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ORIGINAL PRE-CLINICAL SCIENCE

Molecular markers of programmed cell death in donor hearts before transplantation

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BACKGROUND: In this study we investigate whether pro-apoptotic, pro-inflammatory and other early signaling markers indicative of increased propensity for cell death processes were evident in human donor heart allografts immediately before transplantation, and whether there is an association with primary graft failure.

METHODS: A prospective study was performed utilizing donor left atrial myocardium collected at the time of implantation of hearts from brain-dead donors (BDD, $n = 29$). In addition, to explore the potential of donor hearts from donation after circulatory death (DCD), myocardial samples were obtained during transplantation of lungs from DCD donors ($n = 6$). A comparator reference group ($n = 7$) consisted of left atrial specimens from patients undergoing mitral valve surgery.

RESULTS: Significantly raised levels of caspase-3 specific activity, activated hypoxia inducible factor-1 (HIF-1α) and 8-hydroxy-2'-deoxyguanosine were evident in the transplanted hearts (from BDD) that developed primary graft failure ($n = 11$). DCD hearts did not differ from BDD with regard to mRNA expression levels of FAS, Bax, IL-6 and caspase-3. Although DCD hearts exhibited lower caspase-3 specific activity and activated hypoxia-inducible factor-1 protein, they had higher levels of mRNA for NF-κB, Bnip3 and caspase-1 mRNA. Increased 8-hydroxy-2'-deoxyguanosine levels reflected greater oxidative stress and reactive oxygen species-related DNA fragmentation.

CONCLUSIONS: Our data indicate a significant role of pro-apoptotic and pro-inflammatory activity in allografts that subsequently exhibit primary graft failure. The relatively lower levels of apoptotic and inflammatory activity in DCD hearts suggest they may represent a potentially usable donor cardiac allograft pool. This possibility requires further detailed molecular and clinical research.

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Apoptotic cell death plays a critical role in ischemia-reperfusion (IR) injury, myocardial infarction, dilated cardiomyopathy and heart failure.[1] Despite preservation techniques utilized at procurement and during transport, heart transplantation inevitably subjects the donor heart to IR injury. Primary graft failure (PGF) occurring early after heart transplantation has an incidence of up to 30%, a

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mortality rate of 20%, and is directly related to ischemic time in the transplanted donor allograft.[2,3]

Apoptosis is regulated by extrinsic (FAS or tumor necrosis factor-alpha [TNF-α] receptor) and intrinsic pathways (external or internal signals such as hypoxia), initiating a cascade of effector caspases resulting in programmed cell death.[4,5] The nuclear factor-kappaB (NF-κB) family of transcription factors suppressing ischemia/hypoxia-induced apoptosis and hypoxia-inducible factor-1 (HIF-1α) may have a pro- or anti-apoptotic role depending on cell type and conditions.[6–9] Measurement of 8-hydroxy-2'-deoxyguanosine has been useful in determining the extent of DNA oxidative damage associated with the oxidative stress involved with apoptotic cell death.[10] Other mechanisms of cell death such as necrosis and autophagy also seem to be intimately connected to the apoptotic cascade, with increasing evidence of cross-talk between these intricate pathways.[11,12]

The aim of this study was to prospectively identify whether augmented levels of these markers may be evident early in donor heart biopsies, immediately before transplantation, and to determine whether such augmentation correlates with subsequent onset of PGF in recipients. Given the current interest in the potential of cardiac allograft utilization from donation after circulatory death (DCD), an additional aim was to also detect the presence of elevated markers in myocardium from this cohort and to compare levels to those in brain-dead donors (BDD).

Methods

Patients and sampling

This prospective study was approved by the human research ethics committee of The Alfred Hospital and individual patient consent was waived. Left atrial specimens were collected from donors after cold storage at the time of implantation of hearts from BDD and implantation of lungs from DCD donors at The Alfred Hospital between May 2009 and January 2011. Donor cardiac and pulmonary allografts were procured using standard techniques. Myocardial preservation was initiated with cardioplegic arrest, using St Thomas crystalloid cardioplegia solution with 22.4 mmol/liter aspartate (1 liter, 4° to 10°C). Cardiac allografts were stored in cold Euro-Collins solution and transported on ice. Pulmonary allografts were preserved with Perfadex (6 liters, 4° to 10°C; Vitrolife, Sweden), stored in cold Perfadex, and transported on ice. Total ischemic time was recorded as the time from aortic cross-clamp in the donor to release of aortic cross-clamp in the recipient. Primary graft failure (PGF) was defined as early (within first 72 hours post-operatively) graft dysfunction as demonstrated by right or left or biventricular hypocontractility and dilation associated with hypotension (systolic blood pressure <90 mm Hg) and raised capillary wedge pressure (>20 mm Hg) in the absence of any other identifiable cause, requiring inotropic and mechanical circulatory support.[2,3,13] Post-operative rejection episodes of ISHLT Grade 2R or higher on myocardial biopsy were recorded in all BDD recipients. Clinical data were censored at 12 months after the final enrollment.

Left atrial samples were collected for comparison from a reference group comprised of 7 patients undergoing mitral valve surgery. Samples were collected toward the end of the cross-clamp time and were stored and processed in the same way as the other atrial samples. Surgery was performed with cardiopulmonary bypass and cardioplegia administered as a 6:1 blood solution with our standard cardioplegia, which consists of potassium 10 mmol/liter, magnesium 16 mmol/liter, aspartate 22.4 mmol/liter and lignocaine 0.07 mmol/liter, and has an osmolality of 319 mOsm/liter. The solution was delivered at 5° to 10°C at 10- to 15-minute intervals.

Tissue processing and protein extraction

All tissue specimens were frozen and stored at -80°C until batch analyses for molecular studies were performed. Frozen ground tissue (~50 mg) was homogenized with a hand-held glass homogenizer in 10 volumes of buffer (0.062 mol/liter Tris, 0.154 mol/liter NaCl, 0.2 mmol/liter ethylene-diamine tetraacetic acid, 0.2% NP-40 [Tergitol] and 1/100 mammalian protease inhibitor cocktail [pH 7.4, 4°C]; Sigma-Aldrich). Cellular debris was removed by centrifugation (2,000*g*, 10 minutes, 4°C). Nuclear protein was extracted from ~100 mg frozen ground tissue[14] using reagents (Thermo Scientific).

8-Hydroxy-2'-deoxyguanosine

DNA was extracted and then analyzed for 8-hydroxy-2'-deoxyguanosine (8-OH-2-deoxyguanosine) content by high-performance liquid chromatography.[10]

RNA extraction

RNA was extracted from tissue (~60 mg) using TRI Reagent (Ambion/LifeTechnologies). Isolated RNA content was estimated using a spectrophotometer (Nanodrop; Ambion). The final RNA pellet was resuspended in 150 μl of RNAse-free water and stored at -80°C.

Real-time polymerase chain reaction

RNA extracts were diluted to 100 μg/ml and reverse transcribed into cDNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Relative gene expression was determined using commercially available TaqMan pre-designed human gene expression assays (ABI) for: HIF-1α (ID: Hs00936371_m1); caspase-1 (ID: Hs00354836_m1); caspase-3 (ID: Hs00234387_m1); FAS (ID: Hs00531110_m1); interleukin-6 (IL-6; ID: Hs00985639_m1); Bnip3 (ID: Hs00969289_m1); NF-κB1 (ID: Hs00765730_m1); and BAX (ID: Hs00180269_m1). Polymerase chain reaction (PCR) amplification and detection was performed on a PCR machine (Model 7900HT; ABI) for 2 minutes at 50°C, then 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 60°C and 60 seconds at 95°C. Relative gene expression (between gene of interest and a reference gene GAPDH [ID: Hs99999905_m1]) was determined from real-time quantitative PCR using the $2^{-\Delta\Delta Ct}$ method as per the ABI protocol.

Caspase-3

The activity of caspase-3 in tissue samples was determined via caspase assay (Promega), calculated from the *p*NA calibration curve ($r^2 = 0.995$) and expressed as picomoles *p*NA liberated/hour.