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Detection of cardiomyocyte apoptosis in forensic autopsy cases

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Abstract The purpose of the present study was to determine reliable parameters for the detection of apoptotic cells for use as a diagnostic marker during the early stage of acute myocardial infarction (AMI) in forensic autopsy cases. Myocardial tissues taken from forensic autopsy cases were examined by immunohistochemical and molecular-biological methods using the terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labelling (TUNEL) and the DNA laddering methods. In cases of AMI with a time period between 2 h from onset to death and 20 h postmortem time, the nuclei of cardiomyocytes were stained positive with the TUNEL method and DNA fragmentation of myocardial cells was detected by agarose gel electrophoresis. Similar findings were obtained in cases of carbon monoxide (CO) intoxication. However, no apoptotic cells were found in other cases such as methamphetamine (MAP) intoxication, tetrodotoxin intoxication, alcohol intoxication, asphyxia, head injury, heart injury or myocarditis. These findings suggested that it would be possible to apply TUNEL-positive cells as a diagnostic marker during the early stages of AMI.

Keywords Apoptosis · TUNEL method · Cardiomyocyte · Forensic autopsy · Myocardial infarction

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Introduction

It is widely acknowledged that apoptosis and necrosis are two fundamental types of cell death with different characteristics [1]. Apoptotic cell death is characterised either by histological parameters such as cell shrinkage, condensation of chromatin and formation of apoptotic bodies at the electron microscope level, or by fragmentation of intranucleosomal DNA to multiples of 180–200 bp fragments [2]. These fragments give a characteristic DNA ladder pattern on agarose gel electrophoresis and can also be detected by the immunohistochemical method [3, 4]. However, necrotic cell death shows damaged organelles and dispersion of cytoplasmic elements into the extracellular space [5].

In recent years, the role of apoptosis in cardiomyocytes has been debated among researchers [6, 7, 8, 9, 10, 11]. In particular, there are a variety of controversies regarding the role of apoptosis in myocardial infarction (MI). The reason for this is that it has long been assumed that MI followed necrotic cell death [12]. However, according to studies of human autopsy specimens, apoptosis appeared to be predominantly localised in the hypoperfused border zone between the central infarct area and non-compromised myocardial tissue [7, 13]. Moreover, Veinot et al. [14] examined the time of appearance and extent of apoptosis in human acute myocardial infarction (AMI) and compared these with necrotic cell death. They found that there was widespread apoptosis in the infarcted area only a few hours before the appearance of coagulative necrosis in heart tissue of autopsied cases of AMI, suggesting that apoptosis may be the early and predominant form of cell death in infarcted cardiomyocytes.

Taking into consideration the above observations, we conducted the present study to examine whether apoptotic cell death could be detected by the TUNEL method using myocardial tissue sections from forensic autopsy cases. In forensic pathology, one of the most important tasks is to determine the cause of death from histological findings using histochemical methods (e.g. haematoxylin-eosin, chromotrope aniline blue staining) and immunohistochemical

markers (e.g. myoglobin, desmin, fibrinogen) [15]. It is, however, occasionally difficult to do so, owing to the effects of post-mortem changes and in some cases, for instance sudden death, it is time- and labour-intensive to obtain specific findings. The diagnosis of heart diseases such as MI have been performed mainly by light microscopy, therefore, it is important to consider the effects of the post-mortem change and to clarify early markers of MI after death. To overcome these difficulties, we evaluated the usefulness of the TUNEL method as a histological diagnostic marker in forensic autopsy cases.

Materials and methods

Forensic autopsy case selection and tissue preparation

Tissue samples were obtained from forensic autopsies performed at Osaka University Graduate School of Medicine and Nagoya City University Medical School between 1994 and 2001. A total of 40 cases were selected on the basis of the cause of death (Table 1), 12 were from patients who died of AMI and autopsy was performed 5 h–63 h post-mortem. Other samples were from patients who died from methamphetamine (MAP) intoxication (4 cases), CO intoxication (3 cases), tetrodotoxin intoxication (2 cases), alcohol intoxication (1 case), asphyxia (9 cases), head injury (3 cases), heart injury (4 cases) and myocarditis (2 cases). All 40 autopsied hearts were fixed with 10% neutral buffered formalin and embedded in paraffin. Sections from paraffin blocks of each sample were stained with haematoxylin and eosin, and examined by light microscopy.

Table 1 Clinical characteristics of patients (*AMI* acute myocardial infarction, *MAP* methamphetamine, *CO* carbon monoxide)

Case No.	Gender	Age (yr)	Time lapse between onset and death	Time from death to autopsy (h)	Cause of death	TUNEL staining	DNA Ladder
1	M	64	Less than 30 min	29	AMI	–	–
2	M	60	Less than 30 min	36	AMI	–	–
3	M	64	Less than 2 h	10	AMI	+	+
4	M	63	2 h	63	AMI	–	–
5	M	49	Less than 2 h	20	AMI	+	+
6	M	61	Less than 2 h	5	AMI	+	+
7	M	45	2 h	15	AMI	+	+
8	M	50	Less than 30 min	24	AMI	–	–
9	F	55	Less than 2 h	8	AMI	+	+
10	M	43	Less than 30 min	12	AMI	+	+
11	F	65	5 h	26	AMI	–	–
12	M	62	4 h	32	AMI	–	–
13	M	26	7 h	10	MAP intoxication	–	–
14	F	47	Unknown	4	MAP intoxication	–	–
15	F	40	4 h	17	MAP intoxication	–	–
16	M	33	24 h	27	MAP intoxication	–	–
17	F	82	Unknown	10	CO intoxication	+	–
18	M	23	Less than 1 h	6	CO intoxication	+	–
19	M	24	Less than 1 h	13	CO intoxication	+	–
20	M	35	7 days	17	Tetrodotoxin intoxication	–	–
21	M	55	3 days	24	Tetrodotoxin intoxication	–	–
22	M	44	Unknown	30	Alcohol intoxication	–	–
23	M	75	Less than 3 h	18	Asphyxia	–	–
24	F	68	Unknown	20	Asphyxia	–	–
25	F	86	Less than 30 min	48	Asphyxia	–	–
26	F	39	Less than 1 h	11	Asphyxia	–	–
27	F	47	Less than 30 min	56	Asphyxia	–	–
28	F	37	Less than 30 min	25	Asphyxia	–	–
29	M	10	Less than 4 h	25	Asphyxia	–	–
30	M	25	Unknown	87	Asphyxia	–	–
31	F	25	Less than 2 h	6	Asphyxia	–	–
32	M	19	Less than 30 min	22	Head injury	–	–
33	M	22	Unknown	27	Head injury	–	–
34	F	29	Less than 30 min	10	Head injury	–	–
35	M	20	Less than 30 min	12	Heart injury	–	–
36	M	67	30 h	7	Heart injury	–	–
37	M	67	Less than 30 min	5	Heart injury	–	–
38	M	29	Less than 30 min	12	Heart injury	–	–
39	M	18	Unknown	12	Myocarditis	–	–
40	M	17	Unknown	11	Myocarditis	–	–

Genomic DNA extraction and gel electrophoresis

To confirm the histochemical features of fragmentation of intranucleosomal DNA to multiples of 180–200 bp fragments, the presence of the DNA ladder pattern was determined on agarose gel electrophoresis [7].

The stored heart tissues were minced with a sterile mini cordless grinder in sterile phosphate buffered saline (PBS). The suspensions were centrifuged at 3,000 rpm for 15 min and the supernatants were removed. After incubation with proteinase K (Wako Pure Chemical, Osaka, Japan) (125 μg/ml) in a lysis buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 20 μg/ml pancreatic DNAase-free RNAase (Sigma, St. Louis, Mo.), 0.5% SDS, and 100 mM EDTA at 50°C for 3 h, protein contaminants were removed by phenol/chloroform extraction and precipitated with ethanol. The pellets were resuspended in TE buffer (Tris-HCl pH 7.6 and 1 mM EDTA) and treated with RNAase for 3 h at 37 °C. Genomic DNA was re-extracted by phenol and chloroform followed by ethanol precipitation, and then dried and stored at –20 °C in 10 mM Tris-HCl (pH 7.6). Extracted DNA was quantified by measuring its optical density at 260 nm (a value of 1.0 corresponds to approximately 50 μg/ml DNA) and 20 μg of each DNA was subjected to electrophoresis on a 2% agarose (NuSieve GTG Agarose, Takara, Japan) gel with ethidium bromide staining.

In situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) method

The TUNEL protocol in the present study for detecting DNA fragmentation was based on previously published procedures with slight modifications [16]. After removal of the hearts at autopsy, small portions were fixed in buffered formalin solution and embedded in paraffin. Deparaffinised and hydrated sections were incubated with 20 μg/ml proteinase K for 15 min at room temperature, and washed 5 times in distilled water. To inactivate endogenous peroxidase, the sections were incubated with 0.3% H_2O_2 in methanol for 10 min at room temperature. The sections were rinsed with distilled water and immersed in TdT buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT and biotinylated dUTP in TdT buffer were then added to cover the sections and incubated at 37 °C for 60 min. The reaction was terminated by transferring the sections to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The sections were rinsed with distilled water, covered with a 0.5% aqueous solution of skimmed milk (Yukijirushi Nyugyo, Tokyo, Japan) for 10 min at room temperature, rinsed in distilled water and immersed in PBS for 5 min. The sections were covered with avidin-peroxidase (Sigma, St. Louis,Mo.), which was diluted 1:20 in distilled water, incubated for 30 min at 37 °C, washed in distilled water for 60 min and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) for 10 min at room temperature. Sections were counterstained by placing them in haematoxylin for 3 min, and were then dehydrated through alcohol solutions of increasing concentrations and toluene. Slides were coverslipped and then examined by light microscopy. Positive controls for each section were deparaffinised, processed through proteinase K and underwent endogenous inactivation in the same manner. Each section was then pretreated with DN buffer (30 mM Tris pH 7.2, 140 mM potassium cacodylate, 4 mM $MgCl_2$, 0.1 mM DTT). DNAase I (10 mg/ml, Sigma), dissolved in DN buffer, was added to cover the section. After 15 min of incubation at room temperature, the section was washed with distilled water and continued to be processed through DNA nick end labelling. Negative controls for each section were treated in the same manner except that they received TdT alone in TdT buffer without biotinylated dUTP.

Results

To detect DNA fragmentation by apoptosis in cardiomyocytes, we studied serial sections of heart tissues by TUNEL staining.

In a total of 12 patients who died of AMI (samples were taken between 5 and 63 h post-mortem), TUNEL-positive cells were observed in 6 patients (cases 3, 5, 6, 7, 9 and 10) who died within 2 h from onset to death and whose samples were taken between 5 and 20 h post-mortem (Fig. 1, Table 1). Furthermore, TUNEL-positive cells were present in the central areas and border zones of histologically infarcted areas, whereas no TUNEL-positive cells were present in the remote, non-infarcted areas. In another 6 AMI patients (cases 1, 2, 4, 8, 11 and 12) where samples were taken 24–63 h post-mortem, no TUNEL-positive cells were observed. In addition, in cases of CO intoxication, TUNEL-positive cells were observed in a widespread area (cases 17, 18 and 19). In other cases such as those of MAP intoxication, tetrodotoxin intoxication, alcohol intoxication, asphyxia, head injury, heart injury, and myocarditis, no apoptotic cells were found (cases 13–16, and 20–40).

Agarose gel electrophoresis of genomic DNA isolated from hearts showed a laddering pattern of apoptosis in patients who died of AMI when samples were taken 5–20 h post-mortem (Fig. 2), but showed no laddering pattern in other cases including the case of CO intoxication.

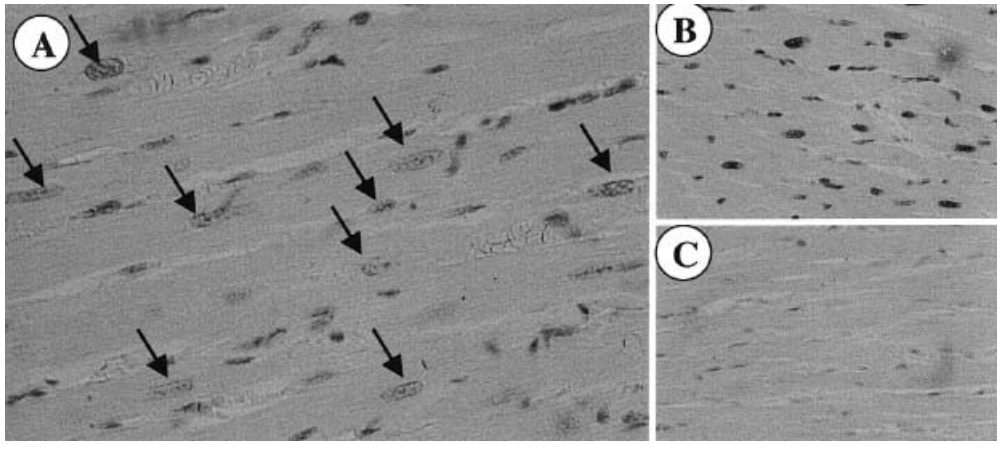

Fig. 1 A–C Light micrographs of TUNEL-stained myocardium (case 3). **A** TUNEL-positive cells (*indicated by arrows*) are seen in the infarcted myocardium. **B** Positive control with the TUNEL method. This shows the figure that each DNA in the nuclei was digested completely by DNAase I, so that the nuclei were strongly stained. **C** Negative control with the TUNEL method. This was treated in the same manner except that they received TdT alone in TdT buffer without biotinylated dUTP, so that no nuclei were stained. Original magnification **A–C** × 200

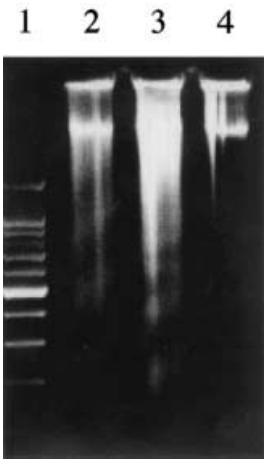

Fig. 2 Agarose gel electrophoresis of myocardial DNA. *Lane 1* 100 bp ladder marker. *Lanes 2* and *3* are from the hearts of patients who died of AMI (*Lane 2*, case 1; *Lane 3*, case 3). *Lane 4* is from the heart of a patient who died of CO intoxication (case 12). Note that the AMI sample taken within 20 h post-mortem shows a clear ladder pattern (See Table 1)

Discussion

In forensic autopsy specimens, it is occasionally difficult to diagnose AMI because of the effects of post-mortem changes and it requires at least 4 or 5 h to be able to find typical morphological changes in MI. Brinkmann et al. [15] reported that a selected set of histochemical and immunohistochemical methods would improve the detection of early myocardial infarction/ischaemic damage although one single criterion could not resolve the diagnostic problem. For these reasons, we tried to determine new criteria of AMI and apply them as a diagnostic marker in an early stage of AMI in forensic autopsy cases. The findings of the present study revealed that forensic autopsy specimens from patients who died of AMI (samples were taken within 20 h post-mortem and the time lapse between onset and death was within 2 h) were positive with TUNEL staining and showed a DNA laddering pattern. All TUNEL-positive cells were observed in the central infarcted areas and border zones between infarcted areas and normal areas. Similarly, Ito et al. [7] reported that the infarcted myocardial cells, located sporadically among normal myocardial cells or along boundaries between ischaemic and non-ischaemic areas, were stained positive with the TUNEL method and showed specific DNA fragmentation with the ladder pattern on agarose gel electrophoresis. Furthermore, Saraste et al. [13] reported that apoptotic cardiomyocytes were observed particularly in the border zones of histologically infarcted myocardium, whereas very few apoptotic cells were present in the non-infarcted myocardium. These observations on MI have confirmed the contribution of apoptosis to cardiomyocyte death. Moreover, there is increasing evidence that ischaemia-induced apoptosis precedes necrosis, suggesting the possibility of inhibition of substantial cardiomyocyte death by inhibiting apoptosis. In addition, light microscopic examination of AMI specimens revealed some histological changes such as eosinophilic changes (cases 3, 5, 6, 7 and 8), pycnosis (cases 3 and 9), contraction band necrosis (cases 3, 6, 7, 11 and 12), and coagulation necrosis (cases 5, 6 and 11). In short, TUNEL-positive cells were observed even in the apparently normal areas of the border zones. The TUNEL technique detects double-stranded breaks in DNA, which is widely used as a method that identifies apoptotic cells and fragmented DNA by endogenous endonucleases is the biochemical hallmark of apoptosis. When compared to the immunohistochemical study of early myocardial damage previously reported by Ortmann et al. [17], TUNEL-positive cells seemed to be as useful a marker as the early depleted myoglobin although the methodology was rather different. Especially, it seems to be useful in AMI cases that show a characteristic DNA ladder pattern on agarose gel electrophoresis. Therefore, if specimens are taken within 20 h of death, it is suggested that TUNEL-positive cell would be a new diagnostic marker during an early stage of AMI (within 2 h) where microscopic examination reveals few specific findings.

In contrast, all AMI specimens taken after 20 h from death were negative with TUNEL staining and in addition, the DNA laddering pattern was not detected. These findings are considered to be the effects of the post-mortem changes. In other words, there is a possibility that no antigens of the cell membrane were activated. In fact, it is difficult in general to activate antigens in the case of an old specimen using immunohistochemical methods [18].

In other cases, TUNEL-positive cells were also observed in cases of CO intoxication. According to previous studies, cardiomyocytes are able to undergo apoptosis during hypoxia [19]. CO, undergoing a series of chemical reactions, causes hypoxia by combining with haemoglobin. This suggests that cardiomyocytes may undergo apoptosis in cases of carbon monoxide intoxication. However, the DNA laddering pattern was not detected. It is suggested that this is a problem due to the quantity of fragmented DNA. In other cases such as MAP intoxication, tetrodotoxin intoxication, alcohol intoxication, asphyxia, head injury, heart injury, and myocarditis, no apoptotic cells were found. With regard to cases of MAP intoxication, a study by Iwasa et al. [16] reported that MAP induced cell death of the thymic and splenic lymphocytes via apoptosis in an acute rat MAP intoxication model. Although they could not detect cardiomyocyte apoptosis, they suggested that MAP and/or its metabolites might directly induce apoptosis of target cells as one of the possible mechanisms. This negative result is in agreement with the present finding of human MAP intoxication. However, according to Graybiel et al. [20], certain oncogenes induced with MAP might act as a trigger for apoptosis, so it should be taken into consideration. Further investigations are needed to clarify these findings for MAP-induced cell death via apoptosis. Therefore, we could not apply TUNEL-positive cells as a diagnostic marker to these present cases.

Ultimately, only AMI cases autopsied within 20 h of death and where the time lapse between onset and death was within 2 h showed a positive result in TUNEL staining and the DNA laddering method. Although the mechanisms underlying this cardiomyocyte apoptosis are unclear, we suggest the application of apoptotic cells as a diagnostic marker during the early stages of AMI in forensic autopsy cases.

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