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(Citation)
Legal Medicine,54:102003

(Issue Date)
2022-02

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90008913

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

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Declarations of interest: None.

Funding: This work was supported by JSPS KAKENHI (Grant Number JP19K10685) to TK.

Acknowledgments

We thank Kyodo Byori, Inc. (Kobe, Japan), Genostaff Co., Ltd. (Tokyo, Japan), Nissei Bilis Co., Ltd. (Koga, Japan), and Kobe University Hospital Advanced Tissue Staining Center (Kobe, Japan) for their excellent technical assistance. We also thank Michelle Kahmeyer-Gabbe, PhD, from Edanz (https://jp.edanz.com/ac), for editing a draft of this manuscript.

Abstract

Vimentin is a type III intermediate filament cytoskeletal protein that is expressed mainly in cells of mesenchymal origin and is involved in a plethora of cellular functions. In this study, myocardial tissues from patients with ischemic heart disease and a mouse model of acute myocardial infarction were subjected to immunohistochemistry for vimentin. We first examined 26 neutral formalin-fixed, paraffin-embedded myocardial tissue samples from autopsies of patients that were diagnosed with ischemic heart disease within 48 hours postmortem. Myocardial cells were negative for vimentin, whereas non-myocardial cells, including vascular endothelium, vascular smooth muscle, fibroblasts, nerve fibers, adipocytes and mesothelial cells, showed positivity. Elevated vimentin expression was observed around myocardial cells undergoing remodeling, suggesting fibroblastic and endothelial proliferation in these locations. By contrast, myocardial foci that were completely fibrotic did not show upregulated vimentin expression. Inflammatory foci including macrophages and neutrophils were clearly visualized with vimentin immunostaining. The same vimentin expression phenomena as those found in human samples were observed in the mouse model. Our study indicates that immunostaining of vimentin as a marker for myocardial remodeling and the dynamics of all non-myocardial cell types may be useful for supplementing conventional staining techniques currently used in the diagnosis of ischemic heart disease.

Keywords: vimentin, immunohistochemistry, mesenchymal marker, myocardial remodeling, forensic pathology

1. Introduction

Vimentin is a type III intermediate filament cytoskeletal protein comprising desmin, glial fibrillary acidic protein (GFAP), peripherin and vimentin [1,2], and is mainly expressed in cells of mesenchymal origin [3]. The vimentin filament network provides architectural support for cells and contributes to the positioning and function of cellular organelles [4-6].

Vimentin filaments are involved in many cellular activities, interacting with other cytoskeletal components to modulate their function, and integrating cellular mechanical functions, including cell migration, adhesion and division [7-9]. Vimentin also plays roles in viral infection and the immune response [3,10].

In this study, we aimed to elucidate myocardial remodeling in ischemic heart disease by evaluating vimentin expression in myocardial tissue from autopsy cases using immunohistochemistry (IHC).

2. Materials and Methods

2.1.

We evaluated 26 neutral formalin-fixed, paraffin-embedded myocardial tissue samples from autopsy cases of patients that were diagnosed with ischemic heart disease within 48 hours postmortem (n = 26; 23 men and 3 women; age range, 36 to 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 autopsy findings, including myocardial findings and coronary insufficiency, and the circumstances at the time of death. After routine hematoxylin and eosin (HE) staining examining a specimen of the entire circumference of the heart or several specimens corresponding to the coronary perfusion zone, one representative section from each patient was subjected to immunostaining.

2.2. IHC

IHC 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 a 3% peroxidase blocking agent, the sections

were incubated with anti-vimentin mouse monoclonal antibody (1:10, N1521, clone: V9; Dako) for 30 minutes. A brown staining pattern was ultimately obtained using 3,3′-diaminobenzidine tetrahydrochloride (Invitrogen, Grand Island, NY, USA). For CD68 and myeloperoxidase (MPO) immunohistochemistry, anti-CD68 mouse monoclonal antibody (HISTOFINE #413791; Nichirei Corporation, Tokyo, Japan) and anti-MPO mouse monoclonal antibody (HISTOFINE #413801; Nichirei Corporation, Tokyo, Japan) were used.

For mouse tissue, anti-vimentin mouse monoclonal antibody (1:500, GTX629743, clone: GT7812; GeneTex, Irvine, CA, USA) was used as the primary antibody. For the secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG goat polyclonal antibody was used (HISTOFINE #414321; Nichirei Corporation, Tokyo, Japan).

2.3. Vimentin/CD31 double fluorescence staining

Primary antibodies were anti-human CD31 mouse monoclonal antibody (1:100, clone: JC70A, NCL-L-CD31-607; Leica Biosystems, Newcastle upon Tyne, UK) and anti-human vimentin mouse monoclonal antibody (1:500, clone: V9, NCL-L-VIM-V9; Leica Biosystems, Newcastle upon Tyne, UK). Secondary antibodies were Opal polymer HRP Ms+Rb (NEL8100001KT, Akoya Biosciences, Marlborough, MA, USA) for CD31 and Alexa Fluor 594 anti-rabbit IgG (H+L) goat polyclonal antibody (1:500, A11005; Invitrogen, Carlsbad, CA, USA) for vimentin. 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. Mouse model of acute myocardial infarction (MI)

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 the anesthesia was

efficient by abolition of the 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 securing 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 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 3 hours later on days 1 and 7, and myocardial tissue was collected and prepared for staining of histological specimens with HE or Azan, and IHC for vimentin. Nine animals were used for modeling: four mice were assigned to the 3-hour infarction group, of which one died, and the best of the remaining three mice was used as the sample; two mice each were assigned to the 1-day infarction and 7-day infarction groups, of which one mouse from each group died during the experiment; and one untreated animal was sacrificed for the examination of normal myocardium. 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 vimentin staining pattern in myocardial tissue

Myocardial cells were negative for vimentin, whereas non-myocardial cells, including vascular endothelium, vascular smooth muscle, fibroblasts, nerve fibers, adipocytes and mesothelial cells (not shown), showed positivity (Fig. 1A, B).

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

Necrotic myocardium was negative for vimentin (Fig. 2A, B). Elevated vimentin expression was observed around myocardial cells that were undergoing remodeling, which suggested that fibroblastic and endothelial proliferation was taking place in these locations (Fig. 2C, D). By contrast, upregulated vimentin expression was not observed in completely fibrotic foci of the myocardium (Fig. 2E, F).

In the inflammatory foci of the post-MI epicardium, infiltrating macrophages and fibroblasts were positive for vimentin (Fig. 3A, B, C). Neutrophilic infiltration of necrotic myocardium was also detected using IHC for vimentin (Fig. 3D, E, F).

Contraction band necrosis was not clarified by vimentin IHC (Supplementary Fig. 1A, B). Myocardial congestion was highlighted because blood cells and endothelium are positive for vimentin (Supplementary Fig. 1C, D). In foci with ongoing remodeling including fibroblastic and endothelial proliferation, double immunofluorescence staining for vimentin and CD31 showed that some of the vimentin-positive cells were CD31-positive endothelial cells (Supplementary Fig. 2). Positivity for vimentin did not change in hypertensive hypertrophic myocardium (data not shown).

3.3. Vimentin expression in a mouse model of acute MI

Next we investigated whether the phenomena observed in human ischemic heart disease samples would also be seen in a mouse model of acute MI. Similar to the human samples, there was positive vimentin expression in the vascular endothelial cells between myocardial

cells. No significant change in vimentin expression was observed in tissues 3 hours after the creation of the infarct. Infiltration of inflammatory cells was highlighted in tissues 1 day after the creation of the infarct. Increased expression of vimentin protein was observed in tissues 7 days after the creation of the infarct, when juvenile fibrous tissue appeared (Fig. 4).

4. Discussion

Postmortem diagnosis of sudden cardiac death due to myocardial ischemia is a major concern and a current challenge in forensic autopsy [11,12]. Postmortem diagnosis is generally based on macroscopic evidence of myocardial necrosis and routine histological findings [11]. However, ischemic heart disease is often difficult and challenging to definitively diagnose histologically on forensic autopsy. The use of immunostaining can aid the analysis of cardiac lesions [13,14].

For several decades, vimentin positivity was used as a marker for mesenchymal cells; nowadays, it is generally considered inconclusive for pathological diagnosis [15]. There is little diagnostic significance in using this antibody apart from limited specific applications, such as distinguishing clear cell renal cell carcinoma from mimics, or endocervical from endometrial adenocarcinoma as part of an IHC panel [16,17].

With an aim to determine whether vimentin IHC could be applied in forensic pathology, we examined vimentin expression in myocardial tissue samples from deceased patients with ischemic heart disease and model mice with acute MI. Analyses of vimentin expression in forensic medicine have been reported by several groups; for example, immunoreactivity for vimentin was investigated in traumatic and hypoxic brain injury [18-20]. Usability of vimentin IHC in forensic samples was even reported to be effective in cases of postmortem decomposition [21]. Vimentin has stable antigenicity and its immunostaining is easy to perform [21,22].

Additionally, vimentin plays a significant role in the transformations in adhesion and motility that occur during the epithelial-mesenchymal transition (EMT), an important developmental process in which epithelial cells acquire a mesenchymal cell phenotype [23-25]. Vimentin expression is currently used as an EMT marker in cancer biology [24].

Cardiac tissue comprises cardiac myocytes and non-myocyte cells, without an epithelial component. Vimentin is a broad, commonly used marker for cardiac fibroblasts [26,27]. Vimentin IHC stains fibroblasts as well as all other non-myocardial cells, including vascular smooth muscle, vascular endothelium, nerve fibers and blood cells, and can be used to clarify the presence of tissue remodeling of the myocardium. Because inflammatory cells are also vimentin-positive, the dynamics of active fibroblasts and the inflammatory process can be clarified simultaneously. Regarding the evaluation of fibrotic areas following an ischemic insult, activated fibrotic nests containing juvenile fibroblasts can be visualized more clearly using vimentin than with conventional special stains such as Azan.

Vimentin staining results in our human postmortem tissues were as follows: 1) necrotic myocardium was negative for vimentin; 2) vimentin expression was increased during myocardial remodeling following ischemic insult, and disappeared once the remodeling process was finished; 3) inflammatory foci, including macrophages and neutrophils, were made more visible by vimentin staining compared with HE staining; 4) myocardial congestion was highlighted via positivity for vimentin by blood cells and endothelium; 5) contraction band necrosis was not observed; 6) the pattern of vimentin positivity was not different in hypertensive hypertrophic myocardium (data not shown).

We also investigated whether the vimentin staining patterns observed in human samples would be found in a mouse model of acute MI. Indeed, the same phenomena, namely vimentin positivity in the area where fibroblasts appear and the repair process takes place, were 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 risk of death in mice. As observed in human samples, inflammatory foci were highlighted by vimentin staining. Early

lesion of acute MI was not highlighted by vimentin IHC.

IHC analysis of endothelial markers in ischemic heart disease has been suggested to be useful [14]. In diagnostic pathology, a staining results of several antibodies are used as a panel. Although the specific phase of myocardial remodeling that follows ischemic heart disease can be identified by standard HE histology, an IHC panel of mesenchymal markers including fibroblasts and endothelium may facilitate a more detailed characterization of the specific phase of myocardial remodeling. Furthermore, analysis of mesenchymal dynamics in myocardial tissue through immunostaining for vimentin may be useful from a different perspective. The time course from initial onset to death can be estimated from evaluations of myocardial remodeling, which may be useful in the forensic pathological diagnosis of ischemic heart disease. This study has reaffirmed vimentin immunostaining as a useful mesenchymal adjunctive marker for the visualization of myocardial remodeling in ischemic heart disease.

In conclusion, our study suggests that that immunostaining of vimentin as a marker for myocardial remodeling and the dynamics of all non-myocardial cells may be a valuable supplement to conventional staining techniques used in the diagnosis of ischemic heart disease.

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Figure legends

Figure 1 Basic vimentin staining pattern in myocardial tissue.

Myocardial cells were negative for vimentin (A); non-myocardial cells, including vascular endothelium, vascular smooth muscle (S), fibroblasts, nerve fibers (N), adipocytes, and mesothelial cells (not shown) showed positivity (B). (AB: magnification x100)

Figure 2 Vimentin expression in human myocardial tissue with ischemic heart disease (positivity in remodeling foci)

Hematoxylin and eosin staining (A, C, E) and vimentin immunohistochemistry (B, D, F) were used. A, B: Vimentin expression in necrotic myocardium; necrotic myocardium was negative for vimentin. C, D: Vimentin expression in ongoing remodeling of the myocardium; upregulated vimentin expression was observed around myocardial cells undergoing remodeling. E, F: Vimentin expression in completely fibrotic foci was not upregulated. (A-F: magnification x100)

Figure 3 Vimentin expression in human myocardial tissue with ischemic heart disease (positivity in inflammatory foci)

Results of hematoxylin and eosin staining (A, D), vimentin immunohistochemistry (C, F), CD68 (B) and myeloperoxidase (MPO) immunohistochemistry (E). A–C: In inflammatory foci of post-myocardial infarction epicardium, macrophages and fibroblasts that appeared within the area were positive for vimentin. D–F: Neutrophils infiltrating necrotic myocardium were positive for vimentin. (ABC: magnification x40, DEF: magnification x100)

Figure 4 Vimentin 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 vimentin immunohistochemistry (C, F, I, L) were used to examine myocardial tissues. A–C: Normal tissue, with vimentin expression in vascular endothelial cells between myocardial cells, similar to human samples. D–F: 3 hours after inducing acute MI, there was no significant change in vimentin expression. G–I: 1 day after inducing acute MI, there was no fibrosis; infiltration of inflammatory cells are highlighted (arrows). J–L: 1 week after inducing acute MI, there was elevated vimentin expression in juvenile fibrous tissue growth with vascularization. (A-L: magnification x100)

Table 1

Clinical characteristics of the 26 study patients with ischemic heart disease.
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 identification of cases used for the figures.
m, male; *f,* female; *AMI,* acute myocardial infarction; *IHD,* ischemic heart disease

Supplementary Figure 1

Tissue samples of human myocardium with ischemic heart disease stained with phosphotungstic acid hematoxylin (A), vimentin immunohistochemistry (B, D), and hematoxylin and eosin (C). A, B: Contraction band necrosis was not visualized by immunostaining for vimentin. C, D: Myocardial congestion was highlighted by immunostaining for vimentin. (AB: magnification x100, CD: magnification x40)

Supplementary Figure 2

Double immunofluorescence staining for vimentin and CD31. Human myocardial tissue with fibroblastic and endothelial proliferation (A: hematoxylin and eosin staining) subjected to immunofluorescence staining with monoclonal antibodies against CD31 (B: green) and vimentin (C: red). Nuclei were stained with DAPI. Some of the vimentin-positive cells were also CD31-positive endothelial cells (D: merging of B and C). (A-D: magnification x100)

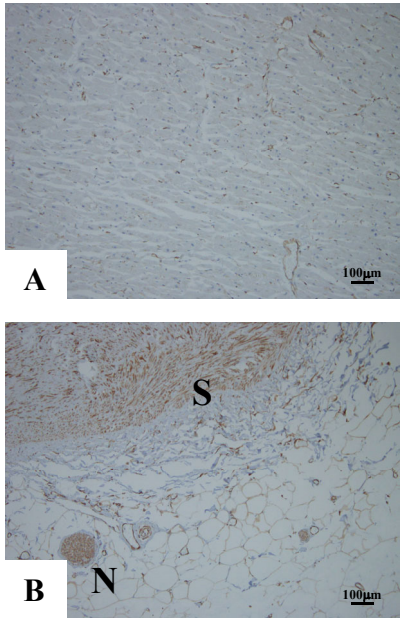


Fig. 1

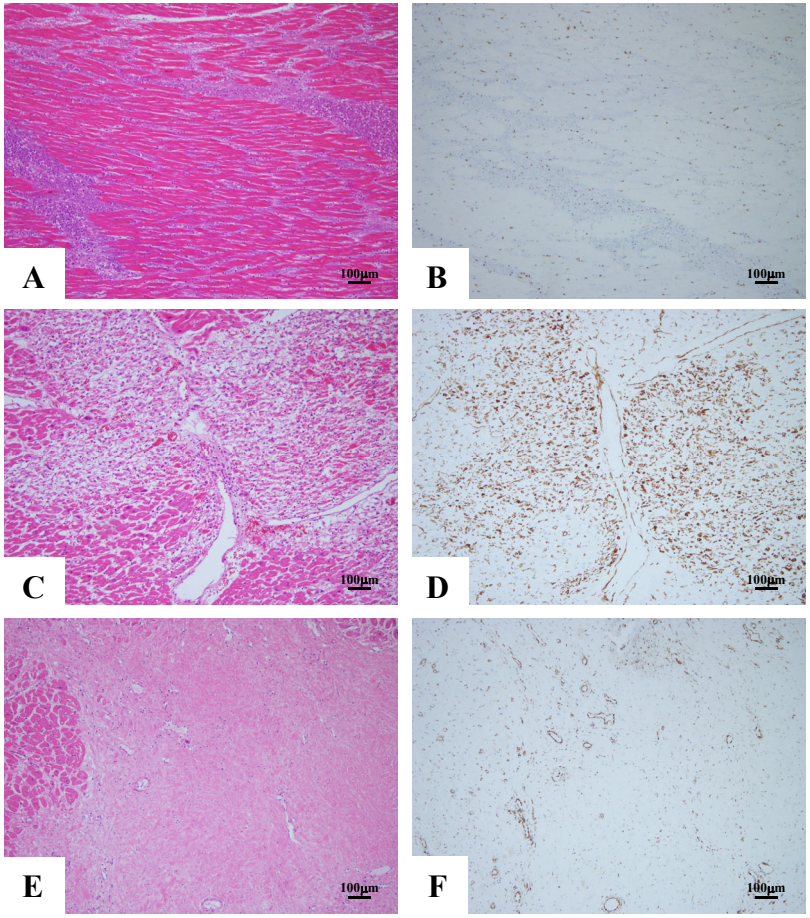
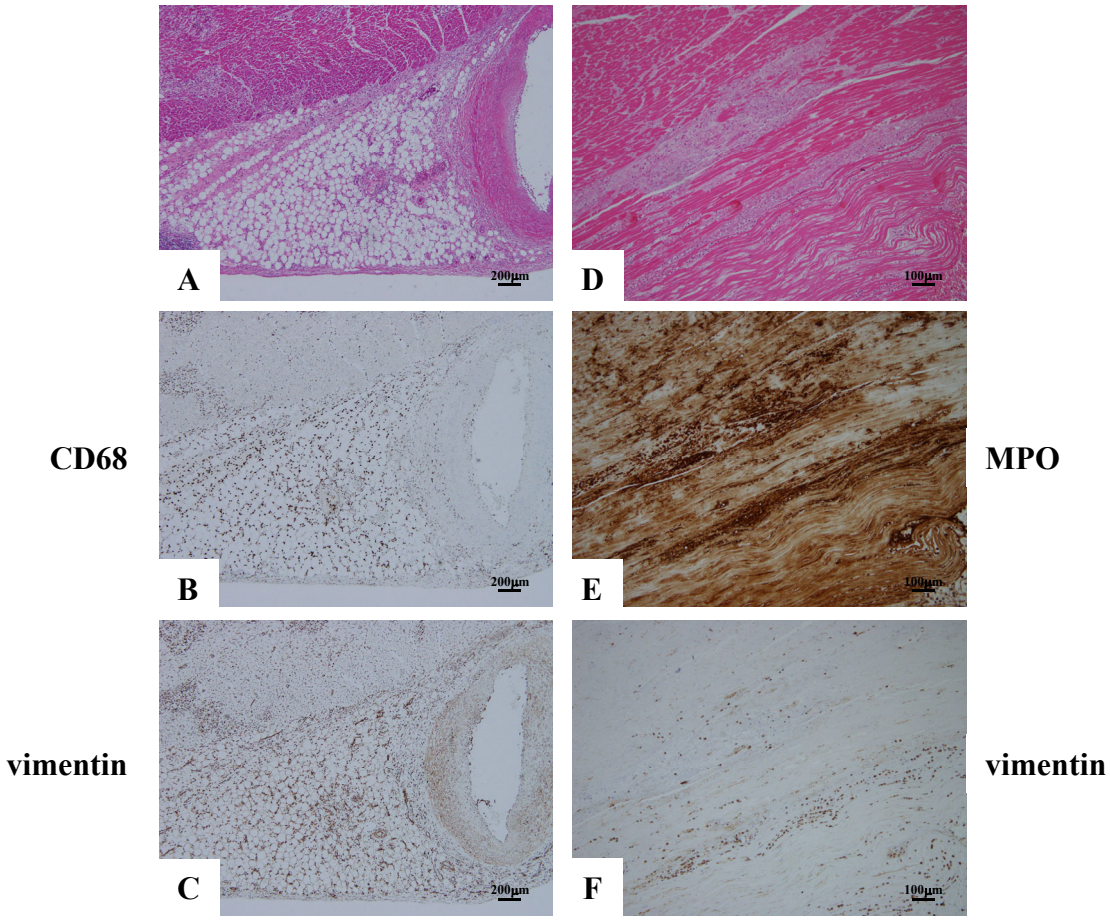


Fig. 2


A
200μm
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CD68
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E
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Fig. 3

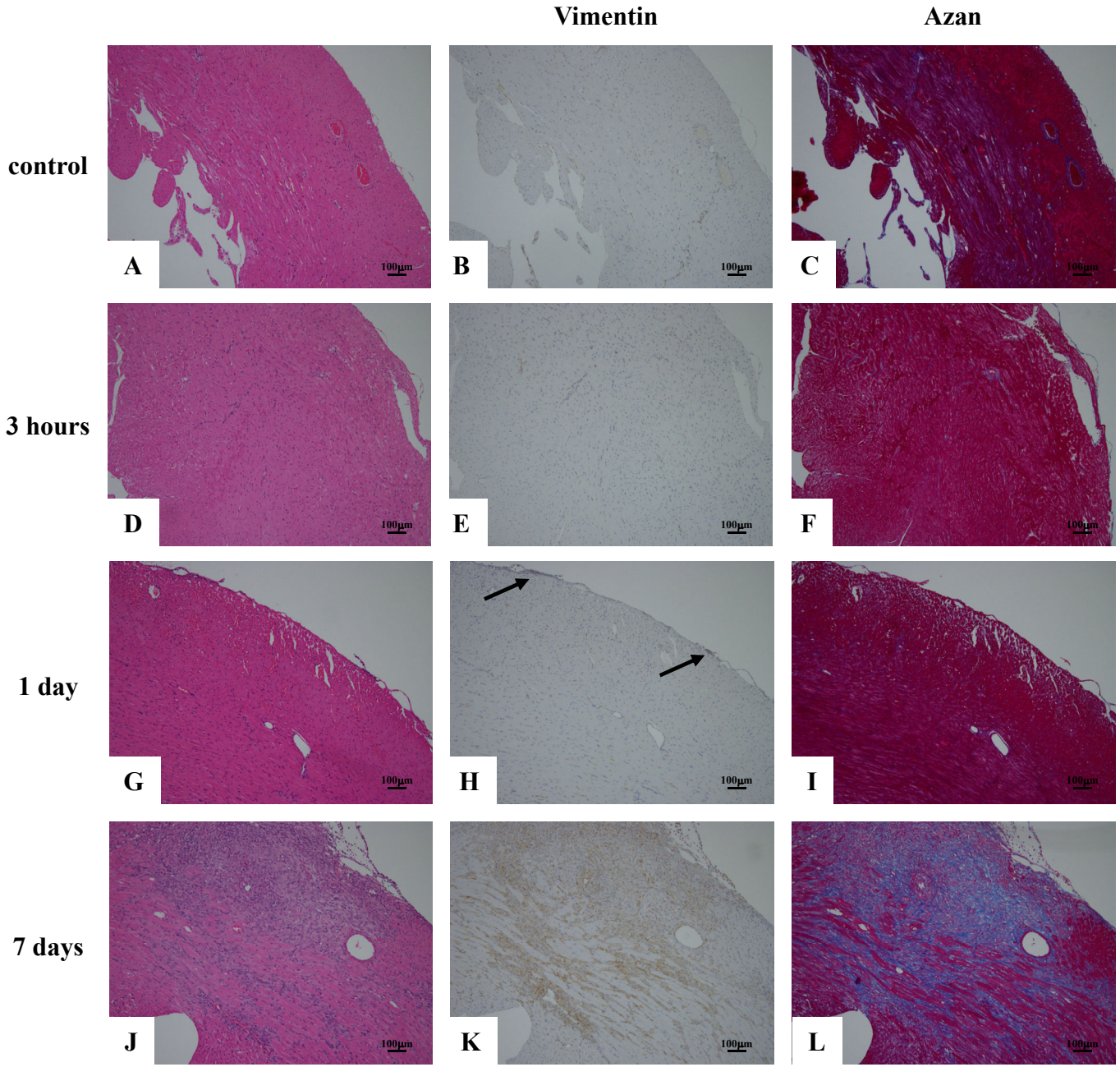
Vimentin
Azan
control
3 hours
1 day
7 days
A
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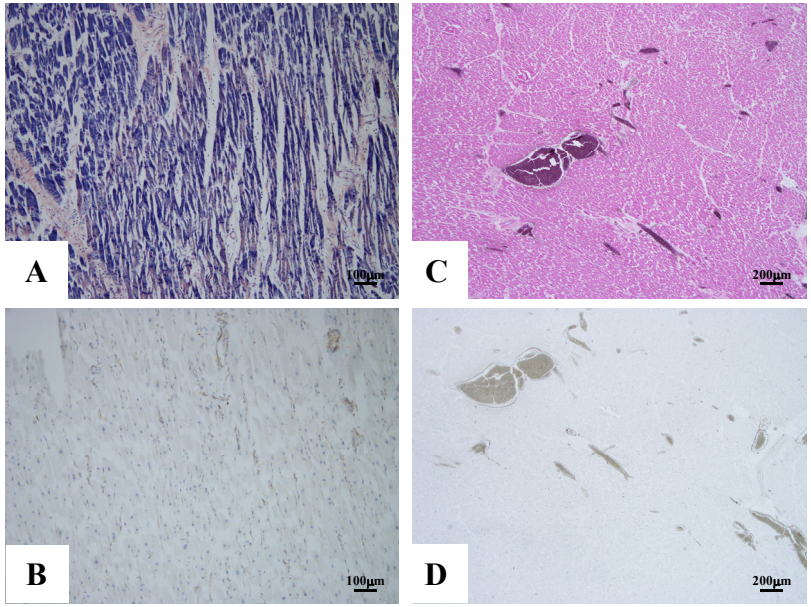
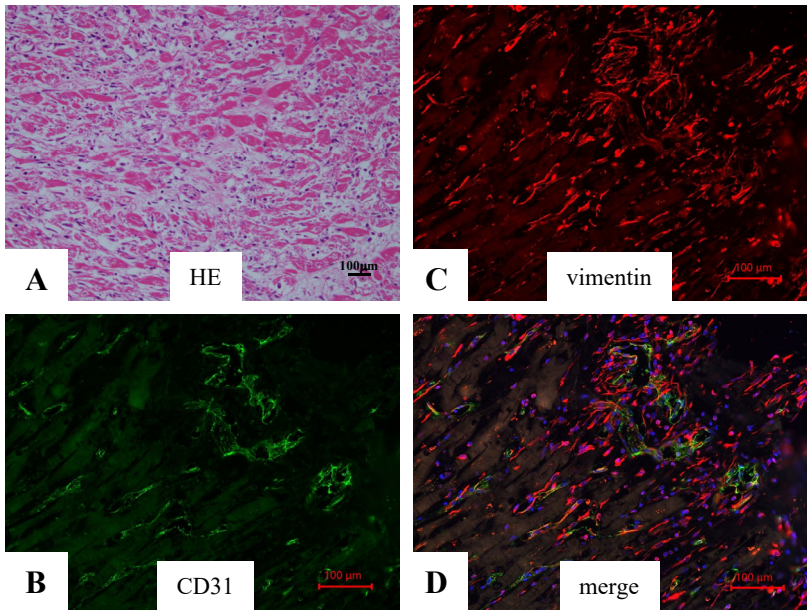
Fig. 4



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. 3D-F
2	48	m	IHD	moderate			
3	68	m	IHD	severe			
4	54	m	AMI, recurrent	severe			
5	51	m	AMI, recurrent	mild		○ several hours	Fig. 2EF
6	63	m	IHD	severe			
7	78	m	AMI, cardiac tamponade	severe			Fig. 2A-D, 3A-C
8	49	m	IHD	severe			
9	36	m	IHD	mild	○		Supplementary Fig. 1CD
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			
14	75	m	IHD	moderate	○		
15	56	m	IHD	mild			
16	85	m	IHD	mild	○		
17	54	f	IHD	moderate			
18	55	m	AMI, cardiac tamponade	mild		○ a few days	Supplementary Fig. 2
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	○		
26	82	f	AMI	mild	○		Supplementary Fig. 1AB