Ontogeny of MMPs and TIMPs in the Murine Neocortex

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ABSTRACT: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have been implicated in normal brain development, adult stroke, and, more recently, perinatal brain injury. Here, our objective was to obtain comprehensive and comparative data on the ontogeny of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the neocortex of male and female mice belonging to various strains, from embryonic life to adulthood. We used gelatin zymography, ELISA, and real-time PCR analyses. MMP-2, MMP-9, and TIMP-1 activity and/or expression peaked during embryonic life and the early neonatal period, whereas TIMP-2 peaked during the first two postnatal weeks. Comparable results were obtained in all the mouse strains except BALB/c, where MMP-2 levels were considerably lower at all ages compared with the other strains. No gender effect was observed on any of the study parameters. This comprehensive study will serve as a basis for future investigations into the role for MMPs and TIMPs in normal brain development and prenatal brain injury. (*Pediatr Res* 65: 296–300, 2009)

Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases capable of degrading the extracellular matrix (ECM). Matrix degradation occurs during tissue remodeling and inflammatory reactions. MMPs cleave many other important molecules including growth factors, cytokines, and chemokines. Therefore, the role for MMPs extends far beyond the normal and disease-related processes involving structural ECM changes (1).

MMPs and their naturally occurring inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) are expressed by all the main nervous-system cell types including neurons, astrocytes, oligodendrocytes, and microglia (2–5). MMPs (most notably MMP-2, -3, and -9) and TIMPs are essential for coordinated ECM remodeling (6–9). ECM remodeling is probably very important for CNS development, as extensive cellular migration and remodeling are required (10,11). In addition, studies of the spatiotemporal expression of many MMPs (including MMP-2, -3, -9, -12, -14, and -24) suggest a role in various steps of brain development (review in Ref. 1). For example, oligodendrocytes express MMP-9 during migra-

tion and process extension (5), and MMP-9 is involved in myelination (12,13). Similarly, MMP-9 is expressed during mouse neurogenesis, suggesting a role in neuronal production (14). Finally, MMP-2, -3, -9, and -24 are strongly expressed in the developing cerebellum (review in Ref. 1). Interestingly, TIMPs exert physiologic effects in the CNS independently from their inhibitory actions. They appear to be involved in neuronal death, axonal sprouting, and synaptic mechanisms underlying learning and memory in mice (15–17). Excessive expression of MMPs and TIMPs may result in tissue damage. Accumulating evidence supports a major role for MMP-2 and -9 and their respective inhibitors in the pathogenesis of various injuries to the adult or immature brain. Recent studies in mouse null mutants showed a pivotal role for MMP-9 in recovery from traumatic brain injury (18). MMP-9 gene suppression has beneficial long-term effects on neurovascular remodeling and behavioral recovery after stroke (19) and exerts neuroprotective effects against hypoxic-ischemic injury in the immature brain (20). Similarly, structural and functional CNS recovery after injury is impaired in MMP-2 null mice, which exhibit increased glial scarring and reduced axonal plasticity (21). Increased TIMP-1 expression was found in the adult rat CNS after kainate-induced excitotoxic seizures (22), suggesting a neuroprotective effect of this inhibitor. TIMP-1 and TIMP-2 gene transfer *via* an adenoviral vector in a mouse model of global cerebral ischemia was neuroprotective (23). Thus, MMPs and TIMPs—most notably MMP-2, MMP-9, TIMP-1, and TIMP-2—may be involved both in normal CNS development and in hypoxic-ischemic and inflammatory brain damage. Few comprehensive descriptions of the ontogeny of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the mouse brain are available (14,24). Here, we used zymography, ELISA, and real-time PCR for a comparative descriptive study of the ontogeny of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the neocortex of male and female mice belonging to various strains, from embryonic life to adulthood.

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Abbreviations: E, embryonic day; ECM, extracellular matrix; MMP, matrix metalloproteinases; P, postnatal day; TIMP, tissue inhibitor of metalloproteinases

METHODS

Animal specimens. Experimental protocols were approved by our institutional review board, met INSERM guidelines, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All animals were kept in a ventilated room under controlled conditions of lighting (12-h light/dark cycle) and temperature ($22 \pm 2^\circ\text{C}$) and were given free access to food and water. We used five mouse strains: C57BL/6, C3H, BALB/c, FVB, and Swiss. The animals were killed by decapitation. Gender was determined on the day of birth (P0) and confirmed after death, by visual inspection. The neocortex was dissected at the following embryonic (E) and postnatal (P) ages: E14, E17, P0, P5, P10, P15, P21, P30, and P60. The specimens were immediately frozen in isopentane and stored at -80°C until further use.

Gelatin zymography. MMP-2 and MMP-9 activities were determined by gelatin zymography. Frozen brain specimens were homogenized and solubilized in lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, and Complete Mini EDTA-free Cocktail Protease Inhibitor (Roche, Meylan, France). Homogenates were centrifuged at 14,000 rpm for 10 min at 4°C . Total protein concentrations in the supernatants were determined using Biuret's method. Brain samples (100 μg protein) diluted in TAE buffer were run on a 9% polyacrylamide gel containing 0.1% gelatin in Tris-glycin SDS, using standard conditions.

Then, the gel was incubated with zymogram-renaturing buffer (2.5% Triton $\times 100$), with gentle agitation, for two 30-min periods at room temperature. The gel was incubated in fresh zymogram-renaturing buffer overnight at 37°C . Coomassie blue G 250 diluted in fixative (methanol/acid acetic/water: 50/10/40) followed by destaining (methanol/acetic acid/water: 50/10/40) was used to reveal protease activity. Human recombinant MMP-9 and MMP-2 (Millipore, Bedford, MA) served as internal standards. Areas of gelatinolytic activity were measured using automated image analysis (Vilbert-Lourmat, Marne-la-Vallée, France).

Enzyme-linked immunosorbent assay. MMP-2, TIMP-1, and TIMP-2 levels were determined using ELISA. Quantikine human-mouse MMP-2, human TIMP-2, and mouse TIMP-1 commercial ELISA kits (R&D System, Lille, France) were used as directed. No sample dilution was required.

Expression of MMP mRNAs. Total RNA was extracted according to a protocol derived from the original procedures of Chomczynski and Sacchi (25) and consisting of two independent total RNA extractions separated by a DNA set treatment (DNA-free kit, Ambion, Austin, TX). RNA quality and concentration were assessed based on relative absorbance at 260 versus 280 nm, and RNA integrity was checked by electrophoresis on 1.5% agarose gel with ethidium bromide. Total RNA (900 ng) was subjected to reverse transcription using the Iscript kit from Bio-Rad (Hercules, CA). Negative controls (samples without reverse transcriptase) were individually amplified by PCR using the various study primer sets, to ensure absence of genomic DNA contamination. To specifically amplify mRNA encoding various mouse proteins, we designed specific primer sets (sense and antisense, respectively) using Oligo6.0 and M-fold software (Invitrogen, Carlsbad, California). The primer sequences are given in Table 1. Preliminary experiments showed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were extremely stable in *in vivo* samples and treatment conditions (26). Therefore, we chose this housekeeping gene to standardize our quantitative experiments. Real-time PCR was set up using SYBR green-containing supermix from Bio-Rad for 50 cycles of a three-step procedure, including a 20-s denaturation step at 96°C , a 25-s annealing step at 61°C , and a 20-s extension step at 72°C . Amplification specificity was assessed by melting curve analysis. The cycle threshold (CT) values obtained by real-time PCR were quantified using a relative standard curve. For this purpose, 8 dilutions (obtained by 2-fold serial

dilution) of a pool of cDNAs were quantified. Results were expressed as the target/internal standard (GAPDH) concentration ratio of the sample divided by the target/internal standard concentration ratio of the calibrator.

Statistical analyses. Quantitative data were expressed as means $\pm$ SEM for each experimental group. Statistical analysis of the data were performed using one- or two-way ANOVA with Dunnett's or Bonferroni's *post hoc* comparison tests (GraphPad (4) Prism version for Windows; GraphPad Software, San Diego, CA).

RESULTS

Matrix metalloproteinase-2. By gelatin zymography and ELISA, neocortical MMP-2 activity and concentration were highest in the embryonic period and at birth (P0) then decreased gradually until adulthood (Figs. 1A and 2). These declines in MMP-2 activity and level occurred in all five mouse strains (Fig. 2, ANOVA 1). In BALB/c mice, however, MMP-2 levels were considerably lower at all ages studied, compared with the other strains (Fig. 2F, ANOVA 2). No gender effect was found for MMP-2 levels by ELISA in any of the mouse strains (Fig. 1B). In Swiss mice, the results of real-time PCR analysis of MMP-2 mRNA (Fig. 3A, ANOVA 1) were generally comparable with those obtained by gelatin zymography and ELISA (Figs. 1A and 2B, ANOVA 1) with, however, a few differences, such as longer lasting mRNA expression (until P5 instead of E17). No gender effect of real-time PCR data were noted (data not shown).

Matrix metalloproteinase-9. Gelatin zymography (Fig. 1A) showed weak MMP-9 activity between E14 and P5. After P5, no MMP-9 was detected in any of the strains (data shown only for Swiss mice). No gender effect was observed (Fig. 1B). Given the lower sensitivity of ELISA compared with gelatin zymography (nanograms *versus* picograms) and the very low MMP-9 activity by gelatin zymography, MMP-9 ELISA was not performed. The results of real-time PCR analysis (Fig. 3B) of MMP-9 mRNA were comparable with those obtained by gelatin zymography. No gender effect was noted for real-time PCR results (data not shown).

Tissue inhibitors of metalloproteinase-1. TIMP-1 mRNA expression assessed by real-time PCR was highest between E14 and P10 then decreased gradually until P60 (Fig. 3C, ANOVA 1). ELISA showed a similar profile with strong TIMP-1 protein expression during a shorter period (P5 *versus* P10) (Fig. 4). No gender effect was observed (data not shown).

Tissue inhibitors of metalloproteinase-2. TIMP-2 protein could not be evaluated by ELISA because the human TIMP-2 ELISA performed poorly and no mouse TIMP-2 ELISA kit

Table 1. Sequences of primers used in quantitative real time PCR

Genes	Primer sequences	Amplicon sizes (bp)	Spanning regions	NBI access
GAPDH	5'-GGCCTTCCGTGTTCTAC-3' OH, 5'-TGTCATCATACTGGAGGTT-3' OH	80	1093–1173	XM111622
MMP-2	5'-AACTTCCGATTATCCCATGAT-3' OH 5'-GGCCAGTACCAGTGTCAGTA-3' OH	87	1585–1672	NM008610
MMP-9	5'-TGTTCCCGTTCATCTTTGAG-3' OH 5'-ATCTGGTCATAGTTGGCTGT-3' OH	103	872–975	NM013599
TIMP-1	5'-CAGTAAGGCCTGTAGCTGTGC-3' OH 5'-AGGTGGTCTCGTTGATTCTG-3' OH	109	227–336	NM011593
TIMP-2	5'-GGAATGACATCTATGGCAACC-3' OH 5'-GGCCGTGTAGATAAACTCGAT-3' OH	103	24–127	AY622854

Figure 1. Gelatinolytic activities of MMP-2 and MMP-9 studied by zymography in Swiss mouse neocortex at various embryonic (E) and postnatal (P) ages. The results are representative of three independent experiments. Different animals were studied at each age. M, male; F, female.

was available. Real-time PCR (Fig. 3D, ANOVA 1) showed a gradual increase in TIMP-2 expression during the embryonic and neonatal period to a peak at P5. Then, TIMP-2 expression decreased until P20, when adult levels were reached. No gender effect was observed (data not shown).

DISCUSSION

We report detailed descriptive data on the ontogenesis, in the developing mouse neocortex, of two major MMPs, MMP-2 and MMP-9, and of their endogenous inhibitors TIMP-1 and TIMP-2. Of interest, no gender effect was observed for any of the study parameters.

Ontogeny of MMPs and TIMPs in the mouse neocortex.

Expression and/or activity of MMP-2, MMP-9, and TIMP-1 were strongest during the embryonic stages, supporting a role for these factors in the control of neuronal proliferation and/or migration, in keeping with earlier studies (4,5,13,24,27–34). The expression of these factors during early postnatal life suggests additional roles in neuronal differentiation and/or survival (13,35,36) and in the events involved in gliogenesis, including migration of astrocytes and oligodendrocyte precursors (5,12).

In contrast, TIMP-2 expression peaked during the first 2 postnatal weeks, suggesting a predominant role for this protein in mechanisms involved in the survival and maturation of postmigratory neurons, such as axonal growth and synaptogenesis; and/or in gliogenesis (35). However, we were unable to assay TIMP-2 protein, a fact that hinders the interpretation of our data. More specifically, we have no information on the balance between protein secretion and mRNA for TIMP-2.

The time-course of TIMP-1 expression was comparable with that of MMP-9 expression, suggesting tight control of MMP-9 activity by its endogenous inhibitor. MMP-2 and its inhibitor TIMP-2 differed regarding their expression over time, suggesting a specific role for TIMP-2 in the developing brain, independently from its proteolytic effect on MMP-2. Conceivably, TIMP-2 may act as a constitutive brain-maturation factor (35). To our knowledge, no independent role for TIMP-2 in brain development has been documented to date.

MMP-2 and TIMP-1 mRNAs were expressed for longer periods than the corresponding proteins, suggesting either posttranslational degradation of mRNA or posttranslational regulation.

Our time-course results are consistent with earlier findings. A previous study, conducted in mice aged 1–40 postnatal weeks, showed age-dependent mRNA expression of MMPs and TIMPs, with higher expression levels in the immature brain (24). A broader range of MMPs and TIMPs was examined, compared with our study. Various neuronal tissues in the brain, cerebellum, and spinal cord were investigated, and roles for each MMP and TIMP were suggested based on the results of the spatiotemporal analysis (24). Another study found up-regulation of MMP-9 mRNA in mouse brains during the late embryonic period associated with the development of the neural vascular system (14), in keeping with our results.

Figure 2. MMP-2 protein level assayed by ELISA in the neocortex of five mouse strains at embryonic (E) and postnatal (P) ages. (A, BALB/c; B, C3H; C, C57BL/6; D, FVB; E, Swiss; and F, summary of all strains (ANOVA 2). Square, Balb; up-triangle: C3H; down-triangle: FVB; rhombus: C57BL/6; and circle: Swiss). Six to ten animals were studied in duplicate at each age (males, 3–5; and females, 3–5). Bars represent mean MMP-2 protein concentration $\pm$ SEM. Asterisks indicate statistically significant differences from the P60 group. * p < 0.05, ** p < 0.01 by ANOVA 1 with Dunnett's (A–E) or Bonferroni's (F) posttest.

Figure 3. MMP-2 (A), MMP-9 (B), TIMP-1 (C), and TIMP-2 (D) gene expression studied by real-time PCR in Swiss mouse neocortex at various embryonic (E) and postnatal (P) ages. Six to ten animals were studied in duplicate at each age. Bars represent mean brain MMP-2, MMP-9, TIMP-1, or TIMP-2/GAPDH ratios $\pm$ SEM. Asterisks indicate statistically significant differences from the P60 group. * $p < 0.05$, ** $p < 0.01$ by ANOVA 1 with Dunnett's posttest.

Figure 4. TIMP-1 protein level determined by ELISA in the neocortex of Swiss mice at various embryonic (E) and postnatal (P) ages. Six to ten animals (males, 3–5; and females, 3–5) were studied in duplicate at each age. Bars represent mean TIMP-1 protein concentration $\pm$ SEM. Asterisks indicate statistically significant age differences from the P60 group. ** $p < 0.01$ by ANOVA 1 with Dunnett's posttest.

In adult mice, we found decreasing yet persistent constitutive MMP-2 expression, absence of detectable MMP-9 expression, and large amounts of TIMP-2, in keeping with earlier data (4,24). TIMP-1 was expressed at a lower level than TIMP-2 (4,15,24).

A weakness of our study is the absence of data on the locations of the metalloproteinases or their inhibitors. MMP-2 has been found chiefly in astrocytes, oligodendrocytes, and cortical neurons (1). MMP-9 has been detected in neurons (30), as well as in endothelial cells, which also contains MMP-2 (15). MMP-9 levels are low in astrocytes (30). TIMP-1 has been found in neurons and in the hippocampus and cerebellum; whereas TIMP-2 has been identified in cortical neurons and in cerebellar Purkinje and granular cells (1). A major strength of our study is that we used the same protocol for all the ages studied. We collected information on the embryonic, very early postnatal, and late postnatal periods, as well as on adulthood. We conducted gender-specific analyses, and we included both the protein concentrations and the activities of MMP-2, MMP-9, and TIMP-1 investigations.

The physiologic interactions of MMPs and TIMPs with the ECM during brain ontogeny deserve to be investigated. MMPs facilitate cell migration by breaking down the ECM. However, in recent years an increasing number of MMP substrates not found in the ECM were identified, including pro-TGF β ,

pro-BDNF, NGF, and VEGF. These growth factors and neurotrophic factors may influence neural development (review in Ref. 37).

Impact of gender and genetic background on MMP and TIMP ontogeny. Several parameters of brain maturation are influenced by gender in rodents (38,39). However, we found no significant effect of gender on any of our study parameters. To our knowledge, no other study has reported a gender effect on the brain expression of MMPs or TIMPs.

A growing body of evidence suggests that genetic polymorphisms and, more generally, the genetic background may have a major influence on resistance or susceptibility to perinatal brain injury (40–42). Oxygen and glucose deprivation and exposure to the glutamate agonist NMDA caused more cell death in neuronal mice cultures from males than from females (43). In rats on P7, hypothermia protected females but not males from histologic damage and sensory-motor deficits (44). Available mouse strains vary regarding their genetic backgrounds and are therefore useful for investigating the influence of the genetic background. Here, we found that most of the strains exhibited similar ontogenic profiles of brain MMP-2 and MMP-9 expression. However, in the BALB/C strain, MMP-2 levels were very low at all the studied ages. This strain probably exhibits a distinctive pattern of brain development or maturity; thus, evidence of accelerated postnatal brain maturation has been found in hybrid BALB/c mice compared with inbred BALB/c mice (45). It would be of considerable interest to investigate the susceptibility of BALB/C mice to perinatal brain insults comparatively with other mouse strains. This result supports an influence of the genetic background on the expression of molecules such as MMPs that may play a role in the repair of perinatal brain injury.

The continuum of the levels of expression and activity of MMPs during embryonic, early prenatal, and adult life may inform the development of tests for the early diagnosis of abnormal brain development in humans. Thus, mouse studies may identify avenues for future research that may eventually produce information relevant to the developing and aging human brain.

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