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Rejection Severity Directly Correlates With Myocyte Apoptosis in Pig-to-Baboon Cardiac Xenotransplantation

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Abstract

Background— The process by which cardiac myocytes die during xenograft rejection is incompletely understood. The presence of cardiac myocyte apoptosis in discordant xenotransplant models has been noted, yet no investigators have examined whether a relationship between myocyte apoptosis and rejection severity exists. Thus, we chose to further investigate this observation.

Methods— Eight explanted pig-to-baboon cardiac grafts with varying severities of rejection, as determined by hematoxylin and eosin histology, were examined for apoptosis by transmission electron microscopy (TEM) and TUNEL (terminal deoxynucleotide transferase-mediated digoxigenin-dUTP nick-end labeling) immunohistochemistry. In addition, Western blot analysis for the cleavage of the apoptosis regulatory proteins pro-caspase 8 and 3 was performed.

Results— Transmission electron microscopy revealed that a severely rejected graft displayed widespread condensation of nuclear chromatin, which is a characteristic morphologic feature of apoptosis. TUNEL staining verified this observation and allowed for the quantification of myocyte apoptosis in each graft. Subsequent linear regression analysis of the extent of myocyte apoptosis and rejection severity revealed a direct correlation ($R^2 = 0.757$, $p = 0.005$). In addition, Western blot analysis demonstrated that myocyte apoptosis involves the cleavage of pro-caspase 8 and 3.

Conclusions— Myocyte death in rejecting pig-to-baboon cardiac xenografts occurs through an apoptotic pathway and directly correlates with the severity of graft rejection. Further studies aimed at elucidating the apoptotic stimulus are therefore warranted. Moreover, our data suggest that antiapoptotic strategies may be of benefit in the treatment of xenograft rejection.

Rejection of solid organ xenografts is an incompletely understood phenomenon. Although hyperacute rejection of discordant xenografts has been largely overcome,^{1,2} the survival of functioning grafts beyond several weeks remains the exception, due in large part to delayed or acute vascular rejection.^{3,4}

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A great deal of work has been done to elucidate the pathways involved in xenorejection, yet very little work has focused on the ultimate mechanism by which myocytes die during the rejection process. The presence of cardiac myocyte apoptosis in discordant xenotransplant models has been noted incidentally,^{5–8} yet no systematic studies of this phenomenon or the potential mechanisms by which it occurs have been performed. The current study was therefore undertaken to investigate the relationship between cardiac myocyte apoptosis and xenograft rejection severity in a discordant xenotransplant model.

A cell may die by 1 of 2 pathways: necrosis or apoptosis. Necrosis is an energy-independent cell death pathway that ultimately leads to a local inflammatory reaction.⁹ This form of cell death can be initiated by various components of the immune system, including complement.¹⁰ In contrast, apoptosis is a highly conserved and regulated energy-dependent process.⁹ It occurs as part of embryologic development and tissue homeostasis, and is deranged in numerous disease processes. Apoptosis can be stimulated by numerous factors, including growth factor withdrawal,¹¹ ischemia,¹² ionizing radiation,¹³ and death receptor ligation.¹⁴ In addition, immune effectors such as cytotoxic T lymphocytes are capable of inducing apoptosis of target cells.^{15,16}

Apoptosis can be stimulated by death receptor ligation (eg, Fas ligand) or receptor-independent stimuli (eg, ultraviolet radiation).¹⁴ Receptor ligation results in the activation of a proximal set of proteases termed caspases (cysteine-dependent aspartase-specific protease) (eg, caspase 8), which sequentially activate other caspases.¹⁴ In most cases, this caspase cascade converges on the mitochondria to result in the release of proapoptotic factors (eg, cytochrome c), which results in the activation of a distal set of effector caspases, such as caspase 3.¹⁴ These effector caspases ultimately result in the activation of proteases and endonucleases that cleave host structural and regulatory proteins and DNA, resulting in the morphologic and biochemical features of apoptosis, including cell shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation.⁹ In the case of receptor-independent apoptosis, the proximal caspase cascade is often bypassed, and the stimulus acts directly upon the mitochondria, with subsequent activation of effector caspases.¹⁴ Regardless of the stimulus, it is possible to examine a cell for apoptosis morphologically (cell shrinkage and/or chromatin condensation), and/or biochemically (caspase activation/cleavage, DNA fragmentation).

The aims of this study were to determine whether myocytes from discordant cardiac xenografts die by apoptosis, and if so, to investigate whether the extent of apoptosis is related to rejection severity.

MATERIALS AND METHODS

Animals

Transgenic pigs constitutively expressing human CD46 served as cardiac xenograft donors, and adult olive baboons were recipients. All animals were housed and received humane care in accordance with the standards established by the Institutional Animal Care and Use Committee of the Mayo Clinic and Foundation and as described in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Heterotopic Cardiac Transplantation

Donor procedure—A standard technique of heterotopic cardiac xenotransplantation was used.¹⁷ With the animal under general endotracheal anesthesia, a median sternotomy was performed and the donor heart prepared for harvest. After heparinization, cold crystalloid cardioplegia solution (15 ml/kg) (Plegisol; Abbott Laboratories, North Chicago, IL) was

infused antegrade into the aortic root after cross-clamping the aorta. The heart was excised in a standard fashion. A small atrial septal defect was created to improve atrial blood washing.

Recipient procedure—Under general endotracheal anesthesia, through a lower midline abdominal incision, the infrarenal abdominal aorta and inferior vena cava were isolated, and the porcine heart graft was implanted by anastomosis of the donor aorta to the recipient abdominal aorta end to side, and the donor pulmonary artery to the recipient inferior vena cava in an end-to-side fashion. The transplanted heart is therefore beating and perfused, but not loaded.

Immunosuppression

All recipients received tacrolimus, sirolimus, and dexamethasone. Treatment with anti-CD20 monoclonal antibody (17 mg/kg IV) was begun on preoperative Day 14, and was continued every 7 days thereafter until graft explant. Infusions of NEX1285, an α -galactosylpolyethylene glycol conjugate that binds to immunoglobulin (Ig) M and IgG Gal(α 1–3)Gal antibodies, were provided daily after transplantation until graft explant.

Monitoring and Assessment of Graft Function

Cardiac xenograft function was assessed by palpation, echocardiography, and intramyocardial electrography. Blood samples were collected twice weekly for hematology, clinical chemistry, and drug level determination. Xenograft explant was performed at the time of graft failure, which was defined as cessation of beating confirmed by palpation and echocardiography, or at the time of necropsy in the case of animal death. At the time of explantation and recipient euthanasia, a complete postmortem examination, including histology of the brain, heart, lungs, liver, kidneys, and any other grossly abnormal organ, was performed.

Histopathologic Examination

Representative sections of left ventricle were fixed in formalin or quick frozen in liquid nitrogen for storage at -80°C in OCT media (Tissue-Tek; Sakura Finetek, Torrance, CA). After fixation, paraffin sections underwent hematoxylin and eosin staining to determine severity of rejection as a percentage of myocardium. Changes considered to represent xenograft rejection included myocyte vacuolization, interstitial edema, intramyocardial hemorrhage, loss of myocyte nuclei, homogenization of cytoplasm, loss of cross striations, vascular congestion, and thrombosis.¹⁸ In addition, cryostat sections were immunostained for deposition of IgG, IgM, and C5b by means of standard indirect immunoperoxidase avidin-biotin complex methods. The overall rejection severity for each animal was expressed as a percentage on the basis of extensive review of the clinical record, histology, and immunopathology.

Transmission Electron Microscopy

Frozen left ventricle samples were fixed in 1% glutaraldehyde and 4% formaldehyde in phosphate-buffered saline. After fixation, samples were rinsed in phosphate-buffered saline and postfixed in 1% osmium tetroxide for 1 hour. Samples were rinsed in distilled water and stained en bloc with 2% uranyl acetate (30 minutes, 60°C), and rinsed again in distilled water. Samples were then dehydrated progressively with increasing amounts of ethanol in propylene oxide and embedded in multi-component epoxy resin (Spurr kit; EBS Sciences, Agawam, MA). Sections of 90 nm were cut and stained with lead citrate, then examined by transmission electron microscopy (TEM) (JEOL ExII; Jeol, Peabody, MA).

TUNEL Immunohistochemistry

TUNEL (terminal deoxynucleotide transferase-mediated digoxigenin-dUTP nick-end labeling) staining was performed on formalin-fixed, paraffin-embedded tissue from all 8

animals (Molecular Histology, Little Rock, AR). Quantification of apoptotic cells was performed by a panel of 3 reviewers, blinded as to sample origin. TUNEL-positive and -negative cells were enumerated, and TUNEL positivity was expressed as a percentage of total cells. The scores of the 3 reviewers were averaged to derive a final percentage.

Western Blot

Frozen left ventricular tissue was homogenized on ice in RIPA buffer (150 mmol/liter NaCl, 1 % Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium do-decyl sulfate, 50 mmol/liter Tris pH 8.0) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN). Homogenates were centrifuged at $700 \times g$ for 5 minutes at 4°C to remove cellular debris, and the supernatant was stored at -80°C. Supernatant protein concentration was determined (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 40 µg of protein. After transfer to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) and a 1-hour block with 2% bovine serum albumin at room temperature, the membrane was incubated overnight at 4°C with rabbit polyclonal primary antibodies for caspase 8 (Chemicon International; Temecula, CA; 1:100) or caspase 3 (Cell Signaling Technology; Beverly, MA; 1:500). After incubation with an anti-rabbit–horseradish peroxidase–conjugated secondary antibody (1:10,000, 1 hour, room temperature; Amersham Bioscience, Piscataway, NJ), proteins were visualized with enhanced chemiluminescence (Amersham Bioscience).

Statistical Analyses

The relationship between myocyte apoptosis and rejection severity was analyzed by linear regression.

RESULTS

Cardiac Myocytes From a Rejected Graft Have Morphologic Features of Apoptosis

The morphologic features of cardiac myocytes from a graft with a rejection severity of 90% were examined. By means of TEM, we observed widespread condensation of nuclear chromatin, which is a characteristic morphologic feature of apoptotic cells⁹ (Figure 1A). Moreover, the apoptotic cells had structural features indicating that they were myocytes. For comparative purposes, we used tissue from an animal that died as a result of hemorrhage shortly after transplantation. Histologic examination confirmed that this graft displayed no appreciable features of rejection (rejection severity of 0%). TEM examination displayed no nuclear chromatin abnormalities, nor were any other morphologic abnormalities observed (Figure 1B).

Cardiac Myocyte Apoptosis Correlates With Rejection Severity

On the basis of the TEM results suggesting the presence of myocyte apoptosis, we next performed TUNEL immunohistochemistry on sections of left ventricle from a series of 8 grafts with varying rejection severities (Table 1). TUNEL positivity for each graft was compared with its corresponding rejection severity (Figure 2). In each case, TUNEL-positive cells were phenotypically myocytes. Linear regression analysis of the percentage of cardiac myocyte apoptosis and rejection severity was performed, revealing a strong, direct correlation ($R^2 = 0.757$; $p = 0.005$; Figure 3).

Cardiac Myocyte Apoptosis Involves the Cleavage of Pro-Caspase 8 and 3

To examine the apoptotic pathway by which cardiac myocytes die, we analyzed tissue lysate for the prototypical apoptotic proteins caspase 8 and caspase 3. Lysates were obtained from Animal 7, which displayed extensive apoptosis by TUNEL staining, and from Animal 1, which showed no apoptosis. Western blot analysis demonstrated that myocyte apoptosis is associated

with the cleavage of pro-caspase 8 and 3 (Figure 4). In contrast, cleavage of pro-caspase 8 or 3 were not observed in lysate obtained from a graft with rare TUNEL positivity (Figure 4). Thus, cardiac myocyte apoptosis in xenograft rejection is associated with pro-caspase 8 and 3 cleavage.

DISCUSSION

Similar to previous reports, the data presented here demonstrates that myocyte death in a pig-to-baboon cardiac transplantation model occurs through an apoptotic pathway. Importantly, we also show for the first time that the extent of myocyte apoptosis correlates with the severity of graft rejection.

The significance of determining whether a disease process is mediated through an apoptotic or necrotic cell death pathway lies in potential future therapeutic strategies. Apoptosis has been implicated in the pathogenesis of a number of conditions, including neurodegenerative¹⁹ and infectious diseases,²⁰ heart failure,²¹ cardiac ischemia,²² and sepsis.²³ The use of experimental inhibitors of apoptosis such as soluble caspase inhibitors (eg, z-VAD-fmk),²⁴ the overexpression of antiapoptotic proteins (eg, Bcl-2,²⁵ XIAP²⁶), and siRNA of key apoptotic proteins (eg, caspase 8,²⁷ Fas ligand²⁸) in animal models of these diseases have demonstrated that antiapoptotic approaches are of potential therapeutic benefit. For instance, overexpression of the antiapoptotic protein Bcl-2 has been shown to inhibit apoptosis and promote survival of allogeneic transplanted hepatocytes.²⁹ In addition, antiapoptotic proteins have also been implicated in the development of xenograft accommodation. Studies in hamster heart-to-rat models demonstrate that accommodated grafts display an upregulation of survival/antiapoptotic gene expression, including Bcl-2 and Bcl-xL.^{30,31} In contrast, rejected hearts do not express these antiapoptotic genes, but rather express a predominance of proapoptotic genes including Bax, Bad, and caspase 3.³¹

Apoptosis can be induced by numerous stimuli, such as cytotoxic T lymphocytes, natural killer cells, soluble FasL, growth factor withdrawal, and ischemia. Although our study did not evaluate potential stimuli responsible for the induction of myocyte apoptosis, we demonstrate that apoptosis does occur and that it is directly correlated with the severity of graft rejection. In addition to apoptosis, myocardial histologic features historically associated with coagulative necrosis, including loss of myocyte nuclei, homogenization of cytoplasm, and loss of cross striations, were also observed.¹⁸ Indeed, apoptotic and necrotic cell death are not mutually exclusive events. For example, numerous disease processes, including cerebral³² and myocardial³³ ischemia, demonstrate the simultaneous presence of both apoptosis and necrosis. Although the contribution of each form of cell death to the ultimate pathogenesis of discordant cardiac xenorejection is unknown, our results raise the possibility that antiapoptotic agents, such as those already used in various animal models of apoptotic disease, may be of potential benefit in xenotransplantation.

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Figure 1. Transmission electron micrographs of cardiac xenografts with rejection severities of 90% (Animal 1) (A) and 0% (Animal 7) (B). Note the condensed nuclear chromatin in the rejected graft (arrow), which is absent in the nonrejected graft.

Figure 2.

Sections of explanted cardiac grafts from Animals 1 (A), 5 (B), and 7 (C). The severity of rejection (as determined by hematoxylin and eosin [HE] staining), and extent of apoptosis (as determined by TUNEL [terminal deoxynucleotide transferase-mediated digoxigenin-dUTP nick-end labeling] staining), are indicated below each panel. Asterisks in the HE-stained photomicrographs of B and C indicate broad areas of rejection characterized by loss of myocyte nuclei, homogenization of cytoplasm, and loss of cross striations. Arrows in TUNEL-stained photomicrographs in B and C indicate apoptotic nuclei.

Figure 3. Linear regression analysis illustrating the relationship between the extent of cardiac myocyte apoptosis and graft rejection severity.

Figure 4. Western blot analysis of procaspase 8 and 3 cleavage in a graft displaying no apoptosis (Animal 1), and a graft displaying 90% apoptosis (Animal 7).

Table 1

Recipient Survival and Graft Characteristics*

Animal	Days after transplant	Rejection severity %	Extent of apoptosis %	Cause of death	Major findings at autopsy
1	0	0	0	Hemorrhage	Ischemic changes in kidneys and adrenal glands
2	60	20	40	Aspiration pneumonia	Ischemic changes in kidneys, adrenal glands, and native heart; PTLN (liver)
3	41	30	30	Sepsis	CMV pneumonia; ischemic changes in kidneys and adrenal glands
4	76	40	10	Sepsis	CMV pneumonia; PTLN (liver, small bowel)
5	68	60	60	Sepsis	Central hepatic necrosis (central venous congestion)
6	20	75	40	Hemorrhage	Ischemic changes in adrenal glands
7	92	90	90	Multiorgan dysfunction	Ischemic necrosis in kidneys, liver, and adrenal glands
8	87	100	100	Failure to thrive	Normal

* PTLN, posttransplantation lymphoproliferative disease; CMV, cytomegalovirus.