Int J Legal Med (2010) 124:439–444
DOI 10.1007/s00414-010-0484-y

ORIGINAL ARTICLE

Immunohistochemical detection of MMP-2 and MMP-9 in a stasis-induced deep vein thrombosis model and its application to thrombus age estimation

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Received: 29 March 2010 / Accepted: 28 June 2010 / Published online: 10 July 2010

Abstract We immunohistochemically examined the expression of matrix metalloproteinase (MMP)-2 and MMP-9 using venous thrombi developed by ligation of the inferior vena cava (IVC) in mice. Both MMP-2- and MMP-9- positive cells could be detected in the whole course of thrombus formation after IVC ligation. Morphometrically, their number was greatest 14 days after IVC ligation and thereafter, gradually decreased at 21 days. The number of MMP-9-positive cells was significantly higher than that of MMP-2-positive cells at 1 to 7 days. The average ratio of MMP-9 to MMP-2 (MMP-9/MMP-2 ratio) was >2.0 in all thrombus samples at 1–5 days. After 7 days, the MMP-9/MMP-2 ratio was less than 2.0. These observations implied that an MMP-9/MMP-2 ratio markedly exceeding 2.0 strongly indicates an age of 5 days or less. Furthermore, an MMP-9/MMP-2 ratio of <2.0 probably indicates an age of more than 7 days. The present study demonstrated that the immunohistochemical detection of intrathrombotic MMP-2 and MMP-9 was suitable to estimate the age of venous thrombi.

Keywords Forensic pathology · Thrombus age determination · Immunohistochemistry · Matrix metalloproteinase 2 · Matrix metalloproteinase 9

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Introduction

Deep vein thrombus (DVT) is a major problem as the cause of pulmonary thromboembolism (PTE) from both clinical and forensic aspects. When forensic pathologists encounter cases of DVT-related PTE, they are always required to estimate how old the venous thrombi are, as well as skin wound age determination. Immunohistochemical techniques are widely used in the forensic research fields to estimate the age of skin wounds or brain contusions [1–18]; however, there are only a few forensic studies on thrombus age estimation [19–21].

From pathophysiological aspects, the development and resolution of venous thrombi are very similar to the skin wound healing process, composed of inflammation, proliferation, and maturation. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases; other family members are adamalysins, serralysins, and astacins. The MMPs belong to a larger family of proteases known as the metzincin superfamily. MMPs play an important role in the degradation of extracellular matrix and basement membrane components to allow the migration of vascular smooth muscle cells, and contribute to acute and chronic fibrotic diseases [22–27]. Furthermore, MMP activity is indispensable for the process of thrombus resolution [28–32]. This prompted us to hypothesize that MMPs are candidates for considering the age of venous thrombi.

Thus, in the present study, we examined the intrathrombotic expression of MMP-2 and MMP-9 by immunohistochemical techniques combined with morphometrical analysis, and discussed their suitability as markers for the age estimation of venous thrombi.

Materials and methods

Antibodies

In the present study, the following monoclonal antibodies (mAbs) or polyclonal Abs (pAbs) were used for immunohistochemistry and double-color immunofluorescence analysis: goat anti-MMP-2 pAbs and goat anti-MMP-9 pAbs (Santa Cruz Biotechnology, Inc., Santa Cruz CA, USA), anti-F4/80 mAb (clone BM8; BMA Biomedicals, Switzerland), cyanine dye3 (Cy3)-conjugated donkey anti-rat IgG pAbs, fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG pAbs (Jackson Immunoresearch Laboratories), and biotinylated rabbit anti-goat IgG pAbs (Dako Cytomation, Kyoto, Japan).

Mice

Specific pathogen-free 8- to 10-week-old male BALB/c mice were obtained from SLC (Shizuoka, Japan). All mice were housed individually in cages under specific pathogen-free conditions during the experiments. All animal experiments were approved by the Committee on Animal Care and Use of Wakayama Medical University.

Stasis-induced deep vein thrombus model

Intravenous thrombus was induced as described previously [20, 21]. Briefly, after deep anesthesia with intraperitoneal injection of pentobarbital (50 mg/kg body weight), a 2-cm incision was made along the abdominal midline, and the inferior vena cava (IVC) was exposed and ligated with 3-0 silk suture. The abdominal wall was closed, and 1 ml phosphate-buffered saline (PBS) was injected subcutaneously. At 1, 3, 5, 7, 10, 14, and 21 days after IVC ligation, mice were euthanized by an overdose of diethyl ether, and thrombi with vessel walls were harvested and subjected to further histological analyses. At each time point, five mice were used.

Immunohistochemical analyses

Intravenous thrombi, obtained at the indicated time intervals after ligation, were fixed in 4% formaldehyde buffered with PBS (pH 7.2), transversely cut in the middle of the thrombus, and paraffin-embedded sections (4 μm thick) were made. Immunohistochemical analyses were performed as described previously [33, 34]. Briefly, deparaffinized sections were immersed in 0.3% H_2O_2-PBS for 30 min to eliminate endogenous peroxidase activity. The sections were further incubated with PBS containing 1% normal serum corresponding to the secondary Abs and 1% bovine serum albumin to reduce nonspecific reactions. Thereafter, the sections were reacted with goat anti-MMP-2 pAbs (2 μg/ml) or anti-MMP-9 pAbs (2 μg/ml) at 4°C overnight. After incubation with biotinylated rabbit anti-goat IgG pAbs (2 μg/ml) at room temperature for 60 min, immune complexes were visualized using a catalyzed signal amplification system (Dako Cytomation), according to the manufacturer's instructions.

Semiquantitative evaluation of intrathrombotic of MMP-2 and MMP-9 expression

Intrathrombotic MMP-2 or MMP-9 levels were evaluated semiquantitatively. Briefly, after MMP-2-positive or MMP-9-positive cells were enumerated in five high power fields (×1,000) within the thrombus, the total numbers in the five fields were combined. All measurements were performed by two examiners without prior knowledge of the experimental procedures.

Double-color immunofluorescence analysis

In order to determine the cell type expressing MMP-2 or MMP-9, double-color immunofluorescence analysis was performed as described previously [6, 8]. Briefly, deparaffinized sections were incubated with PBS containing 1% normal donkey serum and 1% bovine serum albumin to reduce nonspecific

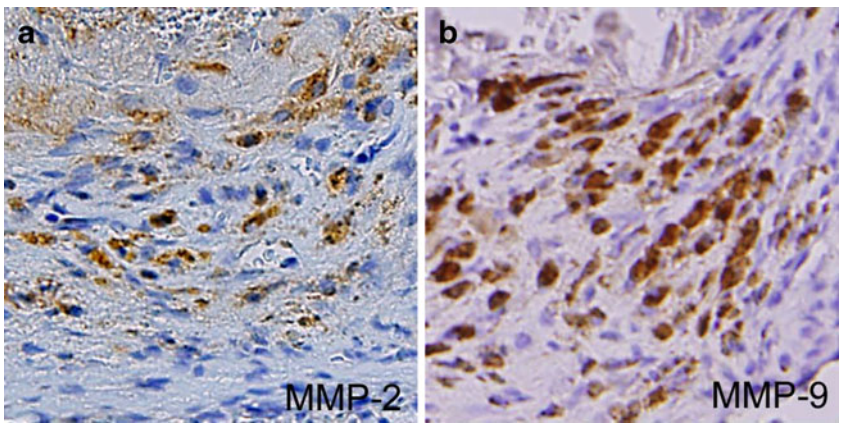

Fig. 1 Immunohistochemical detection of MMP-2 (**a**) and MMP-9 (**b**) 7 days after IVC ligation. Representative results are shown. Original magnification, ×200

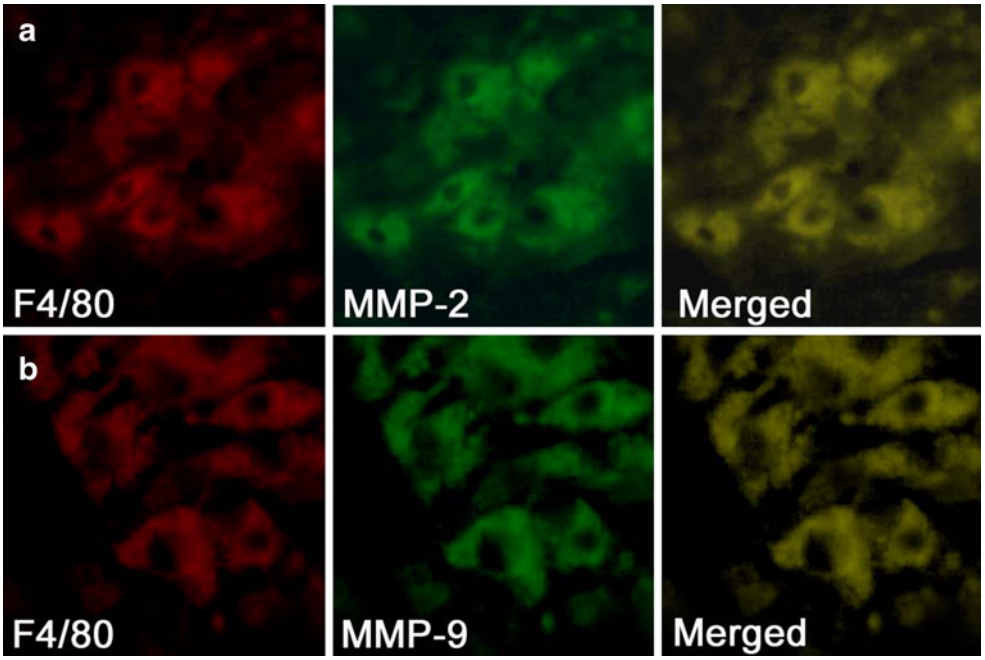

Fig. 2 Double-color immunofluorescence analysis of MMP-2 (**a**) and MMP-9 (**b**) in the thrombi 10 days after IVC ligation. Samples were immunostained with a combination of anti-F4/80 mAb and anti-MMP-2 pAbs or anti-F4/80 mAb and anti-MMP-9 pAbs. Images were digitally merged. Representative results are shown. Original magnification, ×400

reactions as previously described. Thereafter, the sections were further incubated in a combination of anti-F4/80 mAb (2 μg/ml) and anti-MMP-2 pAb (2 μg/ml), or anti-F4/80 mAb (2 μg/ml) and anti-MMP-9 pAb (2 μg/ml). After incubation with Cy3-conjugated and FITC-conjugated secondary pAbs (at each concentration of 15 μg/ml) at room temperature for 60 min, the sections were observed under fluorescence microscopy.

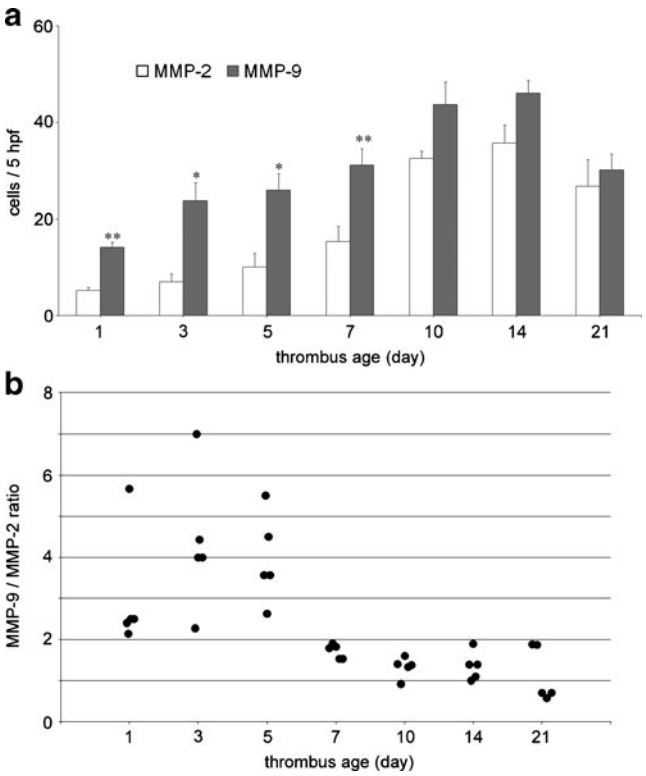

Fig. 3 **a** Semiquantitative analyses of the number of MMP-2- and MMP-9-positive cells. **b** The distribution of MMP-9/MMP-2 ratios in relation to thrombus age (n=5 in each group). Results are the mean± SEM. *p<0.05, **p<0.01

Statistical analysis

All data were presented as the mean ± SEM. Statistical significance was evaluated using Mann–Whitney's U test. P<0.05 was accepted as significant.

Results

Intrathrombotic appearance of MMP-2 and MMP-9

We immunohistochemically examined the intrathrombotic appearance of MMP-2 and MMP-9. During the whole

Table 1 Mean MMP-9/MMP-2 ratios in each thrombus group (n=5)

Thrombus age	MMP-9/MMP-2 ratio Mean ± SEM (range)
1 day	3.2±0.8 (2.1–5.7)
3 days	4.4±1.0 (2.3–7.0)
5 days	4.0±0.6 (2.6–5.5)
7 days	1.8±0.1 (1.5–1.9)
10 days	1.3±0.1 (0.9–1.6)
14 days	1.3±0.3 (1.0–1.9)
21 days	1.3±0.4 (0.6–1.9)

course of thrombi after IVC ligation, both MMP-2- and MMP-9-positive cells could be detected in mononuclear cells, probably macrophages (Fig. 1), and double-color immunofluorescence analysis demonstrated that F4/80-positive macrophages were the main cellular source of both enzymes (Fig. 2). As shown in Fig. 3a, in both enzymes, the positive cell number increased time-dependently until 14 days after IVC ligation, and then decreased. When compared at the indicated time points after IVC ligation, the number of MMP-9-positive cells was higher than the number of MMP-2-positive cells, with significant differences at 1 to 7 days (Fig. 3a). Subsequently, MMP-9/MMP-2 ratios were calculated. All thrombus samples aged 1–5 days showed MMP-9/MMP-2 ratios of more than 2.0 (Fig. 3b and Table 1), whereas at 7 and 21 days, the ratios were less than 2.0 (Fig. 3b).

Discussion

Blood coagulation and the fibrinolysis mechanism are demonstrated by interaction of the vessel walls and blood cells. Similar to skin wound healing, this process is composed of three different phases: inflammation, proliferation, and maturation. Various cells, such as red blood cells, leukocytes, vascular endothelial cells, smooth muscle cells, and platelets, are involved in the coagulation and fibrinolysis systems [35–38]. We demonstrated time-dependent reciprocal changes between the number of macrophages and neutrophils [20], and indicated that the intrathrombotic appearance of neutrophils and macrophages seemed useful for thrombus age estimation. Moreover, we think that many biofunctional molecules derived from leukocytes and endothelial cells are involved in the process of thrombus resolution [39].

MMPs were initially considered to be a group of enzymes with the sole function of regulating extracellular matrix (ECM) composition. ECM degradation is not the sole and possibly not the main function of these proteinases. Indeed, plenty of evidence support the roles of MMPs in physiological processes, such as embryogenesis and wound healing/tissue remodeling, and also in pathological processes, including arthritis, pulmonary diseases, cardiovascular ailments, and cancer [26, 40–43]. Several lines of accumulated evidence have demonstrated that both MMP-2 and MMP-9 played an important role in thrombus formation and resolution [26, 29, 31].

In the present study, we could detect MMP-2- positive and MMP-9-positive cells in the thrombus. Temporal changes in the numbers of these positive cells were very similar to those in intrathrombotic macrophage number [20], and double-color immunofluorescence analysis demonstrated that F4/80-positive macrophages were the main cellular source of MMP-2 and MMP-9. Collagenolysis is indispensable for thrombus resolution [39]. Reduced intrathrombotic collagen contents indicate enhanced collagenolysis. Among several enzymes with collagenolysis activity, MMP-2 and MMP-9 are presumed to have an important role in collagen turnover during thrombus resolution [31]. In the present study, the number of MMP-9-positive cells was higher than that of MMP-2-positive cells at the indicated time intervals and in particular, we found a significant difference in thrombus samples aged less than 7 days. These observations indicated that MMP-9 was more crucially involved in thrombus resolution.

From our observations, MMP-2 and MMP-9 may be useful for thrombus age estimation; however, the sole evaluation of intrathrombotic MMP-2 and MMP-9 expression was seemingly complicated. Previously, we demonstrated that the neutrophil/macrophage ratio in thrombi could give significant information for thrombus age estimation. Thus, from the viewpoint of forensic application, Fig. 2a demonstrated that MMP-9/MMP-2 ratios in thrombi were more useful for the age estimation of venous thrombi. The average MMP-9/MMP-2 ratio was >2.0, with a range of 2.1–7.0 in all thrombus samples aged 1–5 days. These observations implied that an MMP-9/MMP-2 ratio markedly exceeding 2.0 strongly indicated an age of 5 days or less. At later than 7 days, the MMP-9/MMP-2 ratio was less than 2.0 (0.6–1.9), implying that an MMP-9/MMP-2 ratio of <2.0 probably indicates an age of more than 7 days. Our previous study showed that the neutrophil/macrophage ratio in thrombi was useful for thrombus age estimation. In the present study, the majority of positive cells for both MMPs were macrophages. Thus, the combination of MMP-9/MMP-2 ratios with neutrophil/macrophage ratios would provide more detailed information for age estimation of venous thrombi.

Finally, because the present results were obtained from well-controlled animal experiments, it is of course necessary to perform further study using human thrombus samples of various ages. Herein, we provide evidence that immunohistochemical detection of intrathrombotic MMP-2 and MMP-9 is applicable for thrombus age estimation.

Acknowledgments We thank Ms. Mariko Kawaguchi for her excellent assistance in the preparation of this manuscript. This study was financially supported in part by Grants-in-Aid for Scientific Research (A) and (C) from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

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