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Original Article

Immunohistochemical analysis of cardiac troponin inhibitor in an experimental model of acute myocardial infarction experimental model and in human tissues

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ABSTRACT

Acute obstruction of coronary arteries leads to acute myocardial infarction (AMI), which causes unexpected death in humans. However, AMI cannot be easily detected in forensic examinations with traditional hematoxylin and eosin (H&E) staining. We analyzed whether cardiac troponin inhibitor (CTnI) could serve as a sensitive and specific early marker for diagnosing AMI in forensic medicine. We established an AMI model in rabbits by ligating the left ventricular branch and observed CTnI expression with immunohistochemistry after different ligation times. We found increased CTnI staining at the 0.5-h time point and depletion of CTnI staining with a 1-h ligation. The areas in which CTnI staining was depleted as seen with immunohistochemical analysis were consistent with the results of H&E staining. Next, human myocardium tissues from 30 persons who died from AMI and were subsequently examined in our forensic center were studied using immunohistochemistry with an antibody to human CTnI. Areas of infarction also showed depletion of CTnI staining. These findings suggested that immunohistochemical detection of CTnI is earlier, more sensitive, and myocardial tissue – specific as compared with H&E staining. CTnI may serve as an ideal marker for diagnosing AMI in forensic investigations.

Introduction

Acute myocardial infarction (AMI) generally occurs because of severe coronary artery occlusion and is a common cause of sudden unexpected death [6]. Postmortem diagnosis of AMI is a major concern in forensic autopsy cases.

This problem is a current challenge for forensic pathologists, particularly when death occurs within minutes to a few hours after an ischemic insult [4]. This is because myocardial cell death does not occur instantaneously at the onset of ischemia, and at least several hours are required before myocardial necrosis can be identified with standard macroscopic or microscopic examination [10]. Therefore, recognition of early myocardial damage using routine hematoxylin and eosin (H&E) staining is neither specific nor sensitive enough if death of the patient occurred shortly after the onset of the ischemic injury. In fact, once the onset of myocardial ischemia occurs, the structural proteins of the myocardium begin to change [8]. In this regard, the usefulness of immunohistochemical markers for the diagnosis of early myocardial damage has recently been suggested because most markers are serologically detectable even as early as a few minutes after the beginning of symptoms [4,11].

Cardiac troponin inhibitor (CTnI) is the inhibitory subunit that forms part of the regulatory troponin complex. CTnI maps to human chromosome 19q13.4, contains eight exons, and consists of 210 amino acids. CTnI is one of three isoforms, each of which possesses unique amino acid sequences. The other two isoforms are expressed in skeletal muscle and are known as slow-twitch and fast-twitch troponin inhibitors [7]. CTnI is different from both skeletal isoforms because of the presence of an additional 30 amino acid residues at the N terminus. This sequence difference leads to superior specificity of serum CTnI as an indicator of cardiac injury over other current markers such as creatinine kinase-MB (B-brain type and M-muscle type, CK-MB) and myoglobin [12].

CTnI is elevated in serum after AMI because of leakage from damaged myocardial cells [1,5]. Antman et al. reported that measurement of CTnI can be used to detect minor myocardial injury (small infarcts) and stratify the risk of patients with acute coronary ischemia [15]. Hansena et al. determined CTnI expression in the hearts of 46 autopsied individuals using an immunohistochemical technique and suggested that CTnI detection may be a sensitive

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test for the diagnosis of early myocardial infarction [9]. However, no studies have analyzed the expression of CTnI in AMI after different intervals between ischemia and death using adequate experimental models and human cases of myocardial infarction. Therefore, in this study, we established a rabbit AMI experimental model and observed CTnI expression changes after different ligation times with immunohistochemical analysis and compared the results of our immunohistochemical study with the standard histological H&E staining. We also evaluated CTnI immunohistochemical staining in human hearts from autopsies to validate the specificity and sensitivity of CTnI as an ideal marker for early AMI diagnosis.

Materials and methods

The experimental model of AMI

New Zealand White rabbits weighing 1.8–2.8 kg were used. The animals were handled according to the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Department of Laboratory Animal Science at Fudan University School of Medicine.

Surgical procedures were carried out as described [13]. Briefly, New Zealand White rabbits were anesthetized with 30 mg/kg sodium pentobarbital that was administered via a marginal ear vein. The neck was opened with a ventral midline incision, and a tracheotomy was performed. A left thoracotomy was performed in the fourth intercostal space, and the pericardium was opened to expose the heart. The left ventricular branch was ligated with a 2-0 silk thread. Animals were randomly divided into six groups (five rabbits per group). Each group was ligated for 0, 0.5, 1, 3, 6, or 8 h. After the end of each ligation, the hearts were quickly removed, rinsed with phosphate-buffered saline, and placed in 10% neutral buffered formalin for fixation.

Samples from human myocardium tissues

The myocardium tissues of 30 consecutive forensic autopsy cases with AMI (22–53 years of age) were collected during autopsies performed at the Department of Forensic Medicine, School of Basic Medical Sciences, Fudan University, between October 2012 and May 2014 and were analyzed retrospectively. The study was approved by the Ethics Committee of the Fudan University School of Medicine.

Immunohistochemical staining

After fixation in formalin for 48 h, 5-μm-thick paraffin-embedded tissue sections were cut. All samples were dewaxed in xylene, hydrated in a graded series of ethanol, and subjected to antigen retrieval treatment by microwaving prior to blocking with sequential hydrogen peroxide and goat serum. Then samples were incubated overnight with a monoclonal antibody to CTnI (1:2000, MCA1208, Serotec) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (ready-to-use GK507705; Gene Tech Company, Shanghai, China). Finally, the sections were counterstained with hematoxylin.

Results

Establishment of the AMI experimental model

As shown in Fig. 1A, the coronary artery in the rabbit heart is divided into the left anterior descending and left ventricular branches. The left anterior descending branch is slender and goes with the corresponding veins, whereas the left ventricular branch originates from the left circumflex artery and goes without the corresponding vein (Fig. 1B and C). The left ventricular branch was ligated to establish the AMI model. Occlusion of the vessel was visually confirmed by color changes of the myocardial tissue from light red to pale red, indicating successful establishment of the AMI experimental model (Fig. 1D and E).

H&E staining and immunohistochemical analysis

The experimental rabbits were divided into five groups, which were ligated for 0.5, 1, 3, 6, or 8 h. Compared with the control (0-h ligation) group, regional myocardial cells first showed enhanced eosinophilic staining and regular muscle striations (Table 1); then, the striations became blurred and fuzzy, although the myocardial nuclei were not changed. With increasing ligation times, myocardial striations disappeared, and some nuclei disappeared. Ischemic myocardial tissue showed signs of intense eosinophilic staining, indicative of coagulative necrosis (Fig. 2A). We observed expansion of necrotic regions with increasing ligation times. With 6 h of ligation, areas with definite macroscopic signs of acute infarction showed neutrophil accumulation within the myocardial tissue, and subserous fibrin exudation was observed with 8 h of ligation (Fig. 2A).

Immunohistochemical staining showed cytoplasmic CTnI immunoreactivity with pronounced cross-striation in the control myocardium (Fig. 2B). We found increased immunostaining for CTnI with 0.5 h of ligation. After 1 h of ligation, the well-defined corresponding infarcted areas began to show a clear reduction in CTnI expression. As ligation time increased, CTnI immunoreactivity was strongly reduced, and complete depletion was observed after 8 h of ligation (Fig. 2B).

The areas with loss of CTnI expression, as detected with immunohistochemical staining, were consistent with the coagulative necrotic regions observed with H&E staining (Fig. 2C).

Histologic examination and immunohistochemical staining in human tissues

In the human AMI cases, areas with definite macroscopic signs of acute infarction showed microscopic evidence of acute infarction. Histologic examinations of these samples revealed the classic appearance of myocardial coagulative necrosis. The myocytes showed hypereosinophilic staining with pyknosis of the nuclei (Fig. 3A) and accumulation of neutrophils within the myocardial tissue (Fig. 3B). Myocytes showed a lack of cross-striations in the infarcted areas. In some cases, formation of granulation tissue or collagen deposition was observed (Fig. 3C).

Using immunohistochemical staining in regions of normal myocardial tissue, strong cytoplasmic CTnI immunoreactivity was found in the cardiac myocytes (Fig. 3D). However, in infarcted areas, partial or diffuse depletion of CTnI expression was observed (Fig. 3E, arrow). In the regions undergoing repair, granulation tissue was present, and CTnI staining and microvessel formation were completely absent (Fig. 3F, arrow). Suspected areas of myocardial infarction (Fig. 3G) showed enhanced eosinophilic staining in myocardial cell cytoplasm but no obvious changes in the nuclei with H&E staining. In these areas, we observed obvious and complete loss of focal CTnI immunohistochemical staining in myocardial cells (Fig. 3H, arrow). These observations showed the specificity and sensitivity of CTnI in the diagnosis of AMI.

Discussion

In this study, we successfully established AMI experimental models with different ligation times and evaluated CTnI expression using immunohistochemical staining and compared the results with standard H&E staining. We also detected CTnI expression in