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Immunohistochemical analysis of thrombomodulin expression in myocardial tissue from autopsy cases of ischemic heart disease

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Abstract

Thrombomodulin is a transmembrane glycoprotein that is ubiquitously expressed on the surface of vascular endothelial cells. Thrombomodulin exerts its anticoagulant effects by combining with thrombin, activating protein C, and inactivating the coagulation factors FVa and FVIIIa. Clinically, thrombomodulin is also known as a marker of vascular injury because it circulates freely in response to endothelial injury. In this study, myocardial tissue from cases of ischemic heart disease was subjected to immunohistochemistry by thrombomodulin. We examined 40 neutral-formalin-fixed, paraffin-embedded myocardial tissue samples from autopsy cases that were diagnosed with ischemic heart disease (within 48 hours postmortem). Thrombomodulin expression was observed in vascular endothelial cells between myocardial cells and in mesothelial cells of the epicardium. In necrotic myocardium, diffusion of thrombomodulin, which reflected endothelial injury, was observed. Upregulated thrombomodulin expression was observed around myocardial cells under ongoing remodeling, which suggested endothelial proliferation in these locations. Completed fibrotic foci of the myocardium did not show upregulated thrombomodulin expression. In a mouse model of acute myocardial infarction, the same phenomena as that found in human samples were observed by immunohistochemistry of thrombomodulin. Immunostaining of thrombomodulin, as a marker for endothelial injury or myocardial remodeling, may be useful for supplementing conventional staining techniques in the diagnosis of ischemic heart disease in forensic pathology.

Keywords: thrombomodulin, immunohistochemistry, endothelial injury, myocardial remodeling, forensic pathology

1. Introduction

Thrombomodulin, also known as CD141, is a type 1 transmembrane glycoprotein that is mainly expressed on the endothelial cell surface [1]. Thrombomodulin binds to thrombin and forms a 1:1 complex acting as an anticoagulant. Additionally, the thrombin–

thrombomodulin complex activates protein C to produce activated protein C. This process inactivates factors VIIIa and Va in the presence of protein S, thereby inhibiting further thrombin formation [2,3]. Thrombomodulin has a potent anticoagulant effect and is a pivotal determinant of normal coagulation balance [3,4]. Thrombomodulin is ubiquitously expressed on the endothelial cell membranes of arteries, veins, capillaries, and lymph vessels in almost all organs [5]. The placental syncytiotrophoblast shows strong expression of thrombomodulin, where it inhibits thrombin production at sites in contact with maternal blood [5]. Thrombomodulin is also expressed on the surface of platelets, monocytes, dendritic cells, neutrophils, and undifferentiated epithelial cells [6]. Furthermore, thrombomodulin is involved in a wide range of biological processes, affecting tumor cell growth and cancer metastasis [7-10].

Soluble thrombomodulin is circulating in the blood, and it is thought to be caused by cleavage of membrane-bound thrombomodulin expressed on vascular endothelial cells by proteolytic enzymes, such as neutrophil elastase. Clinically, soluble thrombomodulin is a marker of vascular or endothelial injury [11-13].

No previous studies have characterized expression of thrombomodulin in myocardial tissue in forensic cases of ischemic heart disease. Therefore, in this study, we evaluated thrombomodulin expression in myocardial tissue from autopsy cases of ischemic heart disease by immunohistochemistry.

2. Materials and Methods

2.1.

We evaluated 40 neutral-formalin-fixed, paraffin-embedded myocardial tissue samples from autopsy cases that were diagnosed with ischemic heart disease (within 48 hours of postmortem, total n=40; 38 men and 2 women; aged from 36 to 90 years, Table 1). After routine hematoxylin and eosin (HE) staining, one representative section was subjected to immunostaining.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 4-μm tissue sections of paraffin-embedded specimens using EnVision Dual Link System-HRP (Agilent Technologies, Santa Clara, CA, USA). Sections were deparaffinized and pretreated on a hot plate with citric acid buffer (pH 6.0, 100°C) for antigen retrieval. After a 10-minute incubation with 3% peroxidase blocking agent, the sections were incubated with anti-thrombomodulin mouse monoclonal antibody (1:25, clone: 1009; Dako) for 30 minutes. Finally, a brown staining pattern was obtained using 3-3′-diaminobenzidine tetrahydrochloride (Invitrogen, Grand Island, NY, USA). For mouse tissue, anti-thrombomodulin rabbit monoclonal antibody (1:500, clone: EPR18217-209, ab230010; Abcam, Cambridge, UK) was used as the primary antibody. For the secondary antibody, horseradish peroxidase conjugated anti-rabbit IgG (H+L) goat polyclonal antibody was used (HISTOFINE #424144; Nichirei Corporation, Tokyo, Japan).

2.3. *In situ* hybridization for human thrombomodulin mRNA

In situ hybridization was performed with the ISH Reagent Kit (Genostaff, Tokyo, Japan) in accordance with the manufacturer's instructions. The probe was designed from GenBank Accession # NM_000361.2 (Sequence Position: 2131-2918, probe size: 788). Tissue sections (5 μm) were deparaffinized with G-Nox (Genostaff), and rehydrated through an ethanol series and phosphate-buffered saline (PBS). The sections were fixed with 10% neutral-buffered formalin (10% formalin in PBS) for 30 minutes at 37°C, washed in distilled water, placed in 0.2 N HCl for 10 minutes at 37°C, and washed in PBS. The sections were then treated with 25 μg/mL Proteinase K (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 10 minutes at 37°C and washed in PBS. Sections were placed in a Coplin jar containing 1×G-Wash (Genostaff), which was equal to 1×SSC (saline sodium citrate). Hybridization was performed with probes at concentrations of 250 ng/mL in G-Hybo-L (Genostaff) for 16 hours at 60°C.

After hybridization, the sections were washed in 1×G-Wash for 10 minutes at 50°C and in 50% formamide in 1×G-Wash for 10 minutes at 50°C. The sections were then washed twice in 1×G-Wash for 10 minutes at 50°C, twice in 0.1×G-Wash for 10 minutes at 50°C, and twice in TBST (0.1% Tween-20 in TBS) at room temperature (RT). After treatment with 1×G-Block (Genostaff) for 15 minutes at RT, the sections were incubated with anti-Digoxigenin Alkaline Phosphatase conjugate (Roche Diagnostics, Rotkreuz, Switzerland), which was diluted to 1:2000 with ×50G-Block (Genostaff) in TBST, for 1 hour at RT. The sections were washed twice in TBST and then incubated in 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, and 100 mM Tris-HCl, pH 9.5. Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, USA) overnight and then sections were washed in PBS. Sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan) and mounted with G-Mount (Genostaff).

2.4. Thrombomodulin/CD31 double fluorescence staining (cocktail method)

Primary antibodies were a cocktail of anti-thrombomodulin mouse monoclonal antibody (1:50, clone: JC70A, #339M-14; Cell Marque, Rocklin, CA) and anti-CD31 rabbit monoclonal antibody (1:500, clone: EPR3094, ab76533; Abcam). Secondary antibodies were a cocktail of Alexa Fluor 594 anti-mouse IgG (H+L) goat polyclonal antibody (1:500, A11005; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 488 anti-rabbit IgG (H+L) goat polyclonal antibody (1:500, A11034; Invitrogen, Carlsbad, CA, USA). Nuclei were stained with DAPI. On completion of the staining process, the prepared specimens were sealed using Fluoromount (Diagnostics Biosystems, Pleasanton, CA, USA).

2.5. Animal experiments (mouse model for acute myocardial infarction)

C57BL/6J mice aged 9 weeks 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 anesthesia was

efficient by abolition of paw reflex. After hair removal, the surgical site was disinfected using povidone–iodine. Cardiac activity was then monitored by electrocardiography until the end of surgery. After fixing 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) to allow for mechanical ventilation (tidal volume: 200 μL/stroke, respiratory rate: 150 strokes/min). Thereafter, 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. Mice were sacrificed after days 1 and 7, and myocardial tissue was collected and prepared for histological specimens (HE, Azan, immunohistochemistry for thrombomodulin). Two mice were assigned to 1-day infarction and 7-day infarction groups. One of the two mice died during the experiment. To examine normal myocardium, one untreated animal was sacrificed. A total of five animals was used. Animal model production was outsourced to Nissei Bilis (Koga, Japan).

2.6. 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 thrombomodulin staining pattern in myocardial tissue

Thrombomodulin expression was observed in vascular endothelial cells between myocardial cells (Fig. 1A) and in mesothelial cells of the epicardium (data not shown). Using *in situ* hybridization, a positive signal of thrombomodulin mRNA was detected on the endothelium (Fig. 1B). Negative controls prepared by the sense thrombomodulin probe did not display significant signals (Fig. 1C). Double immunofluorescence staining (thrombomodulin and CD31) showed that both proteins were colocalized on endothelial cells (Fig. 1D, E, F).

3.2. Thrombomodulin expression in human myocardial tissue with ischemic heart disease

In necrotic myocardium, diffusion of thrombomodulin expression was observed (Fig. 2A, B). Elevated thrombomodulin expression was observed around myocardial cells under ongoing remodeling, which suggested endothelial proliferation in these locations (Fig. 2C, D). In contrast, completely fibrotic foci of the myocardium did not show upregulated thrombomodulin expression (Fig. 2E, F). In cases of ischemic heart disease within 6 hours of onset with clear borders between infarcted and non-infarcted areas to the naked eye, neither HE staining nor immunostaining for thrombomodulin showed any difference (data not shown). Contraction band necrosis was not clarified by this staining (Supplementary Fig. AB). The positivity of thrombomodulin was not changed in hypertensive hypertrophic myocardium (data not shown). Necrotic myocardium with a patchy distribution, which was relatively difficult to discriminate with HE staining, was highlighted by immunostaining for thrombomodulin (Supplementary Fig. CD). Increased thrombomodulin levels were detected in areas of congestion or interstitial hemorrhage in the myocardium, which presumably occurred as a result of endothelial injury (data not shown).

3.3. Thrombomodulin expression in a mouse model of acute myocardial infarction

We investigated whether the phenomena observed in human ischemic heart disease samples could also be observed in a mouse model of acute myocardial infarction. Diffusion of thrombomodulin protein was observed 1 day after infarction with increased myocardial

eosinophilia and hemorrhage compared with non-infarcted area (Fig. 3E). Increased expression of thrombomodulin protein was observed in tissues 1 week after the creation of the infarct, when juvenile fibrous tissue with vascular growth appeared (Fig. 3H).

4. Discussion

Postmortem diagnosis of sudden cardiac death due to myocardial ischemia is a major concern in forensic autopsy cases [14]. Postmortem diagnosis is generally based on macroscopic evidence of myocardial necrosis and on routine histological findings [14]. However, ischemic heart disease is often difficult and challenging to definitively diagnose histologically on forensic autopsy. The use of immunostaining can aid in the analysis of cardiac lesions [15]. In diagnostic pathology, thrombomodulin is a marker of malignant mesothelioma [16] and is also expressed in urothelial carcinoma [17], but its importance in diagnostic pathology has been declining recently. We examined thrombomodulin expression in myocardial tissue samples from deceased patients with ischemic heart disease and from a mouse model of acute myocardial infarction. We aimed to determine whether thrombomodulin immunohistochemistry for diagnostic pathology can be applied to forensic pathology.

Thrombomodulin is one of the most popular indicators of endothelial injury [18]. Elevated concentration of soluble thrombomodulin are observed in clinical conditions associated with vascular injury, such as atheromatous arterial disease, diabetes mellitus, and various active vasculitis [19,20]. Blood soluble thrombomodulin concentrations are correlated with ischemic heart disease lesions and are a predictor of cardiovascular events [21-26]. With regard to forensic medicine, thrombomodulin levels have been reported by several groups, including changes related to hypothermia [27-30] and time-dependent alterations in expression following traumatic brain injury [31].

Thrombomodulin staining results in the current study were as follows. (1) Fresh infarctions within a few hours of onset were undetectable. (2) The antigen diffused in myocardial necrotic regions. (3) Thrombomodulin expression was increased during myocardial remodeling after ischemic heart disease, and it disappeared once remodeling had finished. (4) Thrombomodulin staining was an effective staining method for detecting myocardial hemorrhage and necrotic lesions that are not easily shown by HE staining. (5) Contraction band necrosis was not observed. (6) The positivity of thrombomodulin was not changed in hypertensive hypertrophic myocardium.

In our study, thrombomodulin expression was elevated in ongoing remodeling foci, which reflected the presence of juvenile fibrous tissue with vascularization. The forensic usefulness of this staining was reported in a study of time-dependent endothelial marker expression after traumatic brain injury [31]. In traumatic brain injury, thrombomodulin positivity was detectable in a cortical contusion with a wound age of 6.8 days at the earliest [31]. This staining reaction is probably related to neovascularization [31].

We examined whether the phenomena observed in human samples could also be observed in mice. We established a mouse model of myocardial infarction and examined the expression pattern of thrombomodulin in mouse myocardium. The same phenomena were observed in the acute and early phases of remodeling. However, the late phase of remodeling, when fibrosis is complete, could not be examined owing to the high risk of death in mice.

With regard to the behavior of other endothelial markers, such as CD31 and CD34, CD31 shows a similar behavior to thrombomodulin, but its expression is maintained even in areas with complete remodeling (unpublished data). CD34 expression levels are continuously maintained throughout the remodeling process (unpublished data). In diagnostic pathology, the staining results of several antibodies are used as a panel for diagnosis. The immunohistochemical panel of these three endothelial markers may allow identification of the specific phase of myocardial remodeling after ischemic heart disease and more detail than that

in conventional methods. Analysis of endothelial dynamics in myocardial tissue from autopsy through immunostaining for thrombomodulin, which is a biomarker of myocardial remodeling, is useful from a different perspective. In conclusion, our observations indicate that immunostaining of thrombomodulin as a marker for endothelial injury or myocardial remodeling may be useful for supplementing conventional staining techniques in the diagnosis of ischemic heart disease.

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Figure legends

Figure 1 Basic thrombomodulin staining pattern in myocardial tissue.

A: Thrombomodulin expression was observed in vascular endothelial cells between myocardial cells. B, C: *In situ* hybridization for thrombomodulin mRNA. B: A positive signal on endothelium was observed (antisense probe). C: Negative controls prepared by the sense thrombomodulin probe did not display significant signals. D, E, F: Double immunofluorescence staining (thrombomodulin and CD31). Human myocardial tissue was subjected to immunofluorescence staining with monoclonal antibodies against thrombomodulin (red) and CD31 (green). Nuclei were stained with DAPI. Both proteins are colocalized on endothelial cells (merge).

Figure 2 Thrombomodulin expression in human myocardial tissue with ischemic heart disease.

Hematoxylin and eosin staining (A, C, E) and thrombomodulin immunohistochemistry (B, D, F) were used. A, B: Thrombomodulin expression in necrotic myocardium. Diffusion of thrombomodulin protein was observed. C, D: Thrombomodulin expression in ongoing remodeling of the myocardium. Upregulated thrombomodulin expression was observed around myocardial cells under ongoing remodeling. E, F: Thrombomodulin expression in completely fibrotic foci, which do not show upregulated thrombomodulin expression.

Figure 3 Thrombomodulin expression in a mouse model of acute myocardial infarction.

Hematoxylin and eosin staining (A, D, G), Azan staining (B, E, H), and thrombomodulin immunohistochemistry (C, F, I) were used. A–C: Normal myocardial tissue. Thrombomodulin expression was observed in vascular endothelial cells between myocardial cells, similar to human samples. D–F: One day after creating acute myocardial infarction. Eosinophilia of the myocardium, with bleeding, was observed. No fibrosis was seen. Diffusion of

thrombomodulin protein was observed. G–I: One week after creating acute myocardial infarction. Thrombomodulin expression was elevated with juvenile fibrous tissue growth with vascularization.

Supplementary Figure

Phosphotungstic acid hematoxylin staining (A), thrombomodulin immunohistochemistry (B, D), and hematoxylin and eosin staining (C) were used. A, B: Contraction band necrosis was not clarified by this staining. C, D: Necrotic myocardium with a patchy distribution, which was relatively difficult to discriminate with HE staining, was highlighted by immunostaining for thrombomodulin (arrows).

Table 1

Clinical information of the forty human cases examined in this study, including age, sex, diagnosis at autopsy, relative coronary atherosclerosis, whether resuscitation was performed, whether myocardial necrosis could be observed, and estimated age of myocardial necrosis, as well as which cases were used for the figure. *m,* male; *f,* female; *AMI,* acute myocardial infarction; *IHD,* ischemic heart disease.

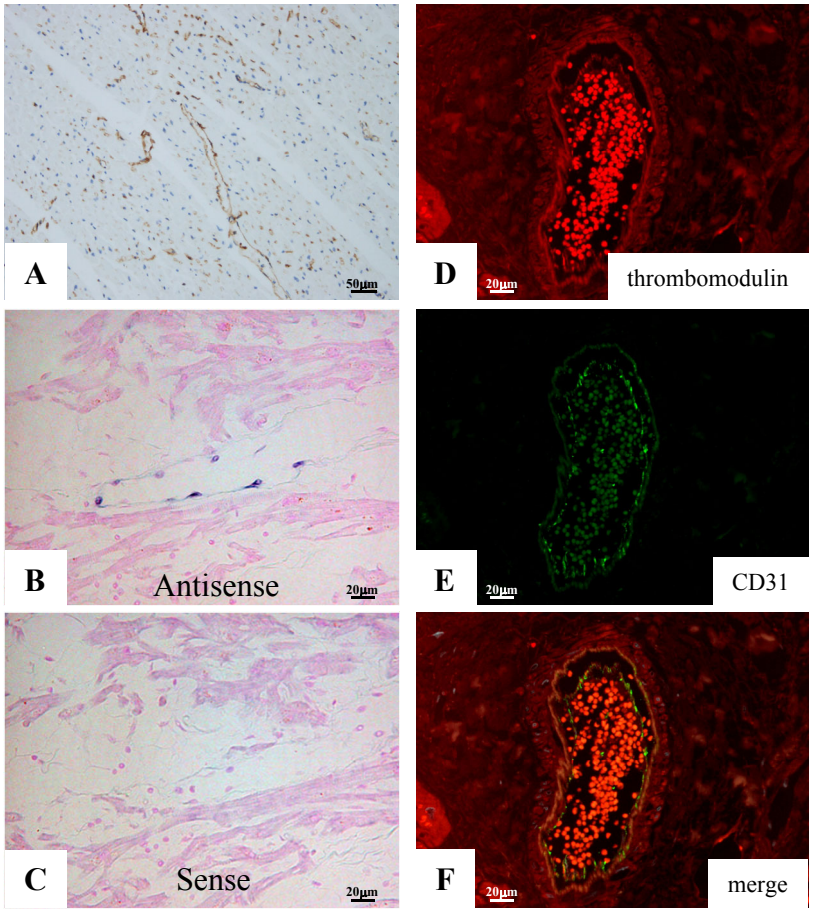
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Fig. 1

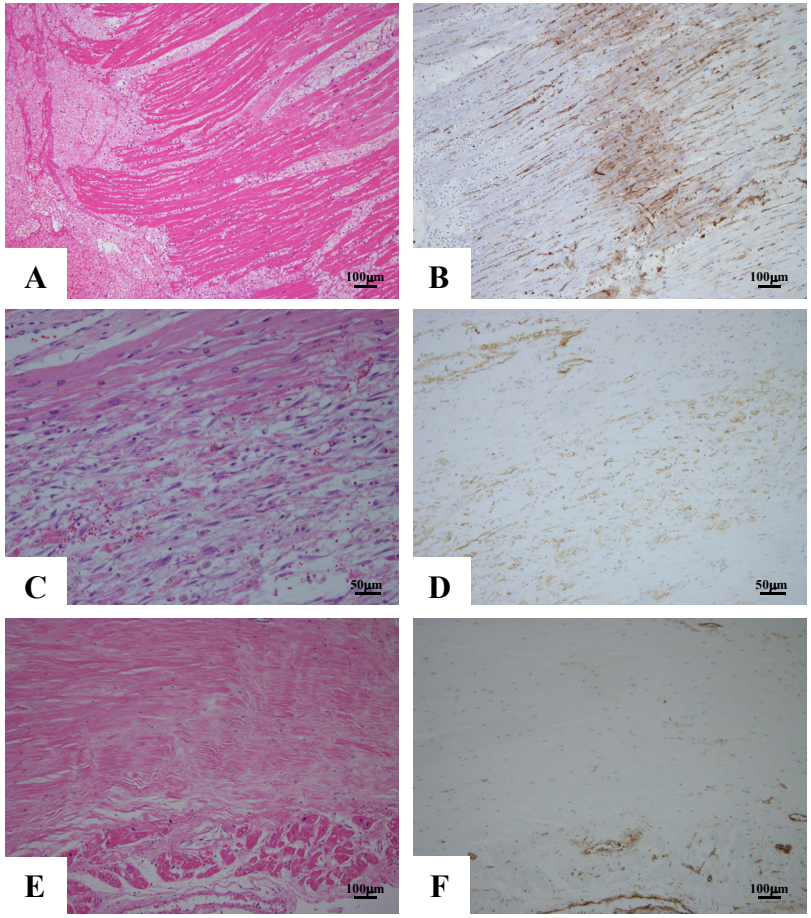


Fig. 2

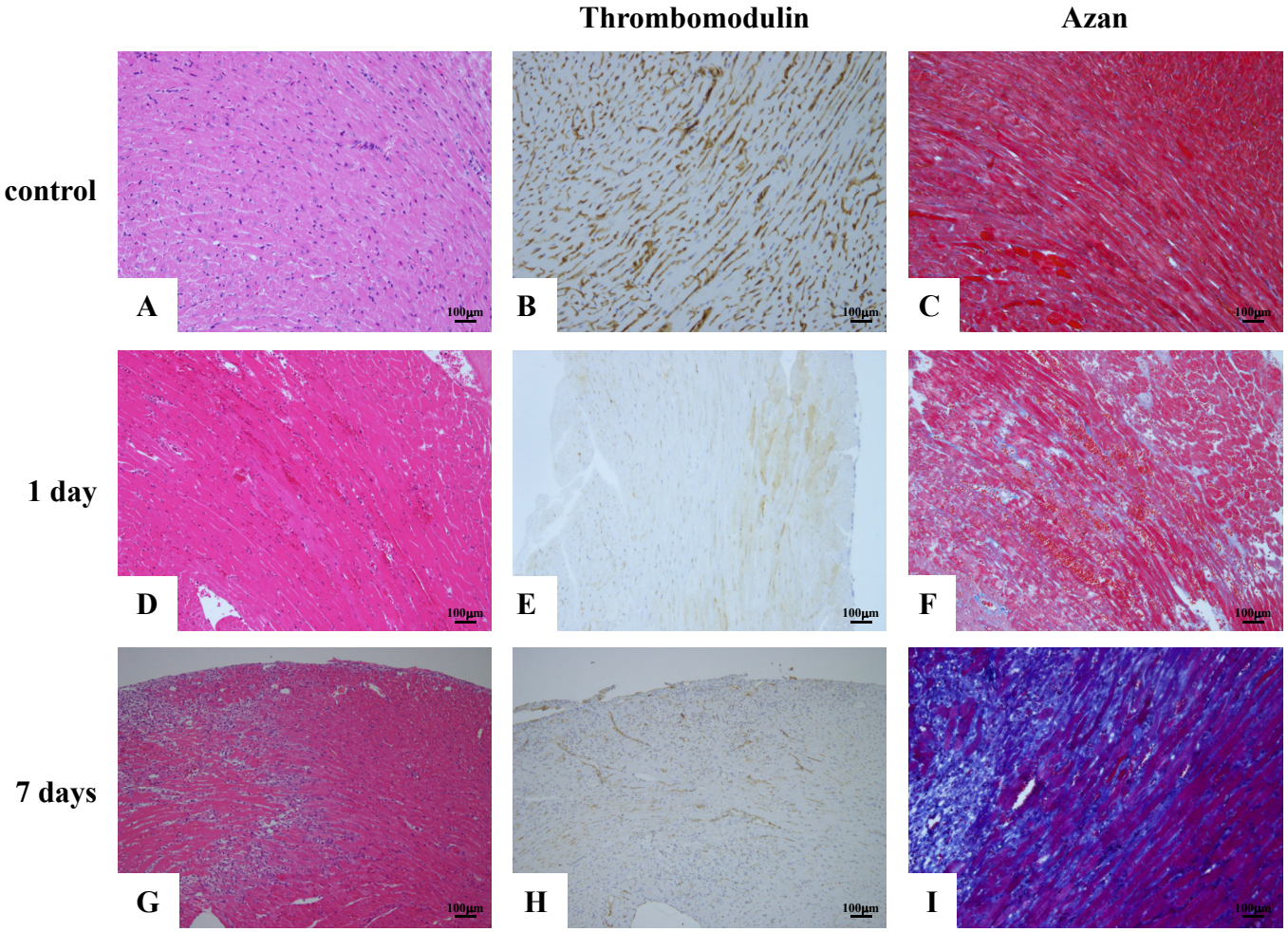
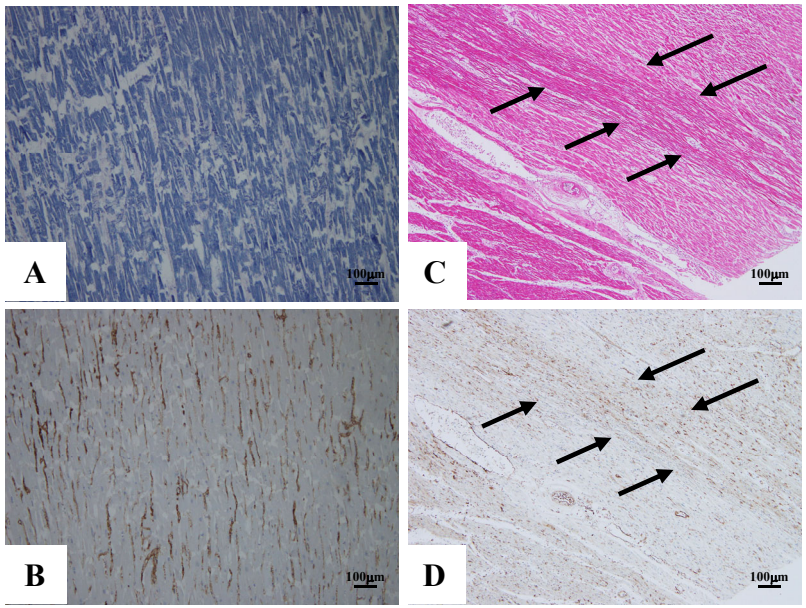


Fig. 3



Supplementary Figure

Case	age	sex	diagnosis at autopsy	coronary atherosclerosis	resuscitation	myocardial necrosis, age of necrosis	figure
1	58	m	AMI	moderate	○		
2	79	m	IHD	severe			
3	48	m	IHD	moderate			
4	45	m	AMI	severe			
5	68	m	IHD	severe			
6	84	m	IHD	severe			
7	67	m	IHD	severe			
8	53	m	IHD	moderate			
9	43	m	IHD	severe	○		
10	54	f	IHD	mild	○		
11	64	m	IHD	severe			
12	46	m	IHD	mild	○		
13	66	m	AMI	severe		○ several hours	
14	54	m	AMI, recurrent	severe			Supplementary Fig. AB
15	51	m	AMI, recurrent	mild		○ several hours	Supplementary Fig. CD
16	82	m	AMI	mild			
17	63	m	IHD	severe			
18	78	m	AMI, cardiac tamponade	severe			Fig. 2ABCD
19	51	m	ami	severe		○ a few days	
20	58	m	IHD	mild	○		
21	49	m	IHD	severe			
22	36	m	IHD	mild	○		
23	47	m	IHD	severe	○		
24	75	m	IHD	mild			
25	75	m	AMI, cardiac tamponade	mild	○	○ a few days	
26	47	m	IHD	severe			
27	67	m	IHD	moderate			
28	75	m	IHD	moderate	○		
29	56	m	IHD	mild			
30	85	m	IHD	mild	○		Fig. 2EF
31	54	f	IHD	moderate			
32	43	m	IHD	mild			
33	55	m	AMI, cardiac tamponade	mild		○ a few days	
34	71	m	IHD	mild			
35	54	m	AMI	severe	○		
36	44	m	IHD	mild			
37	90	m	IHD	mild	○	○ several hours	
38	54	m	IHD	mild	○		
39	55	m	AMI	mild	○		
40	42	m	IHD	mild	○		