JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, November 2012, Vol. 57, No. 6
doi: 10.1111/j.1556-4029.2012.02172.x
Available online at: onlinelibrary.wiley.com

TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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Recognition of Early Myocardial Infarction by Immunohistochemical Staining with Cardiac Troponin-I and Complement C9*

ABSTRACT: The diagnosis of early myocardial infarction (MI) after death, especially in the first few hours (*c.* 6 h) after the onset of MI, poses a challenge to the forensic pathologists. During this time, the damaged myocardium does not show grossly identifiable morphological changes and may not be recognized even with routine histological microscopic examination. However, the infarcted cardiac tissue releases certain chemicals that can be detected microscopically, two of these being cardiac troponin-I (CT-I) and complement C9 (C9). This study utilizes the importance of these two biomarkers immunohistochemically in an attempt to identify this early phase of MI. This study reveals that the early phase of MI of <6 h duration may be detected through immunohistochemical staining with CT-I and C9. The ischemic/infarcted cardiac myofibers in the <6 h group display reduced/absent CT-I staining as well as positive C9 staining.

KEYWORDS: forensic science, forensic pathology, myocardial infarction, hematoxylin–eosin staining, immunohistochemical staining, cardiac troponin-I, complement C9

Myocardial infarction (MI) is one of the main causes of cardiovascular diseases leading to death as identified by World Health Organization (WHO). MI occurs when the cardiac tissue is deprived of oxygenated blood (1,2). This is mainly because of blockages of coronary arteries commonly caused by an amalgamation of fatty acids and white blood cells, known as an atherosclerotic plaque. The restricted blood supply to the heart (ischemia) and subsequent oxygen shortage result in myocardial damage or even cell death (infarction). The resulting necrosis in the cardiac muscle usually occurs in the subendocardium in the population at-large, either when coronary arteries become atherosclerotic although patent or when there is transmural infarct in the wall of left ventricle because of obstruction of a coronary artery (1,2).

Gross and microscopic examination of the infarcted cardiac tissue allows pathologists in assessing the occurrence and the time of an MI with fair accuracy. However, there is a time frame, approximately during the first 6 h in which morphological changes are not pronounced and may not be even recognized microscopically with routine histological staining (3–6).

MI causes not only physical damage on the cardiac tissue, but also physiological changes to the heart. The damaged cardiac tissue releases biochemical markers such as myoglobin, creatine-kinase MB (CK-MB), and cardiac troponin-T and I (CT-I) (7) and also activates the complement system with the protein component called complement C9 (C9) (8). However, some markers are not as specific and sensitive enough to indicate a diagnosis for MI because of limitations in detecting myocardial damage (5–8).

Studies have shown that the specificity of assays of CT-I is relatively greater and more specific in diagnosing MI than that of CK-MB (7,9). Cardiac troponin complex found in the heart is a three-protein complex consisting of CT-I, T, and C (10). The complex plays a major role in the contraction of skeletal and cardiac muscle; however, their amino acid sequence differs in the muscle tissue type, allowing for their specificity (11,12). CT-I is released by damaged myocardium. If an MI occurs, the cell membranes of the cardiac muscles become more permeable and release intracellular components, such as the CT-I, which diffuses into the spaces between the cells and finally into the blood vessels (13,14).

As previously mentioned, MI is hard to detect through macroscopic examination as well as routine histological staining until approximately more than 6 h after the onset of MI (15). Hansen and Rossen (16) conducted trials in 1999 that detected necrotic myocardial tissue by immunohistochemical staining with CT-I. Their results determined that areas of definite infarction consistently showed lack of cross-striations on the muscle cell and loss of CT-I immunoreactivity.

CT-I can also be analyzed as a possible marker in MI through histological and immunohistochemical techniques (17). Jenkins et al. (6) also used CT-I as a marker for myocardial injury because it is localized to and specific to myocardium.

Another marker for MI is C9, a protein involved in the complement system as part of the innate immune system. It provides protection for the host and reduces susceptibility to foreign microorganisms (18). When MI occurs, cardiac cell necrosis triggers the activation of the complement system, causing neutrophilic infiltration in the myocardium (19). Viable myocardium does not activate the complement system because of the lack of cell necrosis (20).

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*Funding for this project was received from University of Windsor, Windsor, ON N9B3P4, Canada.

Received 24 May 2011; and in revised form 6 Sept. 2011; accepted 17 Sept. 2011.

C9 is the last component in the assembly of the C5b-9 membrane attack complex (MAC). The MAC is completed when C9 binds to the C5b678 complex of a target cell and forms the end product C5b-9. The C5b678 complex guides the polymerization of up to 18 molecules of C9. This complex forms a transmembrane pore in the phospholipid bilayer creating a channel that allows for the passage of ions and small molecules, which may enter the cell by osmosis and cause lyses. Insertion of the C5b-9 complex into myocytes may lead directly to cell necrosis. The presence of MAC in damaged cells is evidence that the lytic phase of the complement reaction cascade has gone to completion (21).

Detection of C9 complex has been found to be localized in areas of MI (22). Studies have been performed to detect the complement components by immunofluorescence as early as 2–4 h after ischemia (23). In 1996, a study by Doran et al. (24) successfully demonstrated that MI could be detected through immunohistochemistry with C9. Their C9 slides showed greater advantages in detecting MI than the hematoxylin and eosin (H&E) stains. The MAC complex had been used to localize areas of MI, and these complexes can occur at the cytoplasmic membranous surface of ischemic cells (25). Immunoreactivity with C9 has been reported as a reliable and sensitive method for detecting early myocardial hypoxia (8).

Recently, a study using 26 autopsy cases to determine early acute MI identification using C4D and C9 established that the positively stained necrotic myocytes are apparent before the influx of inflammatory cells, demonstrating utility in early MI (6).

Increased cardiac biomarker level is part of the WHO criteria that sustains their definition of MI. Specifically, the WHO definition of MI is based on the presence of at least two of three criteria including symptoms, electrocardiogram abnormalities, and increased cardiac enzyme levels. The recognition of more accurate biomarkers to detect cardiac necrosis is now playing an increasingly significant role in defining an MI. An acute MI could previously be diagnosed without biochemical evidence of cardiac necrosis. The current consensus definition demands an elevation in cardiac biomarker levels, specifically referring to CT-I. This universal definition of MI insists that any elevation of the CT-I level should be considered to be diagnostic of acute MI in the clinical setting of myocardial ischemia (17).

Considering these factors, the present research is undertaken with immunohistochemical techniques using two important cardiac markers: CT-I and C9. The objective is to determine whether any or both of the two cardiac markers can recognize early MI.

Materials and Methods

Thirty-one autopsy cases were selected from Windsor Regional Hospital records from 2003 to 2007, which presented with symptoms of myocardial ischemic injury. The age groups ranged from 41 to 90 years. The cases were split into two groups: the first where an infarct has occurred *c.* <6 h (Group I), with no infarction seen microscopically in routine histology; and the second where infarction has occurred for more than 6 h (Group II) and microscopically visible with traditional H&E staining. These cases were selected specifically because of heart-related cause of death where MI being the primarily determined or estimated cause of death. The age, sex, and the causes of death reported for each case are summarized in Table 1.

Positive and negative controls were selected for comparison on each immunohistochemical slide made from true infarcts and healthy myocardium. Formalin-fixed tissue samples were collected.

Prepared paraffin blocks and H&E slides of all the cases were retrieved from the hospital records. All the paraffin blocks were cut into 4-μm sections for immunohistochemical staining with CT-I and C9.

Sections for immunohistochemistry with CT-I and C9 were placed in an automated staining system called BenchMark XT (Ventana Medical Systems, Inc., Tucson, AZ). Monoclonal antibody to human C9 was purchased from Hycult Biotechnology (Burlington ON, Canada). The primary antibody for CT-I was purchased from Ana Spec, Inc. (San Jose, CA). All other materials were from Ventana.

The prepared slides were analyzed under a light microscope. The cost of processing one sample/tissue block was *c.* $40.

Samples were considered positive for infarction when more than 10 adjacent cells were immunoreactive. Isolated myocytes reactivity was not considered as a positive classification of immunoreactivity. This was consistent with the widely held view that MI refers to infarctions of 1 cm or more across (26).

Immunohistochemical Staining with Cardiac Troponin-I

The sections were deparaffinated and rehydrated. After blocking with iVIEW inhibitor, which is an endogen peroxidase, and preincubated for 4 min, sections were incubated with mouse anti-troponin-I monoclonal antibody diluted in 1:25 Dako antibody diluents for 24 min. The immunological reaction was visualized by diaminobenzidine (DAB) detection kit, which uses a peroxidase-conjugated avidin-biotin complex technique. The second layer was I-Biotin (Ventana Medical Systems), which was biotinylated goat anti-mouse IgG and IgM and biotinylated goat anti-rabbit IgG in phosphate buffer. Between each steps, the sections were washed with Tris buffer, pH 7.4. The color was developed using DAB, and the sections were counterstained with Harris hematoxylin.

Immunohistochemical Staining with Complement 9

The sections were deparaffinated and rehydrated. After removal of the wax, the slides were washed with antigen retrieval. The sections were then blocked with iVIEW inhibitor and preincubated for 4 min. Sections were then incubated with mouse monoclonal antibody to human C9 diluted in 1:10 Dako antibody diluents for 24 min. An amplification kit was also added to the slides and was incubated for 8 min. The immunological reaction was visualized by DAB detection kit, which is using a peroxidase-conjugated avidin-biotin complex technique. The second layer was I-Biotin, which was biotinylated goat anti-mouse IgG and IgM and biotinylated goat anti-rabbit IgG in phosphate buffer. Between each steps, the sections were washed with Tris buffer, pH 7.4. The color was developed using DAB, and the sections were counterstained with Harris hematoxylin.

Results

The H&E staining showed that none of the Group I cases had any obvious histological alterations demonstrating infarction. A few of the cases did show microscopic changes like wavy myocardial fibers, fragmentation, and some degree of fibrosis (Table 1). The cases in Group II showed more clear and distinct morphological changes like coagulative necrosis and/or neutrophilic influx (Table 1).

The histological micrographs for the H&E staining are shown in the Fig. 1*A*,*D*,*G*. The control heart sample (Fig. 1*A*) showed normal histology with centrally located nuclei in the myocytes and no signs of fibrosis, coagulative necrosis, or neutrophilic influx. The Group I sample (Fig. 1*D*) also did not reveal any significant microscopic

TABLE 1—*Details and comparison of staining of normal and infarcted myocardium with H&E, C9, and CT-I (Group I: <6 h, Group II: more than 6 h).*

S. No	Case/ Sample No.	Age/ Sex	H&E Microscopic Examination	Cardiac Troponin-I Viable-Positive	Complement C9 Viable-Negative
Group I: <6 h					
1.	7	77M	Normal, no change except some fibrosis	Weakly positive	Weakly positive
2.	8	60F	Normal, no changes	Negative-weakly positive	Weakly positive
3.	9	63M	Normal, no changes	Negative-weakly positive	Few isolated positive cells
4.	10	65F	Normal, no changes	Negative-weakly positive	Weakly positive
5.	11	55M	Normal, no changes	Negative	Weakly positive
6.	12	74M	Normal, no change except some fibrosis	Negative	Weakly positive
7.	M-300-07	90M	Old infarcts present, no recent MI, hypertrophic	Negative-weakly positive	Negative
8.	M-122-07	NA	Wavy fibers, remote subendothelial infarct	Negative-weakly positive	Few isolated positive cells
9.	M-125-07	63M	Slight fibrosis, no wavy fibers or contraction bands	Weakly positive	Few isolated positive cells
10.	M-263-07	92F	Ischemic heart disease, remote infarct	Weakly positive	Few isolated positive cells
11.	M-215-07	59M	Ischemic heart disease, no contraction bands, wavy or neutrophils	Negative	Few isolated positive cells
12.	M-157-07	56M	Some interstitial fibrosis, no recent or remote MI	Negative	Few isolated positive cells
13.	M-234-07	84F	Fat infiltration into myocardium	Weakly positive	Negative
14.	M-185-07	50M	Subendocardium infarct, papillary fibrosis	Weakly positive	Negative
15.	M-30-07	61F	Some fragmentation	Negative	Few isolated positive cells
16.	M-82-07	80M	Some fibrosis and fragmentation, wavy fibers	Weakly positive	Few isolated positive cells
17.	M-340-07	42M	Some fragmentation, but overall normal	Weakly positive	Few isolated positive cells
Group II: More than 6 h					
18.	**1**	56M	Coagulation necrosis, neutrophilic influx	Negative	Positive
19.	**2**	77F	Coagulation necrosis, neutrophils influx	Negative	Positive
20.	**3**	82F	Coagulation necrosis, neutrophilic influx	Negative	Positive
21.	**4**	61M	Coagulation necrosis, neutrophilic influx	Negative	Positive
22.	**5**	79F	Coagulation necrosis, neutrophilic influx	Negative	Positive
23.	**6**	58M	Fibrosis, neutrophils	Negative	Positive
24.	M-144-07	57M	Contraction bands present, neutrophils influx	Negative	Positive
25.	M-73-07	67M	Neutrophils influx, coagulation necrosis present	Negative*	Positive†
26.	M-331-07	69F	Wavy fibers, neutrophils present	Positive	Positive
27.	M-36-07	57F	Neutrophils influx, no coagulation necrosis	Weakly positive	Negative
28.	M-006-07	68M	Microorganisms & bacteria present, decomposed heart	Negative	Negative
29.	M-281-07	41M	Fibroblasts in repair stage, eosinophils present, some coagulation necrosis‡	Negative‡	Positive‡
30.	M-59-07	55M	Neutrophilic influx, coagulation necrosis	Negative	Positive
31.	M-311-07	47M	Small necrotic area in subendothelial infarct	Negative	Positive

*Total negative around infarct area, some positive cells in myocardium indicating not all troponin is released.
†Specifically in area of infarct and neutrophils influx.
‡One day old infarct.
MI, myocardial infarction.

morphological changes. The myocardium failed to reveal any coagulative necrosis or neutrophils. However, the myocytes (Fig. 1*D*) were sometimes found fragmented (arrow), which may suggest an early MI. On the other hand, an infarcted heart sample for more than 6 h (Fig. 1*G*) showed no nuclei in the center of the cells (arrow) along with some degree of neutrophilic influx (star), both indicative of early MI.

For CT-I, the control heart sample (Fig. 1*B*) stained positive. All the cases of the Group I showed negative to weakly positive staining for CT-I, whereas more than 85% of the cases of the Group II showed negative staining (Table 1). Figure 1*E* showed an infarcted heart sample of Group I, revealing negative staining on the left side (star) and also areas of weakly positive staining on the right side (arrow). The micrograph of an infarcted heart sample Group II (Fig. 1*H*) showed negative staining indicating that CT-I has been released by the tissue and into the bloodstream, thus staining negative in the myocardium.

The C9 staining was found to be sensitive and specific to the infarcted areas. In Group I, more than 82% of the cases either showed a weakly positive staining or a few isolated cells stained positive (Table 1). In the Group II category, more than 85% showed a positive immunoreactivity (Table 1). The micrographs for the C9 immunohistochemical staining for control heart sample are shown in the Fig. 1*C*, with negative staining. C9 staining of an infarcted heart sample of Group I (Fig. 1*F*) showed isolated cells of the myocardium staining positive. In the heart sample of Group II infarct (Fig. 1*I*), a contrast staining between the viable myocardium and infarcted myocardium was observed. The damaged myocardial cells were stained positive with C9 indicating presence of the MAC. The unstained areas represented viable myocardium.

The immunoreactivity to C9 was observed to be sensitive in detecting individual scattered myocardial cell necrosis. In the case number 9 (Group 1), there were no signs of infarction in the H&E staining although in rare individual cells, positive immunoreactivity for C9 was observed (Fig. 2*A*,*B*). This is a case of 63-year-old man who died from <6-h old acute coronary syndrome, and there was no histological evidence of MI.

Interestingly, in case number M-36-07 of Group II, a weakly positive staining for CT-I appeared around areas of neutrophilic influx and a negative staining for C9. This was a case of 57-year-old female whose cause of death was reported to be an early anterior MI probably due to coronary spasm. The microscopic findings revealed that the anterior myocardium showed focal areas with early influx of neutrophils partially destroying myocardial fibers. Case M-331-07 in Group II was positive for CT-I and also positive for C9. This was a case of 69-year-old female, found unresponsive by husband in her bed with history of hypertension. She complained of nausea and vomiting the evening before. Both the cases might be the marginal time line in the early MI groups. Another case M-006-07 was a decomposed heart where bacteria and small microorganisms were

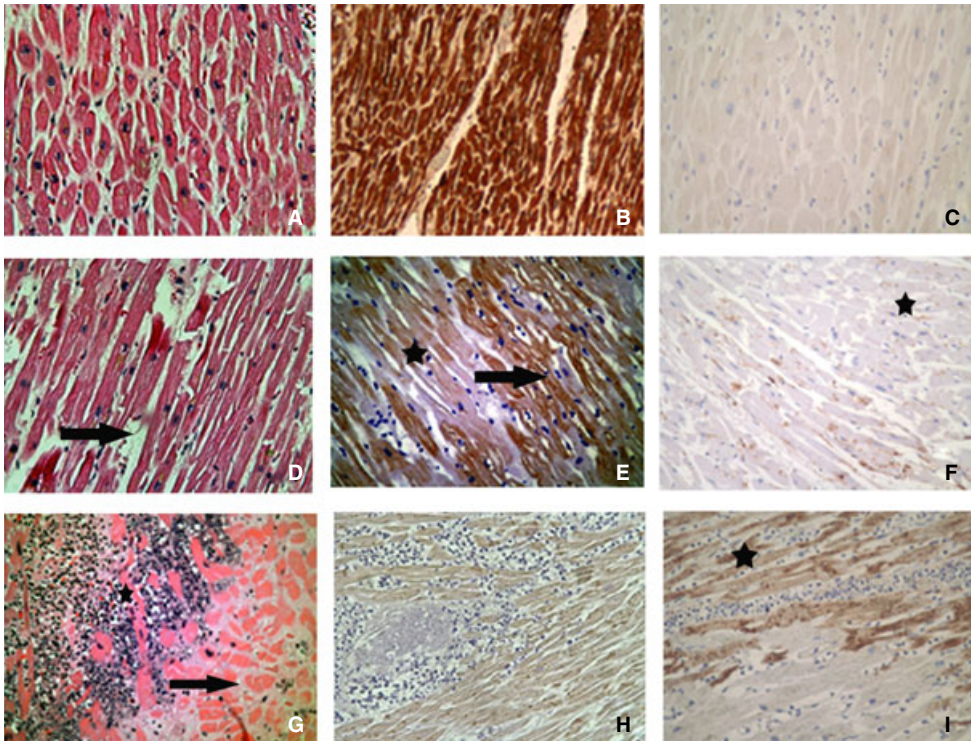

FIG. 1—*Comparison of the H&E staining (A, D, G) with immunostaining for cardiac troponin-I (CT-I) (B, E, H) and complement C9 (C9) (C, F, I) in normal and infarcted myocardium. (A) Normal heart muscle stained with H&E showing central nuclei present in myocytes. (B) Normal heart muscle stained positive for CT-I showing CT-I present in the viable myocardium. (C) Normal heart muscle stained negatives for C9 showing absence of membrane attack complex (MAC) therefore no lyses of myocytes and cells appear normal. (D) Myocardium shows no sign of coagulation necrosis or neutrophils; however, fragmentation of the cells (arrow) with central nuclei is an indication of early myocardial infarction (MI). (E) Weakly positive CT-I immunostaining on right side (arrow) and negative staining on the other side (star) may signify early release of CT-I indicating an early phase of MI and cellular damage. (F) Little areas of the myocardium stained positive for C9 indicating an early formation of the C5b678 complex that could have lysed the areas of the cells and caused injury to the cells. (G) No nuclei present in the center of the cells and presence of neutrophilic influx of cells. Both are indication of positive MI. (H) Negative staining for CT-I, indicating that CT-I has been released by the tissue and into the bloodstream, thus staining negative for its absence. (I) Contrast shown between viable myocardium and infarcted myocardium. Damaged cells stained positive for C9, which indicate the presence of the MAC. Unstained areas are viable myocardium.*

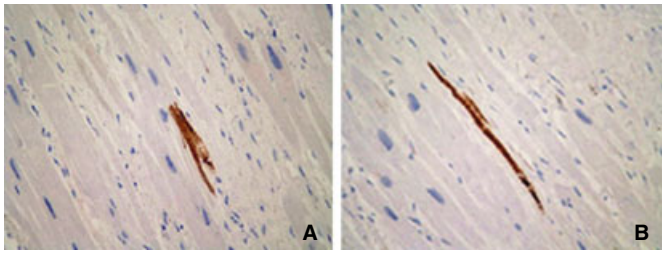

FIG. 2—*Complement C9 (C9) positive immunostaining in the individual cells (A,B) of the case number 9 of the Group I where an suspected myocardial infarct occurred <6 h or so showing the sensitivity of the C9 immunostaining in detecting individual scattered myocardial cell necrosis among viable myocardium.*

present and stained negative for CT-I as well as for C9, possibly due to breakdown of the cells through decomposition.

The case number M-73-07 in Group II where the cause of death was reported as fresh coronary thrombus in H&E showed neutrophilic influx with some viable myocardium (Fig. 3*A*). The infarcted areas were negative for CT-I with some positive cells in the viable myocardium. The latter indicated that not all troponin was released yet from this area of <6-h old MI (Fig. 3*B*). The C9 staining revealed the infarcted cells stained positive, whereas some viable myocardium remained negatively stained (Fig. 3*C*).

Additionally, in case M-281-07, the H&E showed an area of *c.* 1 day old infarcted myocardium with neutrophils and eosinophils while the adjacent area showed a healing MI (Fig. 3*D*). The area showing 1 day old infarct was negative for CT-I (Fig. 3*E*) and positive for C9 (Fig. 3*F*).

Discussion

The diagnosis of an early MI of *c.* <6 h is still a challenge to the forensic pathologists. During this time, the damaged myocardium does not show grossly identifiable changes and may not be recognized even with routine histological microscopic examination. Immunohistochemical staining with two important biochemical markers, CT-I and C9, was able to identify this early phase of MI.

Microscopically, the diagnosis of MI can be made easily if it has lasted more than 6 h, thereby showing the inflammatory cells (neutrophilic influx) and/or coagulation necrosis. Early infarcts of *c.* <6 h revealed some wavy myocardial fibers or fragmentation and hence forth can be very subtle. Although the utility of CT-I and C9 was limited in identifying acute MI of *c.* <6 h, nevertheless the results clearly demonstrated the value of immunohistochemical staining for CT-I and C9 in confirming the presence and extent of this early phase of MI.

The control viable myocardium stained positive for CT-I and negative for C9. The early MI cases of *c.* <6 h (Group I) had no microscopic changes on H&E slides although showed negative or

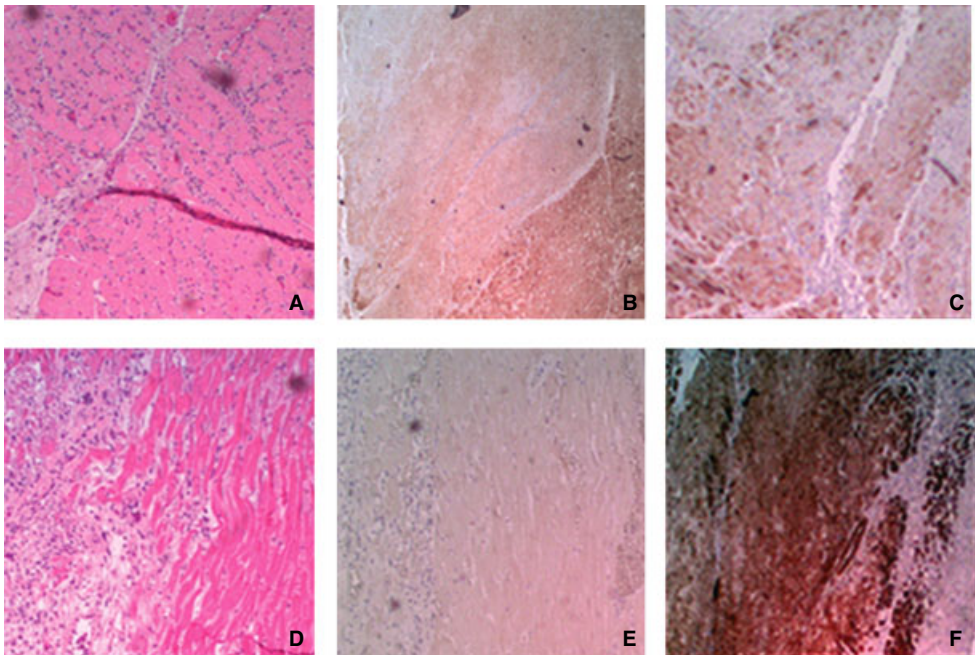

FIG. 3—*Comparison of the staining in two cases (A–C, Case No. M-73-07 and D–F, Case No. M-281-07) of the Group II where myocardial infarction has occurred for more than 6 h. (A) Neutrophilic influx with some viable myocardium. (B) Cardiac troponin-I (CT-I) exiting in the myocytes in viable and infarcted myocardium. (C) Infarcted cells with no nuclei showing positive staining for complement C9 (C9), some viable myocardium showing negative staining. (D) Infarcted myocardium with neutrophils and eosinophils in repair stage to repair old infarct zone with fibrotic connective tissue. (E) No CT-I present, staining negative. (F) C9 staining very positive. Heavily infarcted, little or no surviving myocardium.*

weakly positive staining for CT-I. There were no neutrophils present on any of these cases indicating that an inflammatory response had not yet occurred. Some possible contraction bands were located on certain cases associated with minimal troponin release, but not on all cases. Similarly, wavy fibers were also present on a fair number of the cases. These features are not positive identifiers for early MI. However, their presence may or may not shed some light to the environment of the cells before the necrotic cardiac cells become recognized with H&E staining.

The cases in Group I that demonstrated certain evidences of injury although lacking the neutrophilic influx tested either weakly positive for C9 or showed isolated positive cells. The sensitivity and specificity of C9 immunohistochemical staining can be seen through its ability to recognize individual infarcted cells, which would help determining the presence and extent of early MI. The findings were in agreement with recently published results of Jenkins et al. (6). Patches of CT-I negative staining along with positive C9 staining allowed the cases to be identified as early MI of *c.* <6 h. All cases showing H&E positive for necrotic areas stained negative for CT-I and stained positive for C9, recognizing that the MAC was in progress.

A correlation was seen between patchy, weakly positive CT-I staining, and areas of MI. This loss of CT-I expression could be seen very well in case M-73-07 where there were distinct deprivations in troponin across the area shown in Fig. 3*B*. Although an infarct was already seen in some areas, there was still viable myocardium associated with the specific transition, as well as C9 positive staining for the same area.

Two cases in the Group II stained weakly positive and positive for CT-I. Although contradictory, according to our standard, the presence of neutrophils places them in more than 6 h time frame. However, this initial neutrophilic influx does occur some distance away from the true infarcted zone. The viable myocardium may show neutrophilic influx although no MI occurred in that area thus a CT-I positive staining. Also, both cases might also be in the marginal time line in the categorized MI groups.

With no standardized method of determining early MI of <6 h with H&E staining, research into the biochemical markers with immunohistochemical techniques could provide a vital asset to pathologists in the near future. Also, there being a distinct time frame attached to these processes, the question on whether a numerical duration of time between the first onset of the heart attack and the demise of the individual could be determined. This could potentially assist in determining time of death of an individual suffering from MI.

In conclusion, this study has shown that acute MI of *c.* <6 h may be identifiable. The cardiac myofibers revealed weakly positive/negative staining for CT-I and positive staining for C9 although the conventional H&E staining failed to reveal the evidence of such MI.

Acknowledgments

We would like to express our sincere thanks to Mr. Richard Baillargeon at Windsor Regional Hospital, Ontario, Canada, who performed immunohistochemical staining for this study. Thanks are due to Dr. David Shum, Head Pathology, Windsor Regional Hospital, for providing all the necessary facilities. Thanks are also due to the Chief Coroner's Office of the province of Ontario for permission to use the forensic samples for our research.

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