.

EXPERIMENTAL PROCEDURES

Ischemic model and sampling technique

All animal treatments followed the CMDiK quidelines (Guide for the care and use laboratory animals) and were approved by the First Local Ethics Committee. Male Mongolian gerbils, weighing 50-70 g, were used in the experiments. The ischemic insult was performed as previously described (Domańska-Janik et al. 1999) by 5 min bilateral ligation of the common ca-

rotid arteries under halothane/N_2O anesthesia in strictly controlled normothermic conditions. Animals subjected to ischemia were allowed to recover 3 and 72 h after the insult. Sham-operated animals served as controls. After the recovery period, animals were anesthetized and killed by decapitation. Hippocampi were quickly isolated on ice in the cold room and divided into dorsal (DP) and abdominal (AbP) parts. Manual "unfolding" of dorsal hippocampus, containing CA1 sector, was completed under a binocular scope with the fissura hippocampalis taken as a starting orientation point. The accuracy of this dissection was standarized on cross-sectioned hippocampal slices under the microscope. The remaining tissue was designated as the abdominal part (AbP). The pieces of tissue were frozen in liquid nitrogen and stored in a -80°C freezer until extraction of metalloproteinases.

Matrix metalloproteinases extraction

Extraction of MMPs from the hippocampal sections was performed by affinity chromatography using gelatin-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden), according to the method described by Zhang and Gottschall (1997). Both pieces of tissue (DP and AbP) were manually homogenized (10% w/v) in working buffer composed of 50mM TRIS-HCl, pH 7.6, 150 mM NaCl, 5mM $CaCl_2$, with addition of 0.05% Brij 35, 0.02% NaN_3 and 1% Triton X-100. Homogenate was centrifuged for 5 min at 12 000 x g. A 10 µl aliquot of recovered supernatants was saved and assayed for total protein and the rest was incubated for 60 min with gelatin-Sepharose 4B. After incubation and centrifugation, the gelatin-Sepharose pellet was incubated with elution buffer consisting of working buffer plus 10% dimethylsulfoxide (DMSO). Gelatinolytic activity of MMP in the eluent was measured by standard SDS-PAGE zymography.

SDS-PAGE zymography

The procedure for zymography of gelatinase activity in the tissue samples followed the method described by Zhang and Gottschall (1997). Briefly, tissue extracts normalized for protein concentration were mixed with 2x non-reducing sample buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue) and were subjected to electrophoresis through 10% polyacrylamide gel copolymerized with 0.1% of gelatin at 4°C. A standard sample containing active MMP-2 and MMP-9 enzymes (Oncogene Research Products) was included on each gel as an positive control. After electrophoresis, SDS was removed from the gels by washing with two changes of 2.5% Triton X-100 for 20 min each, in order to renaturate the enzymes. The gels were then incubated for 20 h at 37°C in a substrate buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0,02% NaN_3.

As a control, parallel samples were loaded onto another gel that was incubated in the buffer containing 10 mM EDTA or tissue inhibitor of metalloproteinase, TIMP-1 (3 µg/ml). Following incubation the gels were stained with 0.1% Coomassie Blue R250 (diluted in 40% methanol and 10% acetic acid) and destained until clear proteolytic bands appeared on the contrasting blue background. To quantify the relative changes in MMP expression the gels were digitized, and the area of lysis for each band detected was quantified by computer-assisted planimetry (Gel Expert Program- Nucleotech.Corp).

Results of MMP activities are expressed as means plus/minus the standard deviation (SD). Differences between means were analyzed using one-way analysis of variance (ANOVA). When the ANOVA showed significant differences, comparisons between means were tested by Tukey's post-hoc test. A level of $P<0.05$ was considered statistically significant.

In Situ Zymography

To analyze net endogenous MMPs activity within the brain hippocampus we have conducted *in situ* zymography, according to the method described by Romanic et al. (1998). In brief, the brain tissue from animals subjected to ischemic insult or sham surgery were removed after 72 h and immediately placed on ice. Coronal sections (1 mm) were made through the forebrain by use of a vibratome (752M Vibroslice, Campden Instruments, UK) and rinsed briefly in ice-cold PBS (phosphate buffered saline; pH 7.5). The tissue sections were then directly overlaid on top of gel consisting of 10% polyacrylamide copolymerized with gelatin (0.5 mg/ml) in 50 mM Tris-HCl, 5mM $CaCl_2$, pH 7.4. The tissue sections were incubated on top of the gel for 8 h at 37°C, after which the sections were removed and the gel was incubated further for a total of 18 h. The gels were stained with Coomassie blue and destained with 10% methanol and 7% acetic acid. Gelatinase activity was visualized as a zone of clearing against a blue background.

Western blot analysis of laminin expression

The tissue extracts from hippocampal regions (abdominal and dorsal part) prepared according to Chen and Strickland (1997) were resolved by electrophoresis through a gradient gel (4%-20%). The amount of protein loaded is described in Fig. 3 legend. After SDS-PAGE the proteins were transferred to nitrocellulose membranes (0.45 μm pore size), blocked with 5% nonfat milk for 3 h, and then incubated overnight with specific anti-laminin antibody (Sigma). Bound antibody was detected by the enhanced chemiluminiscent method (ECL).

Protein concentration was measured with Bio-Rad protein assay kit based on the method of Lowry (Bio-Rad Laboratories, Hercules, CA. USA) using an albumin (BSA) standard.

RESULTS

Effect of ischemia and reperfusion on metalloproteinase activity

Zymographic analysis of hippocampal tissue revealed two bands of gelatinolytic activity migrating at approximately 72 kDa and 92 kDa (Fig. 1A). When gels were incubated in the presence of tissue inhibitor of metalloproteinase 1 (TIMP-1) or in the presence of 10 mM EDTA in the medium gelatinolytic bands were no longer seen (not shown), demonstrating that the gelatinases from gerbil hippocampus are indeed MMPs. Based on their molecular weights and their susceptibility to inhibition by TIMP-1 and EDTA, these activities correspond to MMP-9 (gelatinase B) (upper band) and MMP-2 (gelatinase A) (lower band).

Densitometric analysis (Fig. 1B) of the band identified as MMP-2 revealed that transient ischemia during the first 3 h of reperfusion did not affect its constitutive activity. A small transient decrease of activity in the dorsal part was found not to be significant. A marked elevation of gelatinolytic activity to 168% and 188% of control value in abdominal and dorsal parts, respectively, was observed at 72 h of reperfusion. The other investigated metalloproteinase, MMP-9, was not expressed in control hippocampus. The weak broad band of zymographic digestion corresponding to this enzyme appeared in both regions after 5 min of ischemia followed by 3 h of reperfusion. The area of lysis, although a little more clear in the case of dorsal part, after

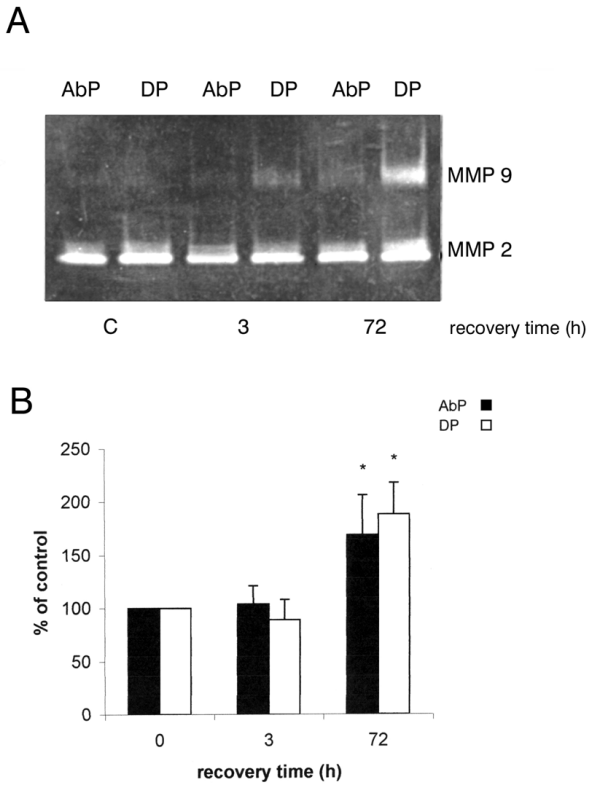

Fig. 1. Zymographic analysis of MMP-2 and MMP-9 activity in dorsal and abdominal parts of the hippocampus after ischemia-reperfusion. A, representative gelatin zymography of dorsal (DP) and abdominal (AbP) part of hippocampus. Tissue extracts (an equivalent of 200 μg of supernatant protein) from sham-operated animals (C) and animals subjected to 5 min ischemia followed by 3 or 72 h of reperfusion were analyzed with the use SDS-PAGE zymograms. The gelatinolytic bands correspond to MMP-9 (upper band) and MMP-2 (lower band); B, statistical analysis of the MMP-2 densitometric data from indicated experimental groups presented as a percent of parallel run of sham-operated controls. The results (mean values ±SD) shown are from 5 independent tissue samples. Statistically significant differences from controls are designated by *, $P<0.05$.

densitometry failed to reach statistical significance when compared to the abdominal region. A significant spatial differences in MMP-9 activity were observed at 72 h of recovery. At this time point activity in the dorsal part was 2-3 times higher than in the abdominal region.

In situ zymography

To analyze net endogenous activity of metalloproteinases (meaning that activity not inhibited by specific tissue inhibitors) we have conducted *in situ* zymography

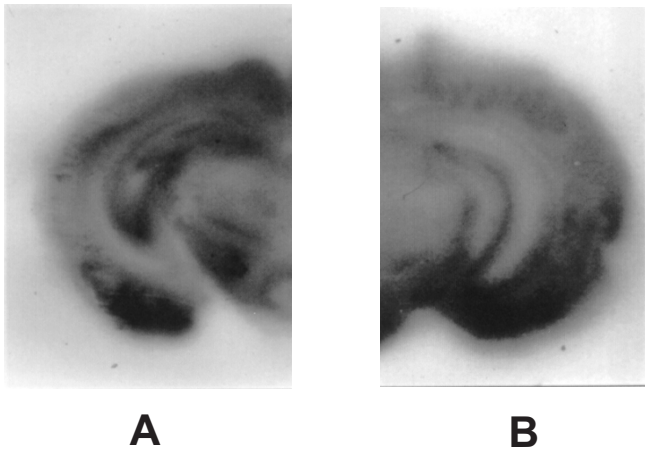

Fig. 2. Ischemia-induced MMPs activity detected by *in situ* zymography. MMPs activity was evaluated in coronal brain sections prepared from sham-operated animals (A) and animals that had undergone 5 min ischemia followed by 72 h of reperfusion (B). Gelatinolytic activity visualized as a zone of clearing is associated with the ischemic hippocampus, mostly with CA1 region. Results are representative of the total number of animals evaluated (n = 4 for ischemia and 3 for sham surgery).

of brain hemispheres. Fig. 2 depicts representative zymograms, which identify gelatinolytic activity in ischemic hippocampus, mostly in CA1 region. In the corresponding nonischemic region the enzymatically digested area is comparatively less clear.

Effect of ischemia and reperfusion on laminin

The level of laminin in dorsal and abdominal parts of hippocampus was evaluated by Western immunoblotting analysis with affinity-purified anti-laminin antibody. Fig. 3 shows a representative immunoblot displaying characteristic beta/gamma 220 kDa laminin chain. The significant attenuation of laminin specific immunoband, confirmed by densitometric analysis (not shown), with simultaneous appearance of the lower molecular weight degradation products was observed at 72 h of reperfusion exclusively in the vulnerable DP region of the hippocampus. In contrast, the generally more resistant to ischemia abdominal part displayed much less, if any, laminin degradation.

DISCUSSION

The current study demonstrates, to our knowledge for the first time, that activation of MMP-9 may be involved in the development of delayed neuronal death after short-term global ischemia. Although accumulating evidence points to an important role of metalloproteinases during stroke in human and animals (Anthony et al. 1997, Clark et al. 1997, Rosenberg et al. 1996, 1998, Romanic et al. 1998, Gasche et al. 1999, Planas et al. 2001), in this pathology they do not necessary reflect a local activity of resident brain cells or their surrounding. Disruption of the blood-brain barrier (BBB) in focal

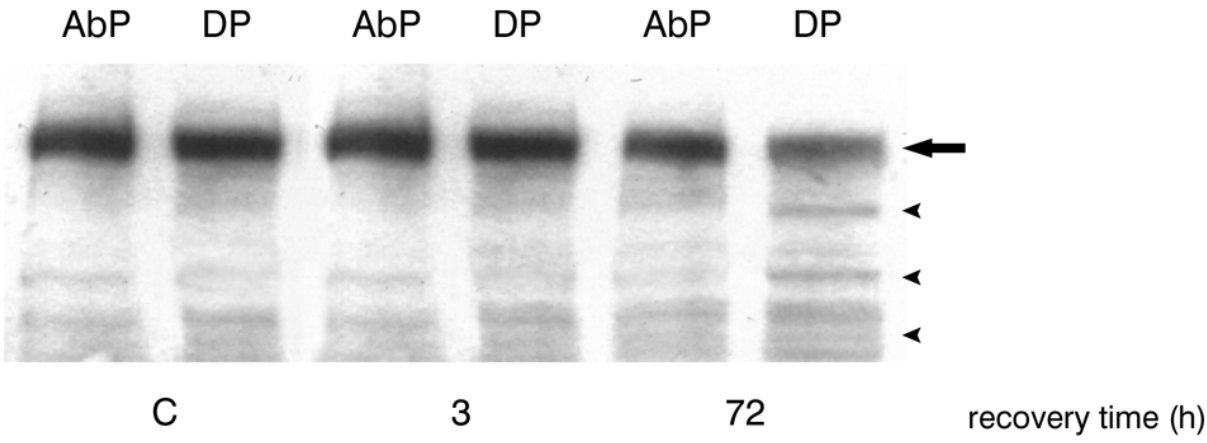

Fig. 3. Expression of laminin in dorsal and abdominal part of hippocampus after ischemia-reperfusion. Representative immunoblot of extract from abdominal (AbP) and dorsal part (DP) of the control sham-operated animals (C) and animals subjected to 5 min ischemia followed by 3 or 72 h of reperfusion. Samples containing 80 μg protein were separated by SDS-PAGE on gradient (4%-20%) gel, immunoblotted with affinity isolated anti-laminin antibody (Sigma) and developed with ECL system. The arrow indicates immunoband corresponding to laminin. The arrowheads show immunoreactive degradation products.

ischemia (Fujimura et al. 1999, Gasche et al. 1999, Heo et al. 1999) and the resulting infiltration by mononuclear cells to the brain tissue can provide the additional source of MMPs activity. In our study the employed model of transient global ischemia excludes the possibility of BBB breakdown and inflammatory cells' participation. The reaction of MMPs can be considered as a local tissue response to ischemic insult. Moreover, this reaction was found to differ significantly in two investigated hippocampal regions of various susceptibility to the injury, simultaneously with the other signs of lethal signalling cascade induction (Domańska-Janik et al. 1999, Friberg et al. 1999, Domańska-Janik et al. 2001, Ziemka-Nałęcz et al. 2002).

Our direct *in situ* zymographic assay has demonstrated that proteolytic activity of metalloproteinases precipitates in the ischemia-injured hippocampus at 72 h of reperfusion. Furthermore we have noticed different reactions depending on the type of MMPs involved. Despite that the constitutive activity of MMP-2 significantly increased in the late postischemic phase, the pattern of this response does not show any spatial preferences. In contrast, postischemic induction of MMP-9 was observed selectively in the dorsal part of the hippocampus. This may represent a specific component of detrimental tissue reaction to injury which correlates with time and space of postischemic neuronal loss. Furthermore, the enhancement of MMP-9 activity seems to parallel the pattern of microglia activation evident also in CA1 region after similar ischemic insult (Domańska-Janik et al. 2001). The reactive microglia might be a direct source of this protease as suggested previously by other data showing the MMP expression in microglia in different experimental models (Gotschall et al. 1995, Gotschall and Deb 1996, Cross and Woodroofe 1999).

The exact mechanism of MMP-9 induction and activation after ischemia has not been yet defined. It has been established that the primary mode of MMPs regulation in various pathophysiological conditions may occur either *via* gene transcription, the proenzyme activation or release of metalloproteinase inhibition exerted by endogenous tissue inhibitor- TIMP (Nagase 1997). It has been shown that inducible MMP-9 gene possesses an activator protein -1 (AP-1) binding sequence in its promoter region (Bian and Sun 1997). Thus it became conceivable that the induction of MMP-9 following 5 min global ischemia might be associated with the increase of AP-1 DNA-protein binding reported during reperfusion (Yoneda et al. 1997, 1998). However, recently we have shown that AP-1 response displays a very similar pattern in both investigated hippocampal sectors after ischemia (Zabłocka et al. 2002) which is not compatible with region-specific enhancement of MMP-9 activity in CA1. Furthermore, in our current experiments we have not found any significant differences in MMP-9 gene expression in post-tischemic hippocampus (manuscript in preparation). This indicates that MMP-9 activation observed in CA1 after ischemia relies on posttranslational modifications of the protein molecule rather than on a gene transcription. It is worthy to point out that MMP-9 is a highly glycosylated protein (Wilhelm et al. 1989), thus potentially susceptible to such posttranslational modification.

Moreover, we should take into consideration that the inactive forms of MMP-2 and 9 are secreted from cells into the extracellular space immediately after synthesis, and then activated by their pro-peptides specific proteases (Murphy et al. 1994, Nagase 1997) - the process connected with the reduction of molecular mass of the enzymes. In our study such proteolytically activated forms of MMP-2 and 9 were not identified as can be judged from an apparent molecular weight detected after SDS-PAGE (92 kDa and 72 kDa). This apparent inconsistency has been often reported (Fini et al. 1996, Rosenberg 1996, 1998, Clark et al. 1997, Mohan et al. 2000). Yet it was found that the latent zymogens might be activated in the *in vitro* assay without any previous cleavage (Kheriff et al. 1998, Zhang et al. 1998), possibly due to the activation by surfactants such as SDS (Springman et al. 1990) during the sample processing. Nevertheless, the relative absence of the MMPs active forms in SDS-PAGE analysis does not rule out the presence of such forms locally *in vivo* as confirmed by our direct zymography on hippocampal tissue. Such very localized proteolytic activation may have escaped our post-SDS-PAGE measurement method, since it was carried out on the total extracts. Additionally, MMPs are rapidly degraded further once the latent forms are locally cleaved and activated (Yu and Stamenkowic 1999). Therefore, only very low, undetectable levels of the MMP-9 may be present in its active form in the tissue at any given time point.

In this study we did not estimate the influence of TIMP-1, a specific inhibitor of MMPs, on postischemic proteolysis. In our hands the commercially available antibodies did not react with TIMP in gerbil brain. Also the reverse zymography method used according to Gasche et al. (1999) for determination of TIMP-1 level in the rat

brain has been determined to be not sensitive enough in the case of gerbil hippocampus. However, the available data (Gasche et al. 1999, Fujimura et al. 1999) have not reported any changes in the level of TIMP-1 after MMPs activation in focal ischemia. If this is the case also after global ischemia remains an open question.

Increased activity of MMP-9 after ischemia correlates with laminin degradation. Several pieces of information have pointed to high expression of laminin in hippocampus (Chen and Strickland 1997, Nakagami et al. 2000). Loss of parenchymal laminin may cause the disruption of cell-matrix contact and by this may contribute to the suppression of survival signals (Murtomaki et al. 1995, Chen and Strickland 1997). The functional role of laminin as an important factor responsible for maintaining cellular viability has been suggested by *in vitro* studies demonstrating that laminin increases long-term survival of mesencephalic dopaminergic neurons (Dong et al. 1994) and prevents excitotoxic apoptosis (Gary and Mattson 2001). In addition, laminin degradation has correlated with the loss of pyramidal neurons after kainate treatment (Chen and Strickland 1997). The present study demonstrates that ischemia-induced increase in MMPs is accompanied by simultaneous degradation of extracellular matrix component- laminin in the dorsal hippocampus. It must be noted, however, that laminin can be also degraded by other enzymes such as plasmin (Tsirka et al. 1997). Nevertheless, focal ischemia in MMP-9 knock outs resulted in decrease of laminin degradation as compared to wild type mice (Asahi et al. 2001).

CONCLUSION

Our data provide first evidence that the MMP pathway may function as a component of delayed neuronal death cascade after transient global ischemia. This has been accomplished by increase of MMP-2 together with MMP-9 gelatinolytic activities resulting in degradation of the laminin in the vulnerable CA1 region of the hippocampus at the time of neuronal death.

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