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ORIGINAL ARTICLE

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Apoptosis in right-ventricle biopsy is not predictive of graft survival

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Abstract Myocardial dysfunction is common in grafted hearts from brain-dead donors, but the mechanisms involved remain unclear, although apoptosis has been suggested to play an important role. In this study, we investigated the presence of apoptotic myocardial cells in donor hearts as compared to control hearts to determine whether pre-existing apoptosis can predict donor heart dysfunction. Apoptosis was studied by in situ DNA fragmentation assay and by Western Blotting for caspase-3, the pivotal executive caspase of the apoptotic pathway. We show that brain-death induced myocardial apoptosis was not predictive of myocardial dysfunction in transplanted hearts.

Keywords Cardiac transplantation · Brain-dead donors · Graft survival · Apoptosis

Introduction

Myocardial dysfunction is common in grafted hearts from brain-dead donors, but the mechanisms involved remain unclear [1], although apoptosis has been suggested to play an important role [1, 2]. Apoptosis is a cellular function necessary for the removal of unwanted cells during normal development or aging. It is controlled through a pathway regulated by aspartate-specific cysteine proteases named caspases [3]. Activation of pro-caspase-3 to caspase-3 is central in the apoptotic pathway. Caspase-3 has many substrates, cleavage of which mediate chromatin condensation and DNA fragmentation [4]. In this study, we investigated the presence of apoptotic myocardial cells in donor hearts as compared to control hearts to determine whether apoptosis can predict donor heart dysfunction.

Materials and methods

Heart tissue specimens

Specimens from a total of 12 hearts (Table 1) were examined using the following experimental protocol. Multiple right ventricular endomyocardial specimens were obtained from hearts from brain-dead (bd) subjects ($n=5$), one was fixed in 10% formalin for histopathological examination and the others were frozen in liquid nitrogen and stored at –80°C. Three of these

Table 1 Medical and biological data of patients (*M* male, *F* female, T traumatic, *H* hypoglycemia, *NA* not applicable, *NP* no clinical problem, *R* rejection, *AI* apoptotic index)

Parameter	Brain-dead donors					Control hearts						
Cases	bd1	bd2	bd3	bd4	bd5	c1	c2	c3	c4	c5	c6	c7
Age	27	19	19	42	21	61	1	47	65	59	80	71
Gender	M	M	M	F	F	M	M	M	F	F	M	F
Cause of brain death	T	T	T	T	H	NA	NA	NA	NA	NA	NA	NA
Transplantation	+	+	+	–	–	NA	NA	NA	NA	NA	NA	NA
Follow up	NP	R	R	0	0	NA	NA	NA	NA	NA	NA	NA
Contraction bands	+	+	–	+	+	–	–	–	–	–	–	–
Coagulation necrosis	–	–	–	+	–	–	–	–	–	–	–	–
Caspase-3 cleavage	+	+	+	+	+	+	–	+	+	–	–	–
AI (%)	19	16	0	5	2.5	0	0	0.6	0	0	1.2	2

hearts were subsequently transplanted. Patients were monitored for development of rejection and associated myocardial dysfunction by endomyocardial biopsies and transthoracic echocardiography at 1, 2, 3, 4, 8, 12, 24 and 52 weeks after transplantation. The biopsies were examined for the presence of rejection and graded according to International Society of Heart and Lung Transplantation criteria [5]. Control (c) left ventricular biopsy specimens ($n=7$) were obtained during surgery for mitral replacement without heart failure. Samples were taken from the apical area and processed as above.

Cardiac histopathology

Formalin-fixed biopsy specimens from all 12 subjects were paraffin-embedded, sectioned and stained with hematoxylin, eosin and safran. They were examined for signs of myocyte degeneration or inflammatory infiltration.

SDS–Page and Western blotting for caspase-3

Myocardial tissue fragments were homogenized in 20 mM Tris pH 8, containing 10% glycerol, 150 mM NaCl, 1% Triton×100, 5 mM EDTA, 1 mM Na_3VO_4, 0.1 M phenylmethylsulfonylfluoride. We separated 20 µg of total protein homogenates on 15% acrylamide gels with 3% stacking gels with 10% sodium dodecyl sulfate. The gels were equilibrated for 30 min in transfer buffer (25 mM tris base pH 7.5, 192 mM glycine, 20% methanol), then transferred to nitrocellulose membranes (Biorad, France) at 500 mA for 1 h. Nitrocellulose membranes were blocked using 3% non-fat dried milk in phosphate-buffered saline containing 0.05% Tween 20 for 1 h, then probed using a 1/1000 dilution of an anti-caspase-3 monoclonal antibody (Ab-1, Oncogene, France). After washing, blots were incubated for a further 1 h with a 1/20,000 rabbit anti-mouse secondary antibodies (Biorad). Protein bands were visualized using the Supersignal Ultra chemiluminescence substrate (Pierce, France). Proteins extracted from a lymphoma were used as caspase-3 positive control.

In situ DNA fragmentation assay

DNA fragmentation was evaluated by TUNEL (TdT-mediated dUTP Nick End Labeling) on 5-µm thick cryocut sections with a POD kit (Roche, France) according to the supplier's instructions, and sections were counterstained with hematoxylin. Negative controls were prepared by substituting phosphate-buffered saline for terminal desoxyribonucleotidyl transferase in the reaction mixture. The apoptotic index (AI) was calculated from the ratio of the positively stained myocardial cells to the whole myocardial cells for each sample at a magnification of 40 and expressed as a percentage.

Results

Patients

The brain-dead donors consisted of three men and two women with a mean age of 25.6 years (19–42 years) (Table 1). Brain-death was traumatic in four cases and followed hypoglycemia in the last case. Three of the hearts were successfully transplanted without dysfunction and followed up for 2 to 11 months (bd1, bd2, bd3). One recipient (bd1) never experienced signs of rejection, and two recipients presented with an episode of acute rejection (bd2 and bd3) (Table 1). Ventricular function, assessed by cardiac echocardiography, was normal except during rejection episodes. The control group, consisted of four men and three women, aged between 1 and 80 years (mean age 45 years).

Cardiac histopathology

The hearts from brain-dead donors showed contraction bands in four cases (bd1, bd2, bd4, bd5). Coagulation necrosis was observed in one case (bd4). In the control hearts, no contraction bands or myocyte degeneration were ever observed (Table 1).

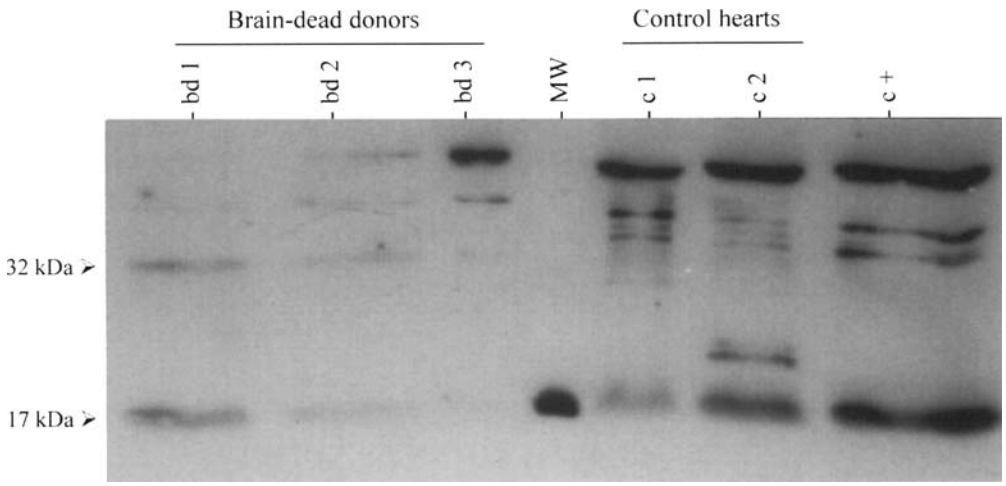

Fig. 1 Detection of caspase-3 activation by Western Blot. Total protein homogenates of myocardial tissues were subjected to 15% SDS-PAGE, blotted, probed with the anti-caspase-3 Ab-1 mAb and revealed by chemiluminescence. Expression of 32 kDa pro-caspase-3 (*top arrow*) and 17 kDa activated caspase-3 (*bottom arrow*) was shown in hearts of the brain-dead (bd 1, bd2, bd3), control heart (c1, c2) and positive apoptotic control subjects (c+)

Caspase-3 cleavage

Activation of caspase-3 was assessed by Western Blot using the Ab-1 monoclonal antibody. This mAb successfully detects both to the intact 32 kD proenzyme and the cleaved 17 kD enzyme. Additional unrelated bands between 45 and 60 kDa may also be detected according to the manufacturer's instructions. Cleavage products of caspase-3 were detected in all of heart biopsies from brain-dead donors. Similar fragments were detected in only three of seven control heart biopsies (Fig. 1).

DNA fragmentation

DNA fragmentation, as visualized by the TUNEL method involving immunostaining of end labeled DNA fragments, was detected in the myocytes from four of five biopsies from brain-dead donors (Fig. 2). The mean of AI was 8.5% (2.5–19%). Only three of seven control hearts biopsies showed a low apoptotic index (0.6–2%) (mean 0.5%) (Table 1).

Discussion

Myocardial dysfunction is common after heart grafting from brain-dead donors, but the mechanisms remain unclear [1]. Myocardial dysfunction can be severe and sometimes precludes the use of the heart for transplantation [6]. Some authors have suggested that apoptosis plays an important role in donor heart dysfunction after brain-death [1, 2].

Several techniques can be used to reveal apoptotic cells. We chose to study apoptosis in myocardial tissues by in situ visualization of DNA fragmentation, a consequence of apoptosis, by TUNEL; and detection of

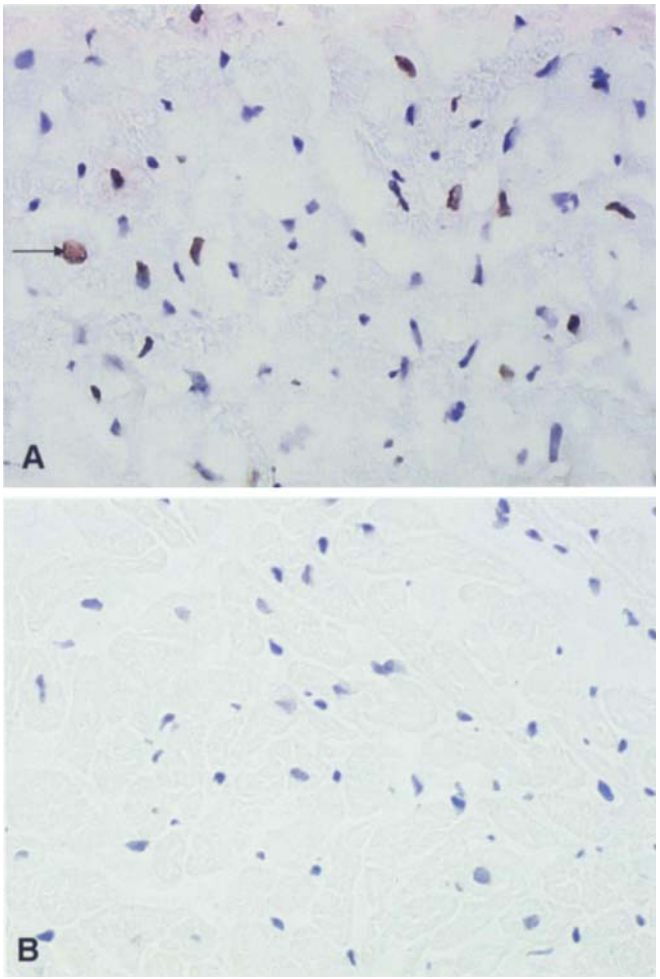

Fig. 2A,B Staining of myocardial sections by terminal in situ nick end labeling method (TUNEL). Brown nuclear staining indicates apoptotic cells (*arrow*). **A** heart of brain-dead donor (bd 5), **B** control heart (c3). Original magnification ×400

caspase-3 activation, the pivotal executive caspase of the apoptotic pathway [4], by Western Blotting.

This study indicates that brain-death induced increased myocardial apoptosis in the right ventricle. These findings can be explained by several mechanisms. Firstly, the catecholaminergic storm in brain-death results in a relative myocardial ischemia; subsequent reperfusion may induce apoptosis [7]. Secondly, rapid ventricular pacing, which can occur during brain-death [8] leads to apoptosis [9]. Finally, catecholamines increase the level of intracellular cyclic AMP, activate the protein kinase C and increase the intracellular calcium concentration [10]; all these molecules are implicated in the regulation of apoptosis [10]. However, our study shows that a high AI in the right ventricle after brain-death does not correlate with subsequent myocardial function in the grafted heart. Tissue samples were taken from the right ventricle, and so do not reflect lesions of the left ventricle, and therefore systolic function. In addition, the apoptotic cells may be replaced by division of myocytes to reconstitute the muscle mass of the myocardium, as has recently been described after myocardial infarction [11].

Left ventricular cardiomyocytes from the control patients showed an AI of less than 2%, similar to the basal ratio of apoptotic myocardial cells reported in the literature [12].

Contradictory results between the two methods were observed in five cases. In three cases, we detected caspase-3 cleavage by Western Blot without signs of DNA fragmentation by TUNEL (bd3, c1, c4). Recently, Miossec et al. [13] showed that activation of caspase-3 could occur in the absence of apoptosis. Furthermore, in two cases (c6, c7), we observed DNA fragmentation without caspase-3 cleavage suggesting that during brain-death, cardiomyocytes may die through a caspase-3 independent pathway.

Based on our preliminary study, brain death induces myocardial apoptosis but does not predict myocardial dysfunction. If this observation is confirmed by a larger study, it implies that systematic detection of apoptotic cells in potential donor hearts for grafting is not justified. To strengthen the observation that apoptosis is a consequence of brain death, the cascade leading to apoptosis as the Bcl2 molecules should be studied.

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