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TUNEL: a useful screening method in sudden cardiac death

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Abstract The primary objective of this study was to investigate if detection of apoptosis in the heart can be used to diagnose early myocardial ischaemia. The material consisted of myocardial tissue from autopsy cases: 10 cases with occlusive, thrombotic coronary artery disease and acute myocardial infarction, 10 cases of sudden cardiac death without coronary artery disease (CAD) and 8 controls without cardiovascular disease and with known causes of death. Necrotic changes in the myocardium were detected with hematoxylin-erythrosin-saffron, Mallory's PTAH stain and with antibodies against complement 9. Apoptotic nuclei were visualised with two different kits using the terminal deoxynucleotidyl transferase-mediated desoxyuridinetriphosphate nick end-labeling (TUNEL) method on histological sections. In the patients with CAD, early myocardial infarction was found in one defined area of the ventricular wall; apoptotic myocyte nuclei were observed not in the necrotic lesions, but evenly spread usually without a gradient, all over the myocardium with a mean number per high power field of 29% (range 3–56%) of the total number of myocyte nuclei. In the sudden cardiac deaths without CAD, necrosis was scarce and distributed both focally and irregularly in both the left and right ventricular walls. With few exceptions, the percentage of apoptotic myocyte nuclei exceeded 20% in all sections (mean 24%, range 0–68%). No difference was seen between patients with CAD and those without CAD ($p > 0.05$). With the TUNEL method, positively stained nuclei were seen very early and extensively all over the myocardium. It is not certain that they represent true apoptosis induced by ischemia, but TUNEL appears to be a useful screening method in cases where sudden cardiac death is suspected.

Keywords Apoptosis · Myocardial infarction · Sudden death · Immunohistochemistry · TUNEL

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Introduction

Sudden cardiac death (SCD) is still a diagnostic challenge to the clinical or forensic pathologist. In the majority of cases, death is probably due to acute coronary artery occlusion by a ruptured placque and/or thrombus [1]. In other cases, especially in young people, the cause might be viral myocarditis, cardiomyopathies, idiopathic arrhythmias, or arrhythmogenic right ventricular dysplasia (ARVD) [2, 3, 4]. Microscopic changes in sudden cardiac death, e.g. edema, wavy fibres and contraction bands, even when due to coronary artery disease (CAD), are often sparsely detected with the common staining methods such as hematoxylin-eosin. With other methods, especially the immunohistochemical ones, early ischaemic lesions and acute myocardial infarctions (AMI) can sometimes be diagnosed [5, 6, 7, 8].

In sudden death in young adults without CAD, a sudden lethal ventricular arrhythmia is often suspected. Some cases might be due to a previously undiagnosed cardiomyopathy, such as ARVD. The increased apoptosis in the right ventricular myocardium reported in ARVD might explain the disease [9, 10], but apoptosis occurs also in myocardial ischaemia [11, 12, 13, 14, 15].

Since both forensic autopsies and clinical autopsies from the clinic of thoracic surgery are carried out at our department, we see numerous deaths from CAD and several cases of SCD every year. We have developed special histological stains as well as immunohistochemical methods for routine use in the diagnosis of these cases.

Aiming to investigate the usefulness of the TUNEL method in the diagnosis of early ischaemic injury, parallel sections from the myocardium in selected cases were studied.

Materials and methods

The study included 10 cases of CAD with early infarction (group A), 10 cases of sudden death without coronary artery or valvular disease (group B) and 8 controls (group C). AMI patients (group A)

were selected from both forensic and clinical autopsies. In the forensic cases the inclusion criteria were occlusive coronary artery disease in combination with either a larger area of coagulation necrosis and/or contraction bands seen with hematoxylin-erythrosine-saffron and/or Mallory's PTAH stains. In the clinical cases there were complementary electrocardiographic and biochemical data. Group B consisted of consecutive autopsies during a period of 1 year. The inclusion criteria were absence of coronary arteriosclerosis, normal heart weight and no other plausible explanation, natural or unnatural, for the death. The majority of the individuals in group B was previously healthy persons without hospital records. Case 11 and 13 had been investigated for irregular heart-beats at the clinic of pediatric cardiology several years previously. The autopsies were performed at the Department of Forensic Medicine in Linköping, Sweden, during 1999 and 2000. The delay between death and autopsy varied between 1 and 4 days. Sudden death was defined according to the WHO criteria, i.e. death within 24 h of onset of symptoms. Cases of previously healthy persons found dead less than 24 h after they were last seen in life were classified as unwitnessed sudden deaths.

Necrosis and apoptosis were visualised with antibodies against the terminal complement complex, i.e. C9 [16] and two varieties of the terminal deoxynucleotidyl transferase-mediated desoxyuridinetriphosphate nick end-labeling (TUNEL) method (Apoptag and Cardiotacs) using immunohistological methods.

Heart tissue was fixed in 4% buffered formaldehyde at pH 7.4 and embedded in paraffin after 3 days. From each heart a complete transversal section was cut about 2 cm below the valvular plane. From this ring six blocks measuring $1 \times 1 \times 0.2$ cm were cut perpendicularly, six adjacent blocks horizontally and 4 μm paraffin sections were stained with hematoxylin-erythrosin-saffron [17] and Mallory's PTAH. Out of these 12 blocks, 4 were selected from each case for immunohistochemistry and TUNEL. In group A, one section from infarction in the left ventricular wall (A INF), one section from the diagonally opposite side of the left wall (A LV 2), and sections from the right anterior (A RV 1) and posterior walls (A RV 2) were studied. In groups B and C two sections from the left ventricle (LV 1 and LV 2) and anterior and posterior right ventricle (RV 1 and RV 2) were studied. Monoclonal C9 antibodies (The Binding Site, Birmingham, UK.) were diluted 1:200. The sections were pretreated with proteinase-K for 1 min and incubated with the antibody solution for 1 h. The antigen antibody reaction was visualised with the avidin-biotin-peroxidase method. The Apoptag (Intergen, New York) and Cardiotacs (RD-systems, Minneapolis, Minn.) kits were applied according to the instructions of the manufacturers. The protocol for Apoptag was slightly modified by substituting the contrast stain methyl green with a 0.01% solution of light green SF (Merck).

Apoptotic cell nuclei were counted over a 1×1 cm eye-piece grid in $400 \times$ magnification, 2×20 fields in each section and the mean was calculated and expressed as the percentage of total cardiac myocyte nuclei per field. The mean number of myocyte nuclei per field was calculated for the left and right ventricular myocardium separately by counting 100 fields selected randomly in the controls. The mean for the left ventricle was 27 and for the right ventricle 19 nuclei per field. In sections displaying myocardial necrosis, nuclei were not counted in the necrotic area.

Statistical calculations were made in the Statview program (Abacus Concepts, Berkley, Calif.) including mean values, standard deviations, simple linear regression and the paired t-test.

This study has been reviewed and approved by the Ethical Committee at the University of Linköping no 00–317.

Results

Morphologically, in the CAD cases (group A, Table 1), fresh myocardial infarction was seen subendocardially over an area of about one-third to one-half of the wall thickness. In the infarction area a combination of contraction band necrosis (100% of the cases) located peripherally, coagulative necrosis and slight infiltration of neutrophils (50%) in the centre of the lesion and positive reactions for C9 (90%) was seen. In the non-CAD cases (group B, Table 2) massive and focal contraction band necrosis was seen (AMI) in three cases and in five cases scarce and multifocal contraction bands in two or more sections, with similarly distributed C9 positive myocytes (GMI). In no case there was any infiltration of neutrophils or coagulative necrosis with eosinophilia. In the controls no ischaemic changes were seen (group C, Table 3).

Resuscitation attempts were done in 6 cases (see [a] in Tables 1 and 2), but this did not appear to have influenced the degree of apoptosis or the degree of contraction band necrosis.

Table 1 A summary of 10 cases of sudden cardiac death with coronary artery disease and myocardial infarction (*PM delay* postmortem delay, i.e. period between death and autopsy, *CB* contraction band necrosis, *CN* coagulation necrosis, *C9 positive* positive for antibodies against complement 9, *AMI* acute myocardial infarction, *MI* section with myocardial infarction, *LV 1* section from left ventricular wall far from the area with MI and showing no morphological signs of necrosis, *RV 1* right ventricle, anterior wall, *RV 2* right ventricle, posterior wall)

Case	Age (years)	PM delay (days)	Diagnosis	CB	CN	C9 Positive	% Apoptosis MI	% Apoptosis LV 1	% Apoptosis RV 1	% Apoptosis RV 2
1[a ε]	74	4	AMI	Yes	No	Yes	29	36	36	9
2	75	4	AMI	Yes	No	Yes	3	39	33	31
3[a ε]	71	4	AMI	Yes	No	Yes	56	54	56	61
4	57	3	AMI	Yes	Yes	Yes	11	34	49	41
5	43	4	AMI	Yes	No	No	24	30	62	35
6[a ε]	80	3	AMI	Yes	Yes	Yes	21	17	52	48
7	65	4	AMI	Yes	Yes	Yes	30	22	63	69
8	57	2	AMI	Yes	No	Yes	37	33	47	67
9[a]	29	4	AMI	Yes	Yes	Yes	30	22	44	58
10	46	4	AW	Yes	Yes	Yes	22	34	52	64
Mean	59.7	3.6					26	32	49	48

[a] Resuscitation with electro-conversion was done
[ε] Clinical autopsies

Table 2 A summary of 10 cases of suspected sudden cardiac death without coronary artery disease (*ARVD* arrhythmogenic right ventricular dysplasia; *GMI* generalised myocardial ischemia, i.e. randomly distributed contraction band necrosis in two or more sections; *LV 1* left ventricle, anterior wall; *LV 2* left ventricle, posterior wall)

Case	Age (years)	PM delay (days)	Diagnosis	Ischaemia Location	CB	CN	C9 Positive	% Apoptosis LV 1	% Apoptosis LV 2	% Apoptosis RV 1	% Apoptosis RV 2
11[a]	14	4	AMI	RV Ant	Yes	No	Yes	7	3	3[b]	1
12	20	4	ARVID	Diffuse	Yes	No	Yes	31	34	59	5
13	16	4	GMI	Diffuse	Yes	No	Yes	3	3	23	1
14	43	4	AMI	LV Post	Yes	No	Yes	27[b]	19	50	27
15[a]	23	4	ARVD	Diffuse	Yes	No	No	32	31	40	23
16	63	4	AMI	LV RV Ant	Yes	No	Yes	46[b]	42	68[b]	68
17	44	4	GMI	Diffuse	No	No	Yes	34	39	30	34
18	24	3	AMI	Diffuse	Yes	No	Yes	54	51	63	38
19	38	2	ARVD	Unknown	No	No	No	0	0	0	2
20	54	3	GMI	Diffuse	Yes	No	No	17	31	6	6
Mean	33.9	3.6						25	25	34	21

[a] Resuscitation with electro-conversion was done
[b] Section with infarction. For the other abbreviations see table notes of Table 1

Table 3 A summary of eight controls who died from non-cardiac causes (*LV* left ventricle; *RV* right ventricle)

Case	Age (years)	PM delay (days)	Diagnosis	CB	CN	C9 Positive	% Apoptosis LV	% Apoptosis RV
21	43	2	Knife wound	No	No	No	0	0
22	56	2	Knife wound	No	No	No	0	0
23	34	4	Hanging	No	No	No	0	0
24	36	4	Overdose	No	No	No	0	0
25	46	2	Gunshot	No	No	No	0	0
26	10	2	Gunshot	No	No	No	0	0
27	36	4	Asphyxia	No	No	No	0	0.5
28	54	1	Drowning	No	No	Yes	0	0.5
Mean	39.3	2.6					0	0.1

For the other abbreviations see table notes of Table 1

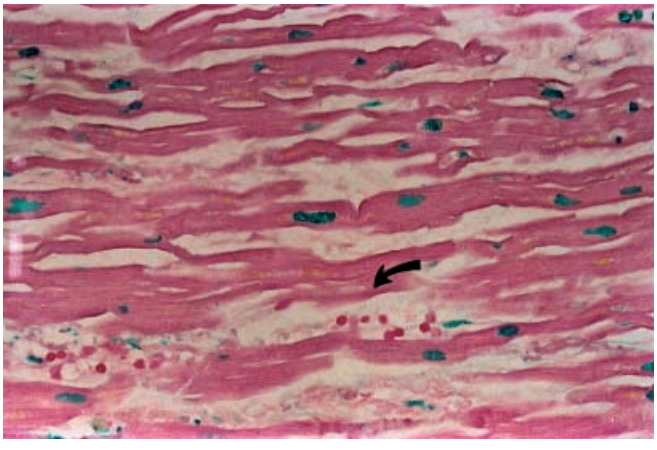

Fig. 1 Subendocardial myocardial infarction in the left ventricular wall. The edge of necrosis is seen in the lowest part of the picture (*arrow*). In the border zone there are extravasated red blood cells, edema and some disintegrated myofibres. In the middle and upper part the edema is less pronounced and the myofibres are intact. Extensive TUNEL-positivity (*green nuclei*) is seen in the vicinity and demonstrated with Cardiotacs (Original magnification × 400)

With the TUNEL method, apoptosis was seen in all sections of group A, but scarcely within necrotic lesions where the cellular nuclei were either absent or severely disintegrated. The mean percentages of apoptotic myocyte nuclei were 26% in the vicinity of infarction (MI) and 32% in the myocardium far away from the infarct (LV2) (Fig. 1) and in the right ventricular myocardium, mean 49% (RV 1) and 48% (RV 2). In sudden cardiac deaths without CAD, diffuse TUNEL positivity was seen in both the left (mean values 25 and 25%) (Fig. 2) and right heart (mean values 34 and 21%). The agreement between the two apoptosis kits (Cardiotacs and Apoptag) was good (r = 0.88). Morphologically, the TUNEL-positive cells did not show any signs of necrosis such as swelling or eosinophilia. The cell nuclei were either of normal size or slightly pyknotic, and sometimes with irregular and marginated chromatin.

The differences in degree of apoptosis between groups A and C, and groups B and C were statistically significant ($p < 0.05$) but comparing groups A and B, no such differences were seen. There was no correlation between the degree of apoptosis, post-mortem delay, age of the patient, or the occurrence of resuscitation attempts.

Fig. 2 TUNEL-positive myocyte nuclei (*brown*) demonstrated with the Apoptag. The section is from the left ventricle in a case of sudden cardiac death without coronary artery disease or myocardial infarction.(Original magnification × 400)

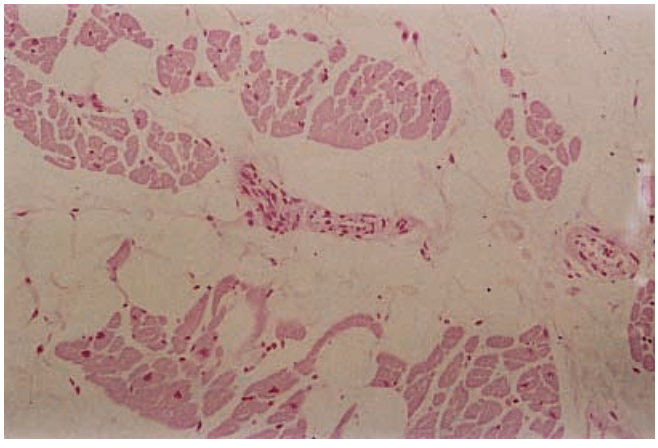

Fig. 3 Control case: a patient with fatty infiltration of the myocardium after death due to drowning. No apoptosis of the cardiac myocytes is visible in a section from the right ventricular wall (Cardiotacs, original magnification × 100)

In the eight controls, TUNEL-positive myocyte nuclei were not seen (Fig. 3), but in a few myocyte nuclei in the subendocardium of the right ventricle in 2 cases. Apoptosis was sometimes detected also in a few interstitial cells.

Discussion

The results of this study suggest that acute myocardial infarction causes generally increased apoptosis of the myocytes in the whole myocardium, not only in the compromised area. This was seen in all 10 CAD cases. Furthermore in 9 of the 10 non-CAD casualties similar changes were seen.

Apoptosis has previously been shown to occur peripherally in acute myocardial infarcts, whereas only necrosis is to be found in the centre of the lesion [11, 12, 13, 14]. Localised areas of pure apoptosis after ischaemic injury have also been seen, however [15] and at least two studies have reported apoptosis far from the infarct lesion [14, 18]. Piro et al. [18] suggested that the extensive apoptosis in non-infarcted areas might be due to mechanical stress. They used the TUNEL method and DNA electrophoresis to corroborate their findings.

In the present study apoptosis was also seen in cases without significant coronary disease or morphological signs of myocardial infarction.

In group A, generalised ischaemia and progressive myocardial dysfunction after infarction might account for the observed apoptotic changes. In group B the pathogenic mechanisms are not clear. It is conceivable that in those cases repeated short episodes of ventricular arrhythmia and subsequent dysfunction may have preceded death. The TUNEL-positivity that was demonstrated in group B was in most cases accompanied by small and scattered foci of C9-positive (necrotic) myocytes indicating episodes of global ischaemia 1 or a few hours before death.

The specificity of the TUNEL method has been questioned in studies on the liver [19], where it was suggested that positive results could be due to post-mortem autolysis. In an experiment in our laboratory, pieces of cardiac tissue were left to decompose in room temperature for various times. The preliminary results indicate that false TUNEL-positivity can occur with the Cardiotacs kit (but not with Apoptag) due to inadequate fixation (unpublished results). Another source of error might be that the TUNEL method does not adequately discriminate between apoptotic and oncotic cells. In experimental studies on ischaemic rabbit myocardium it was found that DNA fragmentation of oncotic myocytes occurred, resulting in TUNEL-positive nuclei [20]. Although differences between rabbits and humans might exist also regarding apoptosis and oncosis, it cannot be ruled out that the TUNEL-positive myocytes that we have observed could be a mixture of apoptotic, autolytic and oncotic cells. In the present study both TUNEL kits gave similar results and the eight control cases were all negative except for a few apoptotic nuclei in two cases. It therefore seems unlikely that the TUNEL-positivity in our cases are due only to post-mortem autolysis or fixation artefacts.

To the forensic and clinical pathologist it is important to find a method that detects early myocardial ischaemia and has a high specificity. One such method is the immunhistochemical demonstration of the terminal complement complex in myocardial infarctions [16]. Experimentally, the terminal complement complex, including C9, has been demonstrated in infarctions as early as 3 h after coronary ligation in the rat [21] and apoptosis has been demonstrated even earlier, i.e. after 45 min in the rat [12]. In cases of sudden cardiac death in humans the exact age of the lesion, if any, is seldom known.

Is extensive apoptosis/oncosis the important feature of SCD? The process of apoptosis is rapid, and is usually completed within 6 h [15]. If our results truly reflect apoptosis, between 20 and 50% of the myocardium would have disappeared within that time. This would certainly explain why these individuals died so suddenly. However, to confirm this, experimental studies are needed.

In conclusion, by use of the TUNEL method on histological sections of the myocardium in patients who died suddenly and unexpectedly, extensive and distinct nuclear positivity, which might represent apoptosis and/or oncosis, was seen in most cases. The TUNEL method appears to be a sensitive and specific screening method when SCD is suspected.

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References

1. Virmani R, Roberts WC (1987) Sudden cardiac death. Hum Pathol 18:485–492
2. Thiene G, Nava A, Corrado D, Rossi L, Penelli N (1988) Right ventricular cardiomyopathy and sudden death in young people. N Engl J Med 318:129–133
3. Goodin JC, Farb A, Smialek JE, Field F, Virmani R (1991) Right ventricular dysplasia associated with sudden death in young adults. Mod Pathol 4:702–706
4. Fornes P, Ratel S, Lecomte D (1998) Pathology of arrhythmogenic right ventricular cardiomyopathy/dysplasia – an autopsy study of 20 forensic cases. J Forensic Sci 43:777–783
5. Ishiyama I, Kamiya M, Rose M, Komuro E, Takatsu A (1982) Fulminant deletion of myoglobin from myocardial fibres in state of acute cardiac failure inducing sudden cardiac arrest. Lancet 25:1468–1469
6. Schäfer H, Mathey D, Hugo F, Bhakdi S (1986) Deposition of the terminal complement complex in infarcted areas of human myocardium. J Immunol 137:1945–1949
7. Leadbetter S, Wawman HM, Jasani B (1989) Immunocytochemical diagnosis of early myocardial ischaemic/hypoxic damage. Forensic Sci Int 40:171–180
8. Ortmann C, Pfeiffer H, Brinkmann B (2000) A comparative study on the immunohistochemical detection of early myocardial damage. Int J Legal Med 113:215–220
9. Mallat Z, Tedgui A, Fontaliran F, Frank R, Durigon M, Fontaine G (1996) Evidence of apoptosis in arrhythmogenic right ventricular dysplasia. N Engl J Med 335:1190–1196
10. Basso C, Thiene G, Corrado D, Angelini A, Nava A, Valente M (1996) Arrhythmogenic right ventricular cardiomyopathy. Dysplasia, dystrophy, or myocarditis? Circulation 94:983–991
11. Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipo-Pulkki L-M (1997) Apoptosis in human acute myocardial infarction. Circulation 95:320–323
12. Kajstura J, Liu Y, Baldini A, Li B, Olivetti G, Leri A, Anversa P (1998) Coronary artery constriction in rats: necrotic and apoptotic myocyte death. Am J Cardiol 82:30K–41K
13. Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, Fujiwara H (1996) Expression of bcl-2 protein, an inhibitor of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. Circulation 94:1506–1512
14. Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, et al, (1996) Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. J Mol Cell Cardiol 28:2005–2016
15. James TN (1998) The variable morphological coexistence of apoptosis and necrosis in human myocardial infarction: significance for understanding its pathogenesis, clinical course, diagnosis, and prognosis. Coron Artery Dis 9:291–307
16. Edston E, Kawa K (1995) Immunohistochemical detection of early myocardial infarction. An evaluation of antibodies against the terminal complement complex (C5b-9). Int J Legal Med 108:27–30
17. Edston E, Gröntoft L (1997) Saffron – a connective tissue counterstain in routine pathology. J Histotechnol 20:123–125
18. Piro FR, di Gioia CRT, Gallo P, Giordano C, d'Amati G (2000) Is apoptosis a diagnostic marker of acute myocardial infarction? Arch Pathol Lab Med 124:827–831
19. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis and autolytic cell death: a cautionary note. Hepatology 21:1465–1468
20. Ohno M, Takemura G, Ohno A, Misao J, Hayakwa Y, Minatoguchi S, Fujiwara T, Fujiwara H (1998) "Apoptotic" myocytes in infarct area in rabbit hearts may be oncotic myocytes with DNA fragmentation: analysis by immunogold electron microscopy combined with in situ nick end-labeling. Circulation 98:1422–1430
21. Väkevä A, Morgan BP, Tikkanen I, Helin K, Laurila P, Meri S (1994) Time course of complement activation and inhibitor expression after ischemic injury of rat myocardium. Am J Pathol 144:1357–1368