Apoptosis and Cellular Activation in the Pathogenesis of Acute Vascular Rejection

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Abstract—Acute vascular or humoral rejection, a vexing outcome of organ transplantation, has been attributed by some to activation and by others to apoptosis of endothelial cells in the graft. We asked which of these processes causes acute vascular rejection by tracing the processes during the development of acute vascular rejection in porcine cardiac xenografts performed in baboons. Apoptosis, assayed by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) expression of activated caspase-3 and proapoptotic genes Bax and Bcl-xL, was not detected until acute vascular rejection was well advanced, and even then, apoptosis was largely confined to myocytes. Activation of the endothelium, as evidenced by expansion of rough endoplasmic reticulum and increased ribosomal antigen and phospho-p70 S6 kinase, occurred early in the course of acute vascular rejection and progressed through the disease process. These findings suggest that acute vascular rejection is caused by an active metabolic process and not by apoptosis in the endothelium. (Circ Res. 2002;91:1111–1116)

Key Words: apoptosis ● endothelial cells ● xenotransplantation ● cardiac transplantation

Acute vascular or humoral rejection is the occasional cause of irreversible rejection of allografts1–4 and a frequent cause of irreversible rejection of xenografts.5–8 Studies in human subjects,9–11 nonhuman primates,14,15 and rodents16–19 suggest that acute vascular rejection is initiated when antibodies and possibly natural killer cells, platelets, and macrophages directed by antibodies of the recipient react with the endothelial lining of blood vessels in the graft, inducing pathophysiological changes in the cells. A critical question is how antibodies, natural killer cells, macrophages, or other elements interacting with the blood vessels of an organ transplant cause rejection.

The pathogenesis of acute vascular rejection has been ascribed to two pathogenic mechanisms that would seem, in principal, to be mutually exclusive. One mechanism involves the activation of endothelial cells in the graft.6,16,20 Activation of endothelium leads to de novo expression of tissue factor and plasminogen activator inhibitor type 1,21,22 causing endothelial cells to become procoagulant, and to the elaboration of cytokines, such as interleukin-1, and cell adhesion molecules, such as E-selectin,23–25 which promote inflammation. Activation of the endothelium might be caused by xenoreactive antibodies and complement,26–28 platelets,13 or inflammatory cells,16,17,29 thus leading to ischemia, thrombosis, and inflammation, the cardinal features of acute vascular rejection. Involvement of endothelial cell activation in the pathogenesis of acute vascular rejection is supported by studies associating the expression of interleukin-1β–converting enzyme, interleukin-2, interferon-γ, tumor necrosis factor-α, and tissue factor with the rejection of xenografts in rodents.30,31

Another pathogenic mechanism for acute vascular rejection involves the apoptosis of endothelial cells in the graft. In rodents30 and nonhuman primates,32 apoptosis of endothelial cells can be induced by ischemia, cytokines, or inflammatory cells and can cause the release of endothelial cells from the underlying matrix,33 loss of vascular integrity, and exposure of the matrix to coagulation and inflammatory elements in the blood. Apoptosis also deprives the endothelium of normal functions, such as inhibition of coagulation and inflammation,34–36 thus inducing ischemia, inflammation, and thrombosis through a pathway distinct from endothelial cell activation. Preservation injury, which has some morphological features in common with acute vascular rejection, is associated with apoptosis,37–40 and therapeutic manipulations that avert acute vascular rejection are associated with the expression of antiapoptotic proteins such as Bcl-2, Bcl-xL, and the zinc finger protein A20.30

Although it is formally possible that endothelial cell activation and apoptosis might occur simultaneously in distinct regions of blood vessels, the predominance of one mechanism over the other would have important implications for understanding how humoral immunity incites disease and what therapies might be effective. Accordingly, we asked whether acute vascular rejection in xenografts might be

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associated predominantly with activation or apoptosis of the endothelium of xenografts.

Materials and Methods

Cardiac Transplantation

Heterotopic cardiac transplants (n=3) from Hartley guinea pigs (200 to 250 g) into Lewis rat recipients (250 to 350 g) (Charles River, Raleigh, NC) were performed as described previously31 by aortic-aortic and pulmonary artery–inferior vena cava anastomosis in an end-to-side manner. To prevent hyperacute rejection, the recipients were given purified cobra venom factor (Quidel) intravenously at a dosage of 60 U/kg at 24 hours before transplantation and 30 U/kg daily thereafter. To prevent cellular rejection, recipients were given cyclosporine (Sandimmune) at a dosage of 20 mg/kg daily.

Heterotopic cardiac transplants from pigs into baboons (Papio anubus) were performed as previously described. Some transplants (n=4) were transgenic for human CD59 and human decay-accelerating factor. The recipients were treated with methylprednisolone (8 mg/kg per day and tapered to 1 mg/kg per day), azathioprine (2 mg/kg per day after a loading dose of 8 mg/kg), and cyclosporine (5 mg/kg per day after a loading dose of 15 mg/kg), as previously described. Two recipients of unmodified porcine hearts were treated with cobra venom factor (60 U/kg per day) and with immunosuppression as described above. Some baboons were depleted of α-Gal and/or 1-3Gal antibodies were detected using affinity-isolated FITC-goat anti–human CD59 and decay acceleration factor transplanted into baboons from which antibody was depleted.

Immunopathology

Immunopathology was performed as previously described. Baboon and rat fibrinogen was detected using FITC-conjugated goat anti-human fibrinogen or FITC-goat anti-rodent fibrinogen (ICN), respectively. Baboon fibrin monomer was detected using mouse monoclonal anti-human fibrinogen β-chain (American Diagnostica, Inc). C4 and the membrane attack complex were detected using FITC-goat anti-human C4 (ICN) and mouse anti-human C5b-9 (clone MBM5, generously provided by Dr A.F. Michael, University of Minnesota, Minneapolis). Porcine major histocompatibility complex (MHC) class II was detected using mouse monoclonal antibodies (generously provided by Dr D. Sachs, Massachusetts General Hospital, Boston). Baboon platelets were detected using a mouse monoclonal anti-human CD41 (clone 5B12, Dako Corp). Mouse monoclonal antibodies were detected using affinity-isolated FITC-goat F(ab’)2, mouse anti-IgG (ICN) absorbed with human and porcine sera and affinity-isolated FITC-rabbit F(ab’)2–anti-IgG (ICN) absorbed with human and porcine sera. Porcine ribosomes were detected with human anti-ribosomal P antigen (Immunovision) detected with FITC-goat anti-human IgG (ICN) absorbed with porcine serum and FITC-rabbit anti-goat IgG (ICN) absorbed with porcine and human serum. Activated p70 S6 kinase was detected with affinity-purified rabbit anti–phospho-p70 S6 kinase (Thr421/422) (Cell Signaling Technology, Inc) and FITC-goat anti-rabbit IgG (ICN) absorbed with porcine and human serum and FITC-rabbit anti-IgG absorbed with porcine and human serum. Activated caspase-3 was detected using rabbit anti-human active caspase-3 (Promega Corp) detected with affinity-purified FITC-goat F(ab’)2–anti-IgG (ICN) and rabbit anti-IgG absorbed with porcine and human serum. Slides were studied using an epifluorescence microscope (DMRD, Leica) equipped with appropriate excitation and emission filters. Digital images were obtained using a high-resolution CCD digital camera (SPOT II, Diagnostic Instruments) and SPOT II software.

Electron Microscopy

Tissue samples were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), rinsed in PBS (pH 7.2), and postfixed in phosphate-buffered 1% osmium tetroxide for 1 hour. Samples were rinsed in distilled water and stained en bloc with 2% uranyl acetate for 30 minutes at 60°C. Samples were rinsed in distilled water, dehydrated in progressively increasing amounts of ethanol in propylene oxide, and embedded in multicomponent epoxy resin (Spurr kit). Sections of 90 nm were cut and stained with lead citrate.

TUNEL Assay

Endothelial cells were assayed for apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis as follows: Confluent endothelial cells, grown on chamber slides, were incubated with human sera (described above), tumor necrosis factor-α (10 ng/mL, Life Technologies, Inc), synthetic melittin (0.75 μmol/L, Sigma Chemical Co), or mouse anti-human α,β, IgG (33 μg/mL, Chemicon International) in culture medium. Any traces of endotoxin were depleted using END-X B15 affinity resin (Associates of Cape Cod, Inc). After treatment, the cells were washed briefly in PBS with 2 mmol/L CaCl2 and 2 mmol/L MgCl2 and then fixed with 1% paraformaldehyde in PBS (pH 7.4) for 10 minutes at room temperature and assayed for apoptosis by labeling free 3’ DNA termini with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (Intergen Co). Labeled DNA was then detected using rhodamine-conjugated anti-digoxigenin antibodies.

Polymerase Chain Reactions

Total cellular RNA from porcine tissues was reverse-transcribed using AMV Reverse Transcriptase (Boehringer-Mannheim) into single-stranded cDNA with an oligo-dT15 primer. Polymerase chain reactions (PCRs) using porcine specific primers were performed after samples were equalized for β-actin. The sequences of primers were as follows: β-actin, ATGTGTTGACCTTCAACAC and CACGTCAACACTTATGAGGGA; Bax, CGAAGGCTCT-TYTTCGCGTGCCA and GCACAGGCCCCTTGACAC; interleukin-1β–converting enzyme, GCACACCCGGCCAGAGCACAGC and CACCCGGACGKCCGCTGAYRATG; porcine inhibitor of apoptosis protein, AGATGGTGCCATTGACCTTCC and CAGATTCCCCAACCAGCTCGAC; heme oxygenase-1, TTAAGCTGCTGTAGGCTTCT and AGGGCCCTTGCAG-CATCTC; and Bcl-xL, GGAGCTGGTACGAACGGGGA and ATCCCGAGAAGACTGCTCAAA. Ampli-Taq DNA polymerase (Perkin Elmer) was used. Samples were electrophoresed in 1% agarose gels, and bands were quantified using Gel Doc 2000 and Quantity One 4.0.3 software (Bio-Rad Laboratories).

Results

Apoptosis in Acute Vascular Rejection

We first sought to determine whether apoptosis occurs in cardiac xenografts during the course of acute vascular rejection. To ensure that the results were not peculiar to one treatment regimen, we studied wild-type porcine xenografts in recipients depleted of complement with cobra venom factor or of xenoreactive antibodies by adsorption with Galα1-3Gal, and we also studied xenografts transgenic for human CD59 and decay acceleration factor transplanted into baboons depleted of neither xenoreactive antibodies nor complement. The development of acute vascular rejection was traced as previously described by visualizing the deposition of fibrinogen and fibrin monomer in biopsies obtained at various times after transplantation (Figure 1).

In hearts destined to undergo acute vascular rejection, fibrin deposition began during the first day and increased progressively until the fifth day (rejection occurred on day 5) after...
transplantation. However, TUNEL analysis revealed very few apoptotic cells early in the course of acute vascular rejection (Figure 1). Figure 1 clearly indicates that deposition of fibrin precedes the detection of apoptotic cells by several days. Although the kinetics of the development of acute vascular rejection and the time of rejection varied from experiment to experiment, the deposition of fibrin always preceded the appearance of apoptotic cells.

Because apoptotic cells do not appear in acute vascular rejection until areas of focal necrosis are also detected, we determined whether apoptosis and necrosis were colocalized. To do so, we asked whether cells had lost membrane integrity, taking advantage of the fact that cells maintain membrane integrity throughout the apoptotic cell death pathway, whereas membrane integrity is lost in necrotic cell death.45 Thus, porcine cardiac xenografts in baboons depleted of anti–Galα1-3Gal antibodies and xenografts transgenic for human CD59 and decay-accelerating factor in baboons depleted of neither xenoreactive antibodies nor complement were dual-labeled for free 3′ DNA termini (apoptosis) using rhodamine-conjugated reagents and for complement component C4 and the membrane attack complex (elements of blood that we commonly detect in necrotic cells) using fluorescein-conjugated reagents. As shown in Figure 2, TUNEL-positive cells were located mainly in regions of focal necrosis, identified by intracellular complement (Figure 2). This would suggest that necrotic cell death precedes apoptotic cell death and may actually be the stimulus for apoptosis.33

Because the pathogenesis of acute vascular rejection has also been studied in rodent models17,19,20,46 and might conceivably differ in such models, we traced the occurrence of apoptosis in guinea pig hearts transplanted into rats treated with cobra venom factor. Figure 3 shows that these xenografts had significant amounts of fibrin within 1 day, but apoptosis was not seen until 3 days after transplantation, when acute vascular rejection was severe (Figure 3).

It is possible that apoptosis of donor endothelial cells occurred early in the course of acute vascular rejection but was not detected by TUNEL. To explore this possibility, we first determined whether tissues obtained early in the course

![Figure 1](image1.png)

**Figure 1.** Tracing the occurrence of apoptosis during the development of acute vascular rejection in porcine hearts transplanted into baboons. Porcine hearts transplanted into baboons underwent acute vascular rejection in 5 to 14 days. Biopsies obtained from the hearts were stained with hematoxylin and eosin (H&E; first row, original magnification ×400), FITC–anti-human fibrinogen (second row, original magnification ×200), FITC–anti-human fibrin monomer (third row, original magnification ×200), and rhodamine isothiocyanate–conjugated anti-digoxigenin (TUNEL; bottom row, original magnification ×400). In the example shown, representative of 5 transplants studied, the first manifestations of rejection (ischemia and formation of fibrin thrombi) can be seen on day 1; however, TUNEL-positive cells are not seen until day 5, the day of rejection.

![Figure 2](image2.png)

**Figure 2.** Localizing apoptosis and necrosis in acute vascular rejection of porcine hearts transplanted into baboons. Biopsies from porcine cardiac xenografts were stained with FITC antibodies specific for human C4 or the membrane attack complex (MAC) of complement, which we commonly detect in necrotic cells because of the loss of cell membrane integrity. The tissues were costained with rhodamine-conjugated anti-digoxigenin (TUNEL). The results shown are representative of 4 transplants studied and suggest that apoptotic cells (TUNEL-positive cells) are mostly found in areas of focal necrosis and that necrosis proceeds apoptosis by at least 4 days. Original magnification ×400.
of acute vascular rejection had an increased amount of activated caspase-3, an enzyme involved in apoptotic DNA cleavage. Tissue samples from all three porcine xenotransplant models as early as 2 days after transplantation had only focal areas of elevated caspase-3 activity, mainly in myocytes, and many fields were entirely free of caspase-3 activity. Thereafter, caspase-3 activity increased until rejection. Figure 4 shows small vessels representative of the time course of acute vascular rejection from one transplant. Caspase-3 activity was seen very rarely and weakly in endothelium; thus, it is not shown in Figure 4 because it would not represent the vast majority of vessels that were studied.

Detecting a Loss of Cells From the Vasculature
Apoptosis of endothelial cells early in the course of acute vascular rejection might have evaded detection if apoptotic cells were shed from blood vessels or engulfed by adjacent cells. To test whether apoptotic cells were shed, we examined tissues from all three porcine xenotransplant models obtained early in the course of acute vascular rejection for evidence of disruption of the endothelial lining. As Figure 5A shows, MHC class II antigen, which is expressed constitutively on porcine endothelium, was continually expressed during the time course of acute vascular rejection (rejection occurred on day 7), and CD41, a platelet antigen, was not detected in spaces between endothelial cells or attached to basement membranes, suggesting that endothelial cells were not shed. In contrast, tissue from cardiac xenografts undergoing hyperacute rejection, a condition in which a focal loss of endothelium typically occurs, revealed a focal loss of MHC class II continuity and a focal increase in platelet antigen along inner walls of blood vessels (Figure 5B). The possibility that apoptotic cells were rapidly cleared from xenotransplants cannot be absolutely excluded; however, the absence of inflammatory cells, which would ingest apoptotic cells, and the absence of apoptotic bodies in the cells composing blood vessel walls would argue against this course of events (Figures 1, 2, and 4).

Gene Expression in Acute Vascular Rejection
We and others have previously shown that proinflammatory and procoagulant genes are expressed in the course of acute vascular rejection in rodents and primates, suggesting that at least some endothelial cells become activated. We explored the possibility that endothelial cells might also express genes characteristic of early events in one or more apoptotic pathway in organs undergoing acute vascular rejec-

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**Figure 3.** Tracing the occurrence of apoptosis during the development of acute vascular rejection of guinea pig hearts transplanted into rats. Guinea pig hearts transplanted into rats underwent acute vascular rejection in 2 or 3 days. Biopsies obtained from the hearts were stained with H&E (top row, original magnification ×400), goat anti-human fibrinogen (middle row, original magnification ×200), and antidigoxigenin (TUNEL; bottom row, original magnification ×400). In the example shown, representative of three transplants studied, the manifestations of rejection (ischemia and formation of fibrin thrombi) can be seen on day 1; however, TUNEL-positive cells are not seen until day 3, the day of rejection.

**Figure 4.** Localization of activated caspase-3 in porcine cardiac xenografts. Biopsies obtained from porcine hearts transplanted into baboons were stained with rabbit antibodies specific for a neoantigen of activated caspase-3. The tissues were costained with rabbit anti–basement membrane antibodies (top row, original magnification ×400) or with H&E (bottom row, original magnification ×200). In the example shown, representative of 6 transplants studied, activation of caspase-3 is seen as early as 1 day after transplantation; however, the enzyme is not found in graft blood vessels but rather in myocytes. Arrows denote small blood vessels.
tion. As Figure 6A (which illustrates a transplant rejected relatively late to allow as much time as possible for apoptosis to develop) shows, acute vascular rejection was not associated with an overall change in the mRNA levels of Bax or Bcl-xL. The development of acute vascular rejection was associated with an increase in mRNA for interleukin-1β–converting enzyme, porcine inhibitor of apoptosis protein (an antiapoptotic protein), and heme oxygenase-1 (Figure 6A); however, these increases were observed only after the lesions of acute vascular rejection were fully manifest (Figure 6B). The increase in interleukin-1β–converting enzyme and porcine inhibitor of apoptosis protein seen late in the course of acute vascular rejection could represent a cellular response to inflammation, inasmuch as production of the corresponding proteins has been associated with inflammatory events as well as apoptotic events.50 Although the kinetics of rejection varied among the transplants, the morphological and molecular characteristics were quite similar.

Endothelial Cell Metabolism in Acute Vascular Rejection

If acute vascular rejection is associated with endothelial cell activation instead of apoptosis, it is reasonable to predict that endothelium in the graft would exhibit evidence of an assembly of ribosomes (typical of activated endothelial cells)51 but not nuclear condensation (typical of apoptosis).52 Hence, we studied the ultrastructure of tissues from organs undergoing acute vascular rejection. These studies revealed a dramatic expansion of the rough endoplasmic reticulum and an absence of nuclear condensation (Figure 7), a picture consistent with metabolically active cells and not apoptosis,39,52 early in the course of acute vascular rejection.

We next sought to determine whether the dramatic assembly of ribosomes seen by electron microscopy is generally characteristic of acute vascular rejection. As Figure 8 (top row) shows, endothelial cells reacted with anti-ribosomal antibody, and reactivity increased progressively during the course of acute vascular rejection of porcine cardiac xenografts. To assess whether the assembly of ribosomes leads to increased protein translation, we determined whether there was a change in the activation of p70 S6 kinase. When phosphorylated, p70 S6 kinase phosphorylates the S6 protein of the 40S ribosomal subunit, which is associated with an enhanced rate of mRNA translation into protein. As shown in Figure 8 (bottom row), endothelial cell reactivity with anti–phospho-p70 S6 kinase antibody increases over the course of acute vascular rejection.

Discussion

The pathogenesis of acute vascular rejection has been ascribed to two seemingly contradictory processes. One theory would hold that acute vascular rejection results from the activation of endothelial cells in the graft, causing proinflammatory and procoagulant changes that eventuate in ischemia and thrombosis, the characteristic features of the disease. The second theory would hold that acute vascular rejection results from apoptosis of endothelial cells or other cells in the graft, causing detachment and loss of cells from blood vessels, leading to vasoconstriction (owing to deficiencies of NO) and thrombosis and inflammation (owing to exposure of the underlining matrix to components of the blood). In the present study, we report that the onset and progression of acute vascular rejection is characterized by heightened endothelial cell metabolism, as evidenced by expansion of ribosomal material in the endothelium, and not by endothelial cell apoptosis. Although the kinetics of acute vascular rejection varied between the three transplant models used, the results were the same. As we discuss below, the distinction between activation and apoptosis of endothelium may have important pathogenic and therapeutic implications.
The earliest sign of organ injury in acute vascular rejection is the swelling of endothelial cells and formation of fibrin deposits along the walls of blood vessels within hours of engraftment. Although these changes could be caused by apoptosis or activation of the endothelium, we show in the present study that only active metabolism (as evidenced by...

Figure 6. Expression of proapoptotic and antiapoptotic genes in porcine cardiac xenografts undergoing acute vascular rejection. Porcine hearts transplanted into baboons underwent acute vascular rejection in 5 to 14 days. RNA from the hearts was tested by PCR for Bcl-xL, Bax, heme oxygenase-1, interleukin-1β-converting enzyme (ICE), and porcine inhibitor of apoptosis protein (PIAP) (A) and was used for evaluation pathology (B). Results shown are representative of 4 transplants studied. A, Expression of proapoptotic and antiapoptotic genes. Total cellular RNA was tested by PCR for Bcl-xL, Bax, heme oxygenase-1, ICE, and PIAP in samples equalized for β-actin (not shown). Changes in expression of heme oxygenase-1, ICE, and PIAP can be seen during acute vascular rejection; however, tissue changes characteristic of acute vascular rejection precede changes in proapoptotic and antiapoptotic genes by at least 6 days. B, Pathology of porcine cardiac xenografts. Porcine cardiac tissues were stained with H&E (first row, original magnification ×400), anti-human fibrinogen (second row, original magnification ×200), or anti-human fibrin monomer (third row, original magnification ×200) before transplantation and 1, 7, and 14 days after transplantation. Tissue changes characteristic of acute vascular rejection can be seen by day 1.

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Figure 7. Ultrastructural analysis of endothelial cells in porcine cardiac xenografts undergoing acute vascular rejection. Tissue from porcine hearts with acute vascular rejection was studied by transmission electron microscopy. A representative micrograph is shown. The endothelial cell nucleus (N) is normal in appearance, and there is an expansion of the rough endoplasmic reticulum (RER), indicating that the endothelium is metabolically active and not undergoing cell death by apoptosis. Original magnification ×4000. Inset shows a segment of normal capillary for comparison.
increased number of endothelial cell ribosomes, typical of endo-
thelial cell activation,31 and by an increase in p70 S6 kinase activity, suggesting an increase in protein translation) is de-
tected. Consistent with this observation, we and others have
found that early lesions of acute vascular rejection are
associated with de novo expression of tissue factor,31 a cell
surface receptor for factor VI, and various cytokines.30 Also
consistent with this concept are observations that apoptotic
cells do not typically exhibit assembly of the rough endoplas-
ic reticulum,52,53 which we observe as a characteristic
feature of acute vascular rejection; indeed, inhibitors of
protein synthesis, such as cyclohexamide,54 staurosporine,55
and ribosome-inactivating proteins,56 help initiate apoptosis
in several different cell types.

Although we found no evidence that apoptosis of endo-
thelial cells occurs early in the course of acute vascular rejection,
that is not to say that apoptosis plays no role in tissue injury
or in the immune response to xenotransplantation. Apoptosis
clearly occurs in myocytes late in the course of acute vascular
rejection and could contribute to the decrease in myocardial
function in the final stages of rejection. Furthermore, apopto-
sis might promote a cross-presentation of autoantigens such
as myosin,57 thus explaining how immunity might be directed
at myosin in the heterotopic transplants but not in the
autologous heart. Finally, although the present study was
conducted in xenotransplants, the aforementioned molecular
changes occur in homologous systems, suggesting that the
results are relevant for allotransplants.

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