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Immunohistochemical analysis of von Willebrand factor expression in myocardial tissues from autopsies of patients with ischemic heart disease

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Abstract

von Willebrand factor (VWF) plays a crucial role in hemostasis and thrombosis. VWF is involved in platelet attachment to the subendothelium, serving as a carrier protein for coagulation factor VIII. In this study, myocardial tissues from deceased patients with ischemic heart disease and a mouse model of acute myocardial infarction were subjected to immunohistochemistry to determine VWF expression. We examined 28 neutral formalin-fixed, paraffin-embedded myocardial tissue samples obtained from the autopsies of patients who were diagnosed with ischemic heart disease within 48 h postmortem. Most myocardial cells were negative for VWF, although some cells showed nonspecific positivity. Elevated VWF expression was observed around myocardial cells undergoing remodeling, suggesting that endothelial proliferation occurred at these sites. In contrast, completely fibrotic myocardial foci did not show upregulated VWF expression. Positivity in fibrin deposition and hemorrhagic sites was observed. The same VWF expression characteristics as those observed in the human samples were observed in the mouse model. VWF immunostaining as an endothelial marker may be a useful supplementation to conventional staining techniques that are currently used in the diagnosis of ischemic heart disease in terms of examining the timing of myocardial remodeling in detail and highlighting the remodeling process.

Keywords: von Willebrand factor, immunohistochemistry, endothelial marker, myocardial remodeling, forensic pathology

1. Introduction

von Willebrand factor (VWF), also known as factor VIII-related antigen, plays a critical role in hemostasis and thrombosis. It is involved in platelet attachment to the subendothelium and acts as a carrier protein for coagulation factor VIII [1-6]. It is a large multimeric glycoprotein that is

synthesized and stored as ultra-large forms in Weibel–Palade bodies in vascular endothelial cells.

The stored VWF is released when endothelial cells are activated by various agonists [1-6].

In this study, using immunohistochemistry (IHC), we aimed to examine myocardial remodeling in ischemic heart disease by evaluating VWF expression in myocardial tissues obtained from autopsies of patients.

2. Materials and Methods

2.1.

We evaluated 28 neutral, formalin-fixed, paraffin-embedded myocardial tissue samples obtained from autopsies of patients who had been diagnosed with ischemic heart disease within 48 h postmortem (n = 28; 25 men and 3 women; age, 36–90 years; Table 1). The autopsies were performed under the Act on the Investigation of Cause of Death and Identification of Bodies Handled by the Police. Ischemic heart disease was determined as the cause of death based on the autopsy findings, including myocardial findings, coronary insufficiency, and the circumstances at the time of death. Additionally, for comparison, the myocardia of two cases of asphyxia (62-year-old man and 88-year-old woman) and two cases of drug intoxication (16-year-old woman and 47-year-old woman) were examined. The tissues were evaluated using routine hematoxylin and eosin (HE) staining, and one representative section from each patient was subjected to immunostaining.

2.2. IHC

IHC was performed on 4- μ m-thick paraffin-embedded tissue sections using EnVision Dual Link System-HRP (Agilent Technologies, Santa Clara, CA, USA). The sections were deparaffinized and pretreated on a hot plate with citric acid buffer (pH 6.0, 100°C) for antigen retrieval. After

incubation for 10 min with 3% peroxidase blocking agent, the sections were incubated with anti-VWF mouse monoclonal antibody (1:50, M0616, clone: F8/86; Dako) for 30 min. A brown staining pattern was ultimately obtained using 3,3'-diaminobenzidine tetrahydrochloride (Invitrogen, Grand Island, NY, USA). For leucocyte common antigen (LCA) IHC, an anti-LCA mouse monoclonal antibody (1:200, 2B11-PD7/26; Dako) was used. For mouse tissues, anti-VWF rabbit polyclonal antibody (1:1000, ACR039A; Biocare Medical, Pacheco, CA, USA) was used as the primary antibody. Horseradish peroxidase-conjugated anti-rabbit IgG goat polyclonal antibody (HISTOFINE #424144; Nichirei Corporation, Tokyo, Japan) was used as the secondary antibody.

2.3. Mouse model of acute myocardial infarction (MI)

Nine-week-old C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). To anesthetize the mice, we subcutaneously injected medetomidine hydrochloride (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg). We verified that the anesthetic dose was efficient by abolition of the paw reflex. After hair removal, the surgical site was disinfected using povidone-iodine. Cardiac activity was monitored using electrocardiography until the end of surgery. After placing the mice in the supine position, the skin was incised to expose the larynx, and an 18G indwelling catheter was inserted orally and connected to a ventilator for small animals (MiniVent; Harvard Apparatus, Inc., Holliston, MA, USA) to allow for mechanical ventilation (tidal volume: 200 μ L/stroke; respiratory rate: 150 strokes/min). Then, the side wall of the thorax was opened to expose the heart, and the anterior descending branch of the left coronary artery was blocked using sutures (7-0 Prolene; Ethicon). The intercostal incision was closed using 6-0 silk sutures, as was the overlying skin. Atipamezole hydrochloride (0.15 mg/kg) was then injected subcutaneously. After spontaneous breathing was confirmed, the mice were weaned from mechanical ventilation. The mice

were sacrificed 3 h later, on day 1, and on day 4, myocardial tissues were collected and prepared for staining with HE or Azan.

Nine animals were used for modeling. Four mice were assigned to the 3-h infarction group, of which one died, and the best individual of the remaining three mice was used as the sample. Two mice, respectively, were assigned to the 1-day infarction and 4-day infarction groups, of which one mouse from each group died during the experiment. One untreated animal was sacrificed for the examination of normal myocardium. Animal model production was outsourced to Nissei Bilis (Koga, Japan).

2.4. Study approval

All experimental protocols involving human subjects were approved by the Ethics Committee of Kobe University Graduate School of Medicine (approval number: 1799). For the mouse model, all aspects of the experimental design and procedure were reviewed and approved by the Institutional Ethics and Animal Welfare Committee of Nissei Bilis (approval number: 1910-07).

3. Results

3.1. Basic VWF staining pattern in myocardial tissue

VWF expression was observed in vascular endothelial cells between myocardial cells (Fig. 1A, B). For comparison, the myocardia of two cases of asphyxia and two cases of drug intoxication were stained for VWF, but no significant change in VWF expression was found (data not shown).

3.2. VWF expression in human myocardial tissue

Most of the necrotic myocardial tissues were found to be negative for VWF (Fig. 2A, B), although some necrotic myocardial tissues showed nonspecific positivity (Fig. 2C, D). Elevated VWF

expression was noted around myocardial cells that were undergoing remodeling, which suggested that endothelial proliferation occurred at these sites (Fig. 2C, D). In contrast, VWF expression was not upregulated in the completely fibrotic foci of the myocardium (Fig. 2E, F). Positivity in fibrin deposition and hemorrhagic sites was observed (Supplementary Figure). Contraction band necrosis was not clarified using VWF IHC (data not shown). Myocardial congestion was highlighted because the blood cells and the endothelium were positive for VWF (data not shown). VWF positivity did not change in hypertensive hypertrophic myocardium (data not shown). Samples with leukocyte infiltration were additionally stained for VWF and LCA. There was a tendency for increased expression of VWF where there was high leukocyte infiltration, although there was non-specific staining in the VWF immunohistochemical staining (Supplementary Figure 2).

3.3. VWF expression in the mouse model of acute MI

We investigated whether the phenomena observed in human ischemic heart disease samples would also be observed in a mouse model of acute MI. We noted weaker VWF expression in the mouse myocardium than that in the human samples. No significant change in VWF expression was observed in the tissues 3 h after the creation of the infarct. Hemorrhagic changes were highlighted in the tissues 1 day after the creation of the infarct. Upregulated expression of VWF was observed in the tissues 4 days after the creation of the infarct, when juvenile fibrous tissue appeared (Fig. 3).

4. Discussion

Postmortem diagnosis of sudden cardiac death due to myocardial ischemia is a major concern and a challenge in forensic autopsy [7,8]. Postmortem diagnosis is generally based on macroscopic evidence of myocardial necrosis and routine histological findings [7]. However, ischemic heart

disease is often difficult to definitively diagnose histologically on forensic autopsy. Immunostaining can be used to aid the analysis of cardiac lesions [9,10].

VWF immunoreactivity is employed as an endothelial marker in diagnostic pathology [11-14]. As a forensic application of VWF IHC, the expression pattern of VWF has been examined in human lung tissues [15,16], and VWF positivity in the capillaries has been suggested to be closely related to the death process involving pulmonary microvascular injury [16]. VWF is a highly sensitive but nonspecific marker of skin wound vitality [17]. VWF expression has been detected in brain injury after a post-infliction interval of at least 3 h, demonstrating vascular responses in brain injury, although only a faint reaction was observed in uninjured brain tissues [18]. On the other hand, VWF is a good indicator of cerebral endothelial injury and activation in severe head trauma [19], and the postmortem interval has been reported to be related to urinary VWF concentrations [3].

To determine whether VWF IHC could be applied in the forensic examination of ischemic heart disease, we examined VWF expression in myocardial tissue samples from deceased patients with ischemic heart disease and model mice with acute MI.

The VWF staining results of the human tissues were as follows: 1) most necrotic myocardial tissues were negative for VWF, 2) VWF expression was upregulated during myocardial remodeling following ischemic insult and disappeared once the remodeling process was complete, 3) positivity in fibrin deposition and hemorrhagic sites was observed because the plasma was positive for VWF, 4) myocardial congestion was highlighted via positivity for VWF by blood cells and endothelium, 5) contraction band necrosis was not observed (data not shown), and 6) the pattern of VWF positivity was not different in hypertensive hypertrophic myocardium (data not shown). On the other hand, for comparison, the myocardia of cases of asphyxia and drug intoxication were stained for VWF, but no significant change in VWF expression was found (data not shown).

The limitation of VWF IHC was its nonspecific positivity (Fig. 2D) because plasma was positive for VWF. VWF positivity is difficult to interpret because of its presence in plasma, which can result in high background staining in necrotic and hemorrhagic tissues [20]. Additionally, VWF IHC cannot be used to detect early lesions of acute MI.

Ischemic conditions induce leukocyte recruitment into the heart [21]. We further evaluated the degree of leukocyte recruitment to compare VWF expression with the degree of leukocyte recruitment. When samples with leukocyte infiltration were additionally stained for VWF and LCA, there was a tendency for increased expression of VWF where there was high leukocyte infiltration, although there was non-specific staining in the VWF immunohistochemical staining (Supplementary Figure 2). Thus, leukocyte infiltration may affect VWF expression in an ischemic myocardium.

We also investigated whether similar VWF staining patterns would be observed in a mouse model of acute MI. We found that the same phenomenon, namely, VWF positivity in the site where the repair process occurs, was observed in the early phase of remodeling. However, the late phase of remodeling, when fibrosis is complete, could not be examined because of the high mortality risk in mice. As observed in the human samples, hemorrhagic foci were highlighted by VWF staining in the mice. Early lesions of acute MI (3 h) were not highlighted by VWF IHC (Fig. 3DE).

We previously examined thrombomodulin expression in myocardial tissues of ischemic heart disease [10]. With regard to the behavior of other endothelial markers, such as CD31 and CD34, the CD31 expression level was elevated, and its expression was maintained even in sites with complete remodeling (unpublished data). CD34 expression levels were found to be maintained throughout the remodeling process (unpublished data).

IHC analysis of endothelial markers in ischemic heart disease has been considered useful for diagnosis [10]. In diagnostic pathology, the staining results of several antibodies can be used as an

IHC panel. Although the specific phase of myocardial remodeling that follows ischemic heart disease can be identified using standard HE histology, an IHC panel of endothelial markers may facilitate a more detailed characterization of the specific phase of myocardial remodeling from a different perspective. VWF IHC also highlights the remodeling process, including hemorrhage and fibrin deposition. The time course from initial onset to death can be estimated by evaluating myocardial remodeling, which may be useful in the forensic pathological diagnosis of ischemic heart disease, although it does not directly lead to the diagnosis of ischemic heart disease.

In conclusion, our study suggests that VWF immunostaining as an endothelial marker may be a valuable supplement to conventional staining techniques used in the diagnosis of ischemic heart disease in terms of examining the timing of myocardial remodeling in detail and highlighting the remodeling process.

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Figure legends

Figure 1 Basic von Willebrand factor (VWF) staining pattern in myocardial tissue

VWF expression was observed in vascular endothelial cells between myocardial cells. A: epicardial side, B: endocardial side.

Figure 2 Von Willebrand factor (VWF) expression in myocardial tissues of humans with ischemic heart disease

Hematoxylin and eosin staining (A, C, E) and VWF immunohistochemistry (B, D, F). A, B: VWF expression in necrotic myocardium; necrotic myocardium was negative for VWF. C, D: VWF expression in ongoing remodeling of the myocardium; upregulated VWF expression was observed around the myocardial cells undergoing remodeling, with nonspecific positivity in necrotic myocardium. E, F: VWF expression in completely fibrotic foci was not upregulated.

Figure 3 Von Willebrand factor (VWF) expression in a mouse model of acute myocardial infarction (MI)

Hematoxylin and eosin staining (A, D, G, J), Azan staining (B, E, H, K), and VWF immunohistochemistry (C, F, I, L) were performed to examine myocardial tissues. A–C: Normal mice tissues with weaker VWF expression than that in human samples. D–F: Three hours after induction of acute MI, there was no significant change in VWF expression. G–I: One day after induction of acute MI, there was no fibrosis; hemorrhagic foci were highlighted. J–L: Four days after induction of acute MI, VWF expression was upregulated in juvenile fibrous tissues, with vascularization.

Table 1

Clinical characteristics of the 28 patients with ischemic heart disease, including age, sex, diagnosis at autopsy, relative coronary atherosclerosis, whether resuscitation was performed, whether myocardial necrosis was observed, and estimated age of myocardial necrosis, as well as identification of cases used for the figures. *m*, male; *f*, female; *AMI*, acute myocardial infarction; *IHD*, ischemic heart disease.

Supplementary Figure 1

Myocardial tissue samples of humans with ischemic heart disease. Hematoxylin and eosin staining (A, C) and VWF immunohistochemistry (B, D). A, B: fibrin deposition was highlighted by VWF immunostaining. C, D: hemorrhage was highlighted by VWF immunostaining.

Supplementary Figure 2

Human myocardial tissue samples of ischemic heart disease with leukocyte infiltration. VWF immunohistochemistry (A, C) and LCA immunohistochemistry (B, D). There was a tendency for increased expression of VWF where there was high leukocyte infiltration, although there was non-specific staining in the VWF immunohistochemical staining.

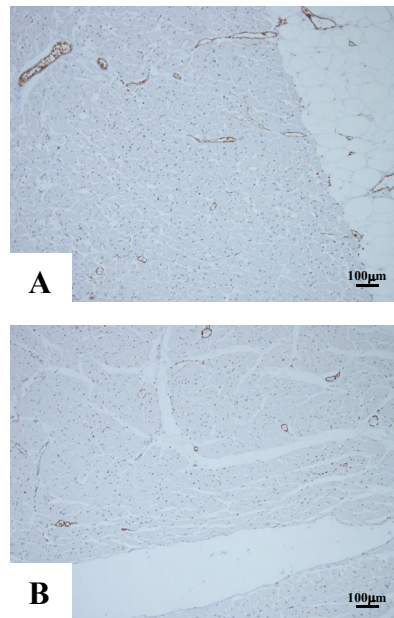


Fig. 1

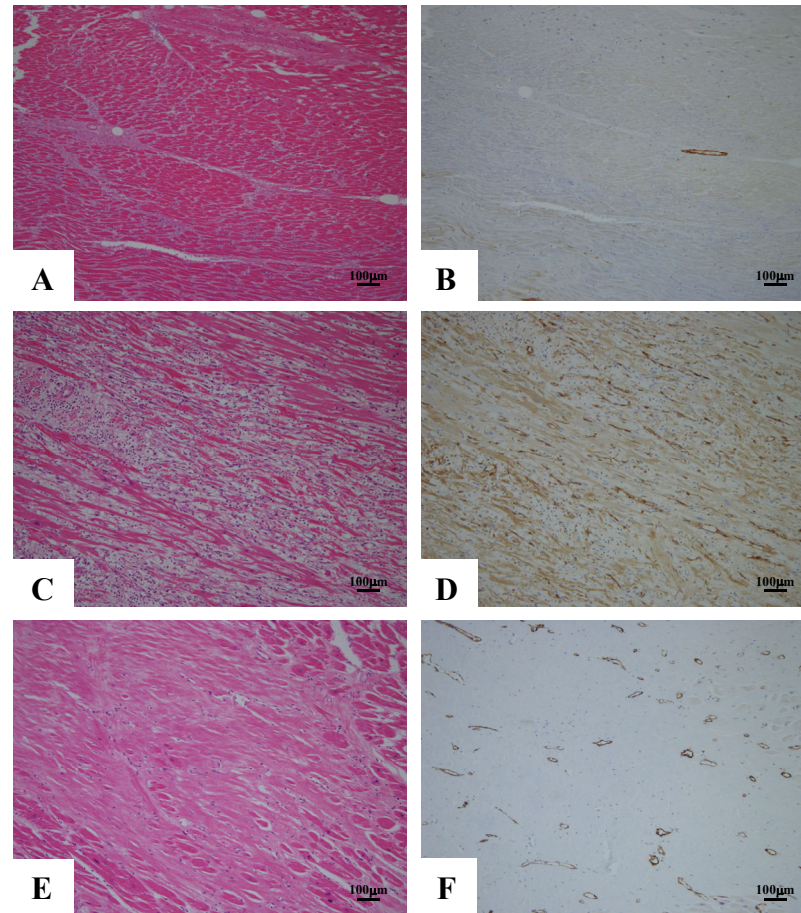


Fig. 2

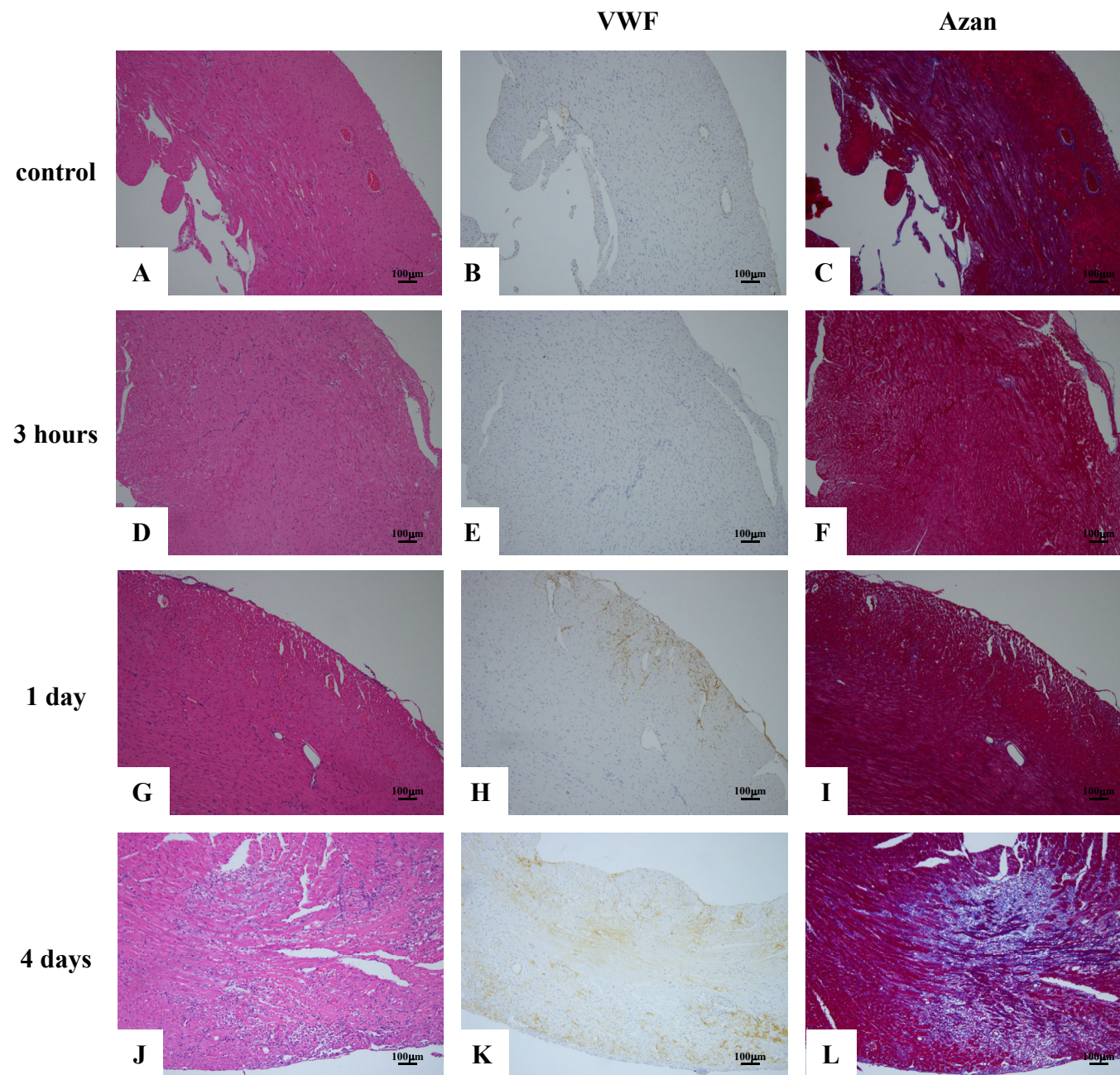
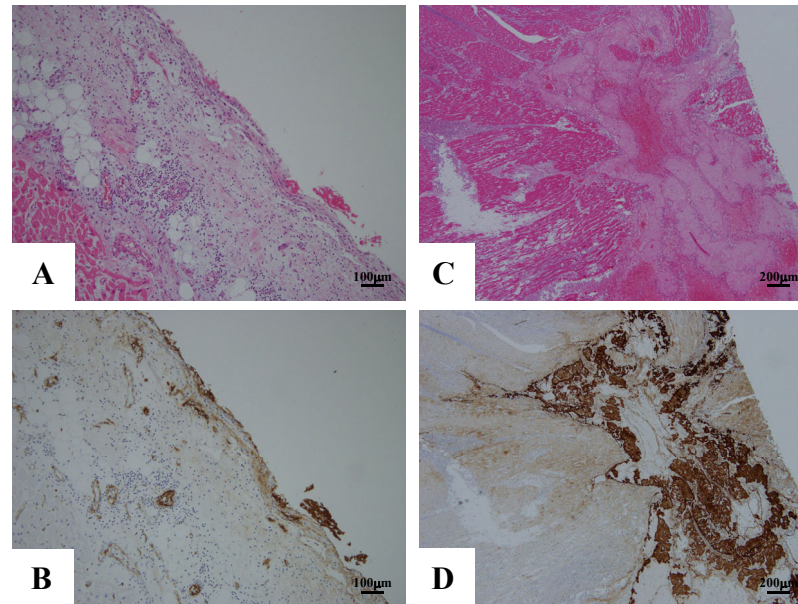
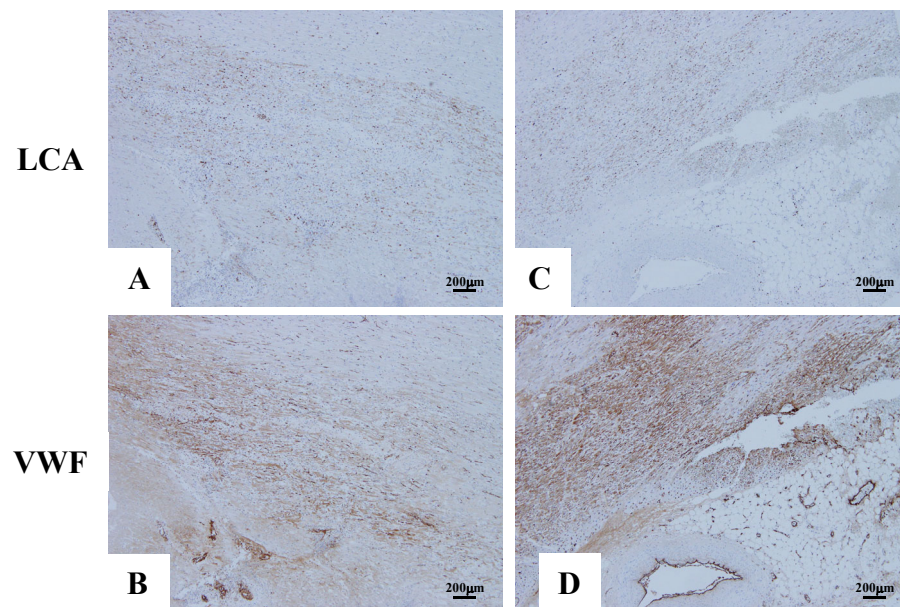


Fig. 3



Supplementary Figure 1



Supplementary Figure 2

Case	age	sex	diagnosis at autopsy	coronary atherosclerosis	resuscitation	myocardial necrosis, age of necrosis	figure
1	63	m	AMI, cardiac tamponade	moderate	○	○ a few days	Fig. 2AB
2	48	m	IHD	moderate			
3	68	m	IHD	severe			
4	54	m	AMI, recurrent	severe			Supplementary Fig. 1CD, Supplementary Fig. 2AB
5	51	m	AMI, recurrent	mild		○ several hours	
6	63	m	IHD	severe			
7	78	m	AMI, cardiac tamponade	severe			
8	49	m	IHD	severe			
9	36	m	IHD	mild	○		
10	47	m	IHD	severe	○		
11	75	m	IHD	mild			
12	75	m	AMI, cardiac tamponade	mild	○	○ a few days	
13	67	m	IHD	moderate			Fig. 2EF
14	75	m	IHD	moderate	○		
15	56	m	IHD	mild			
16	85	m	IHD	mild	○		Fig. 2CD, Supplementary Fig. 1AB, Supplementary Fig. 2CD
17	54	f	IHD	moderate			
18	55	m	AMI, cardiac tamponade	mild		○ a few days	
19	71	m	IHD	mild			
20	54	m	AMI	severe	○		
21	44	m	IHD	mild			
22	90	m	IHD	mild	○	○ several hours	
23	54	m	IHD	mild	○		
24	84	f	IHD	mild	○		
25	42	m	IHD	mild	○		Fig. 1
26	53	m	IHD	moderate			
27	82	f	AMI	mild			
28	60	m	IHD	severe		○ several hours	