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ORIGINAL ARTICLE

Time-dependent appearance of intrathrombus neutrophils and macrophages in a stasis-induced deep vein thrombosis model and its application to thrombus age determination

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Abstract We immunohistochemically examined neutrophils and macrophages in venous thrombi, which developed in the ligation of the inferior vena cava (IVC). Myeloperoxidase (MPO)-positive neutrophils and F4/80-positive macrophages were detected in the whole course of thrombi after IVC ligation. Morphometrically, the number of neutrophils was greatest at 1 day after IVC ligation and, thereafter, gradually decreased with an increase of the post-ligation interval. In contrast, the number of macrophages peaked at 7 days after ligation. The number of intrathrombus neutrophils was significantly higher than that of intrathrombus macrophages at 1 and 3 days, and the average ratios of neutrophils to macrophages (N/M ratios) were 6.8±1.1 (4.8–9.0) and 2.5±0.4 (1.7–4.2) at 1 and 3 days, respectively. After more than 5 days, all samples had N/M ratios of <2.0 (0.2–1.4). These observations suggest that an N/M ratio of >2.0 indicates a thrombus age of 1–3 days. To differentiate between 1- and 3-day-old thrombi, an N/M ratio markedly exceeding 5.0 strongly indicates an age of 1 day. Furthermore, an N/M ratio of 1.0 or less probably indicates an age of more than 5 days. The present study demonstrated that the immunohistochemical detection of intrathrombus neutrophils and macrophages was suitable to determine the age of venous thrombi.

Keywords Forensic pathology · Thrombus age determination · Immunohistochemistry · Neutrophils · Macrophages

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Introduction

From the aspects of pathophysiology, three primary influences predispose thrombus formation, the so-called Virchow's triad: (1) endothelial injury; (2) stasis or turbulence of blood flow; and (3) blood hypercoagulability [1, 2]. The thrombi are mainly divided into two types such as white and red thrombus. The white thrombi, known as mural thrombi, are mainly composed of platelets and fibrin, which often overlay on the injured sites of the arterial walls or cardiac chambers. On the contrary, the red thrombi, being mainly composed of red blood cells and fibrin, often occur in the slower-moving blood of veins [1].

In forensic practice, it is most important to determine the post-infliction intervals of skin wounds or brain injuries, because forensic pathologists are required to judge how wounds or injuries are related to the cause of death [3–12]. Similarly, in cases of pulmonary thromboembolism, forensic pathologists have to determine the age of a thrombus in order to judge the causal relationship between the venous thrombus and trauma. To the best of our knowledge, there are forensic studies on age determination of thrombi [13, 14]. Venous thrombi are resolved by a process of organization and recanalization that is similar to the formation of granulation tissue in healing wounds. The recruitment of inflammatory cells is an important component of both processes. An initial neutrophil population is replaced by monocyte-derived macrophages [15–17] that have the capacity to express a host of chemotactic agents, proteases, and growth factors that orchestrate tissue remodeling and revascularization [18, 19]. Analysis of these sequential phenomena is seemingly useful for staging thrombi.

It had been assumed that the detection of intrathrombus neutrophils and macrophages might give useful information for

determining the age of venous thrombi. Several lines of accumulating evidence established a model of stasis-induced venous thrombi in rodents [20–22]; thus, in the present study, we immunohistochemically examined intrathrombus neutrophils and macrophages using an established model, and discuss its practical suitability as a marker for the age determination of thrombus.

Materials and methods

Antibodies (Abs)

The following monoclonal or polyclonal antibodies (mAb or pAbs) were used in this study: rat anti-mouse F4/80 mAb (Dainippon Pharmaceutical Company, Osaka, Japan), and rabbit anti-myeloperoxidase (MPO) pAbs (Neomarkers, Fremont, CA).

Mice

Specific pathogen-free 8- to 10-week-old male 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 [20–22]. Briefly, mice were deeply anesthetized by 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 ligated with 3-0 silk suture. At 1, 3, 5, 7, 10, 14, and 21 days after IVC ligation, mice were euthanized by an overdose of diethyl ether, and intravenous thrombi were harvested and weighed; thereafter, thrombi were subjected to further analyses. Five animals were investigated at each time interval.

Histopathological and immunohistochemical analyses

Whole of intravenous thrombi obtained at the indicated time intervals after ligation were fixed in 4% formaldehyde buffered with PBS (pH 7.2), and transversely cut at the middle of the thrombus, followed by making paraffin-embedded sections (4 μm thick). After deparaffinization, the sections were stained with hematoxylin and eosin (H&E). Furthermore, immunohistochemical analysis of neutrophils and macrophages was performed, as described previously [23, 24]. 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 non-specific reactions. Thereafter, the sections were reacted with anti-MPO pAbs (Neomarkers) for neutrophils or anti-F4/80 mAb (Dainippon) for macrophages as the primary antibody at 4°C overnight. After incubation with biotinylated secondary antibodies at room temperature for 60 min, immune complexes were visualized using a catalyzed signal amplification system or labeled streptavidin biotin system (DAKO, Kyoto, Japan), according to the manufacturer's instructions.

Morphometry

According to the methods used in previous studies [10, 12, 25, 26], morphometrical analysis was performed for semi-quantitative evaluation of the immunohistochemical findings. Briefly, in each section, five microscopic fields (two central and three peripheral fields) were randomly selected (magnification ×1,000), and the number of neutrophils and macrophages within the thrombi were counted and summed from the five microscopic fields. Moreover, the ratios of neutrophils to macrophages (N/M ratio) were calculated. All measurements were performed blind by two different investigators.

Statistical analysis

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

Results

Thrombus formation

IVC ligation caused intravenous thrombus formation posterior to the ligated point in a time-dependent manner

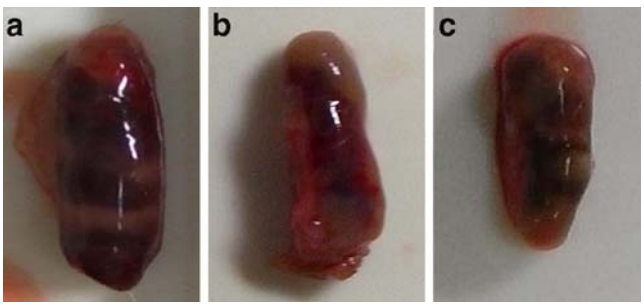

Fig. 1 Macroscopic appearance of venous thrombi after IVC ligation. Representative results are shown here. **a** 5 days, **b** 10 days, and **c** 14 days

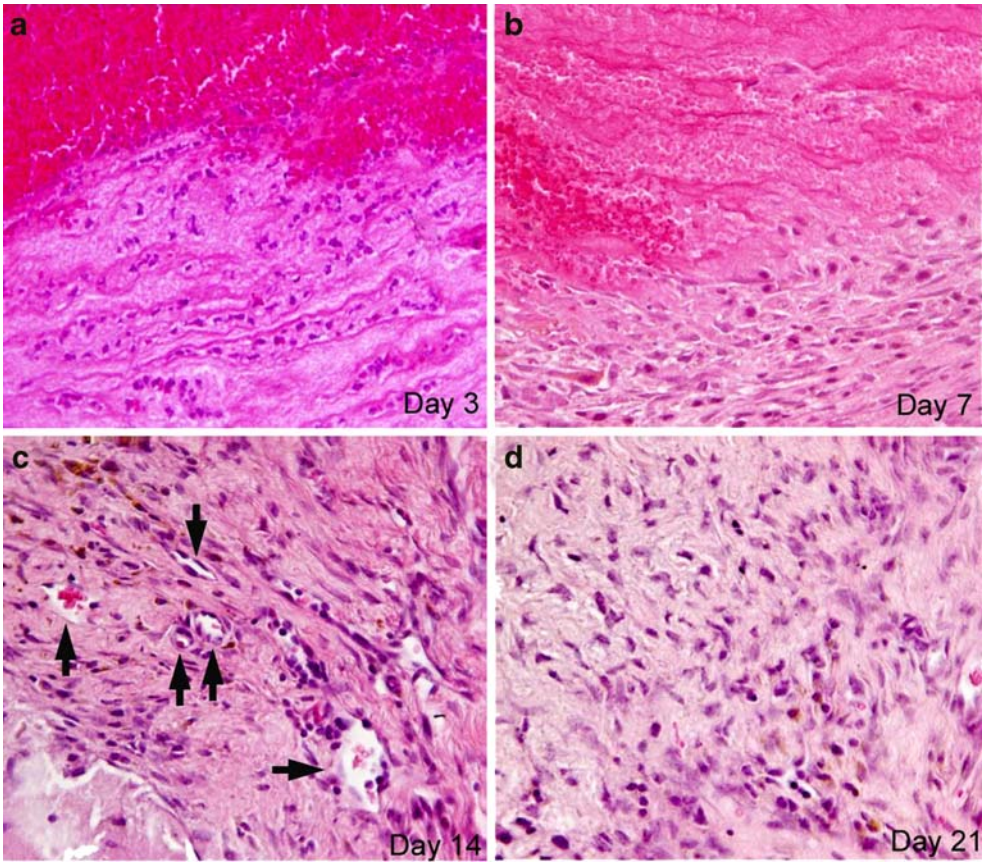

Fig. 2 Histopathological analyses of venous thrombi at the indicated time intervals after IVC ligation. Representative results are shown here. **a** In 3-day-old thrombi, polymorphonuclear leukocytes with erythrocytes were mainly observed. **b** In 7-day-old thrombi, macrophage-like monocytes were seen predominantly. **c** In 14-day-old thrombi, recanalization by angiogenesis (*arrows*) could be confirmed. **d** In 21-day-old thrombi, organization was more evident. Original magnification, ×400

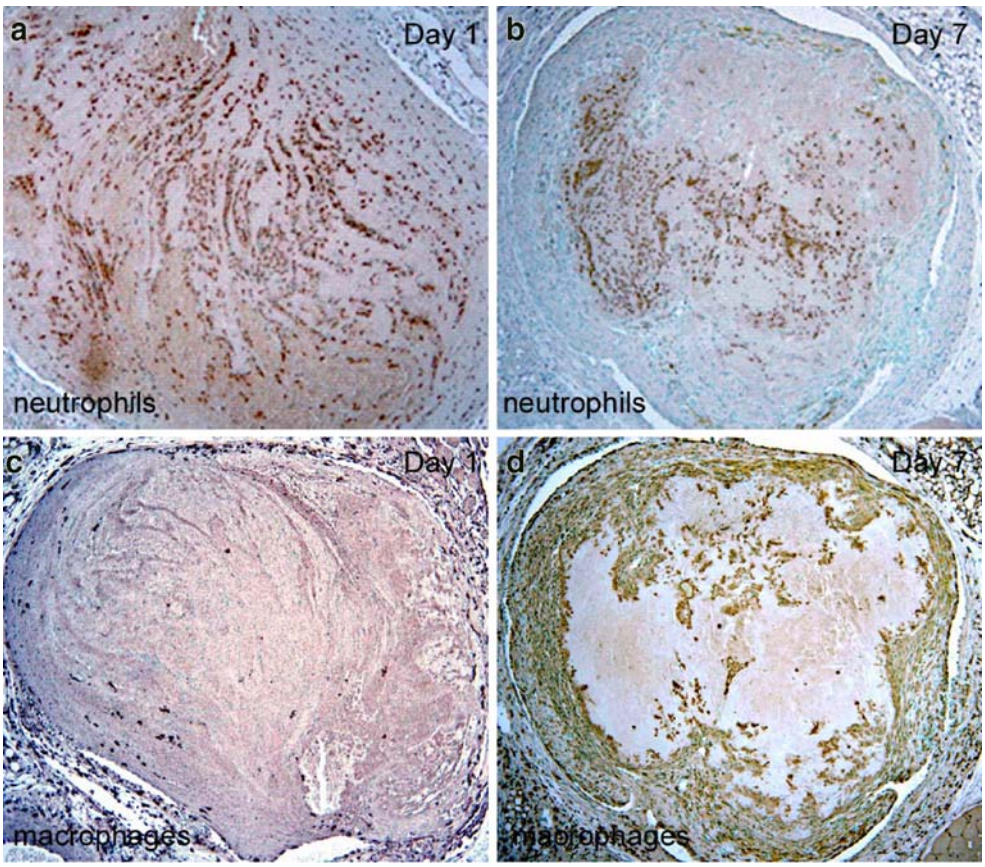

Fig. 3 Immunohistochemical detection of neutrophils (**a** and **b**) and macrophages (**c** and **d**) as described in "Materials and methods". Representative results are shown here. Original magnification, ×100

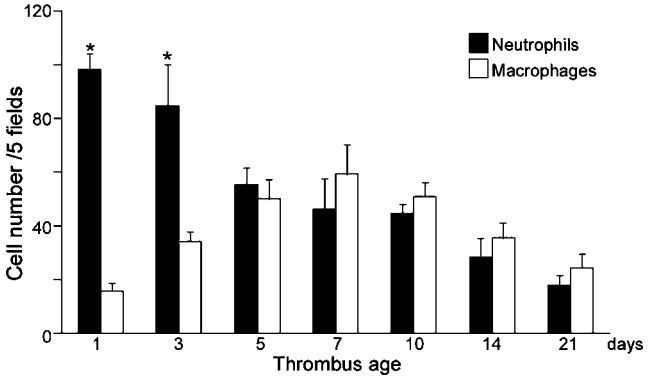

Fig. 4 The numbers of intrathrombus neutrophils and macrophages. All values represent the means±SEM (n=5). *A significant difference was observed statistically (p<0.05)

(Fig. 1) until 5 days after IVC ligation, during which the volume of the thrombus, being reddish in color, increased as evidenced by the thrombus weight of 12.8±0.9 mg; thereafter, the thrombi started to resolve (7.8±0.8 mg at 10 days and 6.2±0.9 mg at 14 days).

Histopathological changes in thrombus formation process

In the early phase after IVC ligation, the thrombi predominantly contained erythrocytes, however, some leukocytes, such as polymorphonuclear cells and monocytes, were also involved in the thrombi. Organic changes such as fibrosis could not be detected in the early phase 1–7 days after IVC ligation (Fig. 2a, b); thereafter, 14 days or more after IVC ligation, organic changes and recanalization could be detected along with the reduction of erythrocytes and leukocytes, compared with thrombi aged 1–7 days (Fig. 2c, d).

Immunohistochemical analyses

Several lines of accumulating evidence demonstrated temporal changes of the intrathrombus leukocyte population

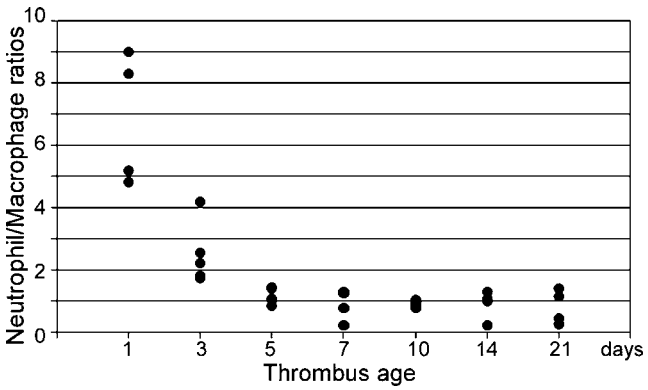

Fig. 5 The distribution of N/M ratios in relation to thrombus age (n=5 in each group). *A significant difference was observed statistically (p<0.05)

[20–22, 27]. MPO-positive neutrophils and F4/80-positive macrophages were detected during the whole course of thrombi after IVC ligation (Fig. 3). In morphometrical analysis, the number of neutrophils was greatest 1 day after IVC ligation and, thereafter, gradually decreased with an increase of the post-ligation interval (Fig. 4). In contrast, the number of macrophages started to increase and peaked 7 days after ligation (Fig. 4). Comparing the numbers of neutrophils and macrophages, the former were predominantly observed 1 and 3 days after ligation (Fig. 4), and N/M ratios were 6.8±1.1 (4.8–9.0) and 2.5±0.4 (1.7–4.2) at 1 and 3 days, respectively (Fig. 5 and Table 1). All thrombus samples aged 1 day (n=5) showed N/M ratios of >4.0, and, four out of the five samples showed a ratio greater than 5.0. In the group of 3-day-old thrombi, all samples (n=5) had ratios of >1.5, among which, four had more than 2.0. At 5 days after ligation, the average N/M ratio was 1.1±0.1. In contrast, after more than 7 days, the number of macrophages was higher than that of neutrophils despite no significant differences, indicating that the ratios were less than 1.0 (Fig. 5).

Discussion

In the field of forensic pathology, there are many studies on the age estimation of skin wounds or brain contusions [28–39]. When the skin or brain is damaged, the tissue repair process starts immediately after injury and is a complicated but well-organized biological phenomenon with concomitant cell migration and proliferation, composed of three different phases, inflammation, proliferation, and maturation. The molecular and cellular pathophysiology of the wound healing process is applied for wound age determination. Recently, the immunohistochemical detection and RNA analyses of several molecules (e.g., extracellular matrix components, growth factors, cytokines, and adhesion molecules) has become a powerful method for forensic practices such as wound age determination or postmortem diagnosis [3–12, 28–45]. To the best of our knowledge, there have been only two forensic studies on thrombus age estimation

Table 1 Means N/M ratios in each thrombus group (n=5)

Age of thrombus	N/M ratio Mean±SEM (range)
1 day	6.8±1.1 (4.8–9.0)
3 days	2.5±0.4 (1.7–4.2)
5 days	1.1±0.1 (0.8–1.4)
7 days	0.9±0.2 (0.2–1.3)
10 days	0.9±0.1 (0.8–1.0)
14 days	0.9±0.1 (0.2–1.3)
21 days	0.8±0.3 (0.2–1.4)

by the use of conventional histopathological methods [13, 14]. Thus, in the present study, immunohistochemical procedures were employed.

The fate of thrombi is similar to the tissue repair process of skin wounds or brain injury. Thrombi undergo some combination of the following four events in the ensuing days to weeks: propagation, embolization, dissolution, and organization and recanalization [1]. In particular, in the process of organization and recanalization, thrombi can induce inflammation and fibrosis, and eventually become recanalized, that is, may reestablish vascular flow, or may be incorporated into a thickened vascular wall. Several lines of accumulating evidence have demonstrated that leukocytes, such as neutrophils and macrophages, are essentially involved in the four processes [19–22].

According to previous studies, the subset proportion of leukocytes recruited at the damaged sites could give significant information about the age determination of a skin wound or brain injury [3, 35]. In those studies, human samples with known post-infliction intervals were employed. There are many studies on the pathophysiology of the development and resolution of intravenous thrombi using a stasis-induced thrombosis model in rodents [19–22], and it is considered that the experimental model mimicked human thrombosis. Thus, the results obtained from this model are seemingly applicable to the age determination of thrombi in forensic practices. The experimental study of Varma et al. [22] demonstrated that the number of intrathrombus neutrophils was greatest at 2 days, and decreased thereafter. The number of macrophages temporally increased, and peaked at 8 days [22]. Our observations were almost consistent with those previous studies.

Considering the mechanism of thrombus formation, intravenous thrombi developed in this stasis-induced model are presumed to be similar to red thrombus rather than white thrombus. Thus, from the viewpoint of forensic pathological application, the present study demonstrated that N/M ratios in thrombi were presumed to be useful for the age determination of red thrombi as follows. In 1- and 3-day-old groups, most samples showed ratios of >2.0, and after more than 5 days, no thrombi had ratios of >2.0. These observations suggest that the N/M ratio of >2.0 would indicate a thrombus age of 1–3 days. Furthermore, in the group of 1-day-old thrombi, four of the five samples had more than 5.0, with a range of 4.8–9.0. In the group of 3-day-old thrombi, N/M ratios ranged from 1.7–4.2; thus, N/M ratios markedly exceeding 5.0 strongly indicate an age of 1 day. Furthermore, there were no significant differences in N/M ratios among the groups of 5–21 days; thus, ratios of 1.0 or less probably indicate an age of more than 5 days.

The previous results [13, 14] were presumed to be less objective than our results, because they were obtained from conventional histopathological methods but not immunohistochemical analyses combined with morphometry. Although further study using human samples obtained from autopsy is, of course, necessary, we provided evidence that immunohistochemical detection of neutrophils and macrophages in venous thrombi could be presumed to give significant information for the age determination of venous thrombi.

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