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 whether acute vascular rejection in 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 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;91:1135-1141.)

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

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cute 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,9–13 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 pathogenetic 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,16,17,20 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.29,30

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

Materials and Methods
Cardiac Transplantation
Animal care and procedures were in accord with NIH and institutional guidelines. 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 previously 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 (Sandoz) at a dosage of 20 mg/kg daily.

Heterotopic cardiac transplants from pigs into baboons (Papio anubus; Southwest Research, San Antonio, Tex) 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 anti-Gal1-3Gal antibodies using a Baxter Autoxepheresis C plasmapheresis device with Gal1-3GalB1-4GlcNAc Sepharose columns, as previously described. 3 to 6 days before and at various times after transplantation. Baboons from which antibody was depleted was treated with methylprednisolone (10 mg/kg per day tapered by 1 mg/kg per day to a maintenance dose of 1 mg/kg per day per day after a loading dose of 15 mg/kg), and cyclophosphamide (1 to 5 mg/kg per day after a loading dose of 10 mg/kg per day for 2 to 3 days), as described in detail.

Immunopathology
Immunopathology was performed as previously described. Baboon and rat fibrinogen was detected using FITC-conjugated goat anti-human fibrinogen or FITC-goat anti-rat 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 C9 (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–anti-mouse IgG (ICN) absorbed with human and porcine sera and affinity-isolated FITC-rabbit F(ab')2–anti-goat 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 and human serum. 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/Ser424, Cell Signaling Technology, Inc) and FITC-goat anti-rabbit IgG (ICN) absorbed with porcine and human serum and FITC-rabbit anti-goat 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-rabbit IgG (ICN) and rabbit anti-goat 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 deoxynucleotidy 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 endotoxins 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-d(T)15 primer. Polymerase chain reactions (PCRs) using porcine specific primers were performed after the samples were equalized for β-actin. The sequences of primers were as follows: β-actin, ATGTTTGAACCTTCAACAC and CACGTCACACTTCATGATGGA; Bax, CGAGAGGTCTT-TYTCTCGRGTGGCGG and GCACAGGCGCTTGGAC; interleukin-1β–converting enzyme, GCACACCGGCCCCAGACCA-CAAG and CCACGGCAKGCCTGRAYATG; porcine inhibitor of apoptosis protein, AGATGTTGCGATTGACCTTTC and CAGATTCACCCACCTCAGC; heme oxygenase-1, TTA-AGCTGGTGATGGCGTCCT and AGGGCCTTCCTGAGACCTTCTC; Bcl-xL, AGATGTGGCCATTGACCTTTC and AGGGCCTTCCTGAGTTTCTC; and TdT, GGAAGCTGAATTCCAGAGGAA and ATCCGGGAAAGATCTTGCTTCAA. AmpliTaq 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 GalL-1-3Gal, 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

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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 \( \times 400 \).

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. Original magnification \( \times 400 \).

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 \( \times 400 \)), goat anti-human fibrinogen (middle row, original magnification \( \times 200 \)), and anti-digoxigenin (TUNEL; bottom row, original magnification \( \times 400 \)). 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.

Because the pathogenesis of acute vascular rejection has also been studied in rodent models, we trace 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 of acute vascular rejection had an increased amount of activated caspase-3, an enzyme involved in apoptotic DNA

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. Therefore, porcine cardiac xenografts in baboons maintain membrane integrity throughout the apoptotic cell death pathway, whereas membrane integrity is lost in necrotic cell death. 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
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 rejection. 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

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.

Figure 5. Integrity of endothelium in porcine cardiac xenografts. Whether apoptotic endothelial cells are lost from blood vessels of porcine cardiac xenografts undergoing acute vascular and hyperacute rejection was tested by examining tissues for loss of continuity of MHC class II antigen and presence of platelets in vessel walls. A, Endothelial integrity in acute vascular rejection. Integrity of the endothelium is demonstrated by the presence of undisrupted fluorescence for MHC class II antigen along the endothelium and the absence of platelet deposition (CD41) early in the course of acute vascular rejection, which occurred on day 7. Arrows denote small blood vessels. Original magnification ×200. B, Endothelial integrity in hyperacute rejection. Loss of integrity of endothelium is demonstrated by disruption of continuity of MHC class II and deposition of platelets (CD41) along endothelium of grafts undergoing hyperacute rejection at 1.25 hours. Arrows denote small blood vessels. Original magnification ×200.
proteins has been associated with inflammatory events as well as apoptotic events. 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) but not nuclear condensation (typical of apoptosis). 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 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 sought to determine 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 antiphospho-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.\(^1,3,15\) 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 increased number of endothelial cell ribosomes, typical of endothelial cell activation,\(^3\) and by an increase in p70 S6 kinase activity, suggesting an increase in protein translation) is detected. 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,\(^3\) a cell surface receptor for factor VI, and various cytokines.\(^3\) Also consistent with this concept are observations that apoptotic cells do not typically exhibit assembly of the rough endoplasmic reticulum,\(^5,2,3\) which we observe as a characteristic feature of acute vascular rejection; indeed, inhibitors of protein synthesis, such as cyclohexamide,\(^4,5\) staurosporine,\(^5\) and ribosome-inactivating proteins,\(^6\) help initiate apoptosis in several different cell types.

Although we found no evidence that apoptosis of endothelial 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, apoptosis might promote a cross-presentation of autoantigens such as myosin,\(^5\) 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.

**Figure 8.** Endothelial cell metabolism in porcine cardiac xenografts undergoing acute vascular rejection. Biopsies from porcine cardiac xenografts were stained for ribosomal P antigen (top row) and anti-phospho-p70 S6 kinase (Thr421/Ser424) (bottom row). An increase in endothelial ribosomal material and activated p70 S6 kinase is seen during the course of acute vascular rejection. Original magnification \(\times 860\).

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**References**

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