Pathways to Acute Humoral Rejection

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Acute humoral rejection, also known as acute vascular rejection, is a devastating condition of organ transplants and a major barrier to clinical application of organ xenotransplantation. Although initiation of acute humoral or vascular rejection is generally linked to the action of antibodies and complement on the graft, other factors such as ischemia, platelets, T cells, natural killer cells, and macrophages have also been implicated. Central to any understanding of the pathogenesis of acute humoral rejection, and to developing means of preventing it, is to know whether these factors injure the graft independently or through one or few pathways. We addressed this question by examining early events in a severe model of vascular rejection in which guinea pig hearts transplanted heterotopically into rats treated with cobra venom factor (CVF) develop disease over 72 hours. The early steps in acute vascular rejection were associated with expression of a set of inflammatory genes, which appeared to be controlled by availability of interleukin (IL)-1. Interruption of IL-1 signaling by IL-1 receptor antagonist (IL-1ra) averted expression of these genes and early tissue changes, including coagulation and influx of inflammatory cells. These findings suggest IL-1 plays an important role in initiation of acute humoral rejection. (Am J Pathol 2004, 164:1073–1080)

Vascular rejection is a challenging problem in organ allotransplantation, and the major impediment to clinical application of xenotransplantation.1 Characterized by focal ischemia, endothelial swelling, and intravascular coagulation, vascular rejection arises over a period of days to weeks in experimental systems, and months in clinical organ transplants2–6 and in xenotransplants.7–11 Various terms including antibody-mediated rejection, acute humoral rejection and acute vascular rejection have been applied to this process. Because of the clinical challenge posed by vascular rejection and the possibility that it might represent a broader set of vascular diseases, there has been much interest in understanding how the condition arises.

Most evidence suggests that anti-donor antibodies, such as those directed against major histocompatibility or blood group antigens, trigger this type of rejection7,12,13; hence, vascular rejection is sometimes called “antibody-mediated rejection.” Consistent with this concept, C4 days deposits are typically found on graft endothelium, reflecting activation of the classical complement pathway by antibodies, and Cd4 is used as a marker of this condition.14–16 As further evidence for the seminal importance of antibodies, depletion of anti-donor antibodies temporarily delays or prevents vascular rejection.7 While these observations strongly suggest that antibodies cause the process, depletion of anti-donor antibodies also induces accommodation, a phenomenon in which a graft develops resistance to injury.8,17 Thus, accommodation might obscure the involvement of factors other than antibodies in the pathogenesis of acute humoral rejection.

While antibodies clearly can trigger vascular disease in organ grafts, some type of disease may occur independent of antibodies. One factor other than anti-donor antibodies might be ischemia-reperfusion injury. Severe ischemia-reperfusion injury soon after transplantation causes recruitment of inflammatory cells and activation of endothelium.18–21 Ischemia-reperfusion injury also stimulates platelets, which activate endothelial cells.22,23 Ischemia-reperfusion injury causes activation of the complement system through classical and alternative pathways.24–26 Because humoral factors could act on a graft independent of antibodies, some have referred to vascular rejection as acute humoral rejection.

In addition to “humoral” factors, recipient leukocytes may interact with donor blood vessels, giving rise to vascular injury. T cells may act on graft endothelial cells,27,28 releasing cytokines that could, like complement, activate endothelium and induce cytotoxicity. Natural killer cells interrupt integrity of endothelium and activate endothelial cells, inducing expression of tissue factor, adhesion molecules, and chemokines.29–31 Macrophages secrete cytokines like tumor necrosis factor (TNF)-α and IL-1β, which activate endothelial cells, and elaborate tissue factor,32 which promotes intravascular coagulation.33 Activated platelets carry cell-surface-bound cytokines particularly IL-1α that may directly stimulate endothelium inducing pro-coagulant and pro-in-
flammation changes thought to underlie vascular or humoral rejection.23,33,34 Because many factors other than antibodies can induce acute vascular rejection of organ grafts, some refer to the process as acute vascular rejection, and we shall use this term below.

To devise an effective way to prevent acute vascular rejection, it is critical to know whether the multiple pathogenic factors mentioned above—anti-donor antibodies, complement, ischemia-reperfusion injury, leukocytes, and platelets—initiate the graft injury independently of one another or whether one or few factors play a dominant role. The present study was designed to distinguish between these two possibilities. We studied the evolution of acute vascular rejection in guinea pig hearts transplanted in rats in which complement was inhibited by CyA. In this model, severe acute vascular rejection develops in 3 days and accommodation, which could confound analysis, is absent. We questioned whether disruption of one “seminal” pathway might alter the tissue injury and course of the disease.

**Materials and Methods**

**Animals**

Male Hartley guinea pigs (200–250 g) and male Lewis rats (250–350 g) served as transplant donors and recipients, respectively. The animals were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and received care in accordance with National Institutes of Health guidelines.

**Cardiac Transplantation**

Rats were anesthetized with ketamine hydrochloride (10 mg/kg i.m.) and sodium pentobarbital (40 mg/kg, i.p.). Guinea pigs were anesthetized with isoflurane by inhalation. Heterotopic cardiac transplants were performed using a modification of the method of Ono and Lindsey.35 Guinea pigs were given 300 units of heparin into the inferior vena cava before harvest. The hearts were flushed with and preserved in 4°C Plegisol (Abbott Laboratories, North Chicago, IL) while the recipients were being prepared. The guinea pig hearts were transplanted by aorta-aortic and pulmonary artery-inferior vena cava anastomosis in an end-to-side manner using 8—0 Prolene from Ethicon (Somerville, NJ) into the abdomen of the recipient. Surgical procedures were carried out under sterile conditions. After reperfusion, all of the transplanted hearts contracted spontaneously.

**Immunosuppression**

To prevent hyperacute rejection and to allow development of acute vascular rejection, the recipients of xenotransplants were treated with purified cobra venom factor (CVF) (Quidel Inc., San Diego, CA) intravenously at a dosage of 60 U/kg 24 hours before transplantation followed by 30 U/kg daily. To prevent cellular rejection, the recipients were treated with Cyclosporine A (CyA) (Novartis Pharmaceuticals Co., East Hanover, NJ) at a dosage of 20 mg/kg daily. The recipients of cardiac allografts received CVF and CyA or CyA alone, as indicated.

**Interleukin-1 Receptor Antagonist**

Human-recombinant interleukin-1 receptor antagonist (IL-1ra), a gift from Amgen, Inc. (Boulder, CO), was administered by continuous infusion (25 mg/kg/day) from a 2-ml osmotic pump (Alza Corporation, Mountain View, CA.). The PE-50 catheters (BD Biosciences, Franklin Lakes, NJ), which were connected with pumps, were put into superior vena cava via the right extra-jugular vein by cut-down.

**Tissue Preparation**

Cardiac grafts harvested from recipients were cut into small pieces. Pieces to be used for immunopathology were placed in OCT compound (TissueTek; Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. Frozen sections were prepared at 4 μm using a Leica CM 3060 cryostat (Deerfield, IL), mounted on positively charged microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA), fixed 10 minutes in 4°C acetone (HPLC-grade, Fisher Scientific) and air-dried for an additional 10 minutes. Sections were then postfixed for 2 minutes in 100 mmol/L Tris-HCl buffered 1% paraformaldehyde containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.2, and rinsed with three changes of PBS, pH 7.2. Goat anti-rat fibrinogen (Cappel/ICN Biomedical, Costa Mesa, CA) conjugated with fluorescein-5-isothiocyanate (FITC), was diluted in PBS containing 1% bovine serum albumin (BSA), applied to specimens for 45 minutes. Sections were then rinsed with three changes of PBS and coverslipped with medium consisting of 25 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma Chemical, St. Louis, MO) 0.5 μg/ml, 4,6-diamidino-2-phenylindole (DAPI; Sigma) and 50% glycerol in PBS, pH 8.6. Slides stored in the dark at 4°C until evaluation using a DMRD fluorescence microscope (Leica, Bannockburn, IL). Digital images were obtained using a high-resolution CCD digital camera (SPOT II; Diagnostic Instruments, Sterling Heights, MI) mounted to the microscope and SPOT II software.

Pieces to be used for light microscopy were fixed in neutral-buffered formalin for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Northern Blot Analysis and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

Pieces of graft tissues, to be used for Northern blots and reverse transcriptase polymerase chain reaction (RT-PCR), were snap-frozen in liquid nitrogen and stored at −80°C. Gene expression in the cardiac grafts was ana-
lyzed by Northern blotting and hybridization with donor and/or recipient specific cDNA probes cloned for this study. When Northern blots were not adequate for the assessment of gene expression, RT-PCR was performed.

The cDNAs of various genes were cloned as follows. Total RNA from guinea pigs or rats was isolated by the guanidinium thiocyanate method. The RNA was subjected to RT-PCR, as described previously, using forward and reverse primers determined from available consensus sequences. The cDNAs were cloned in pGEM-T vector as described by the manufacturer (Invitrogen, Carlsbad, CA). The identity of cDNAs was confirmed by sequencing. The PCR conditions for amplifying cDNA of various genes were 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 75 seconds, for 25 cycles. Guinea pig IL-1β (AF119622) was amplified using oligonucleotides GAGGATGACTTGTCTTTTGA (forward) and AGGTGCTGTGAGTGACT (reverse), generating a 550-bp fragment. Guinea pig IL-1α mRNA was amplified using oligonucleotides GAGGATGACTTGTCTTTTGA (forward) and AGGTGCTGTGACT (reverse), generating a 550-bp fragment. Guinea pig TNF-α (U39839) was amplified using AGCACA-GAGACGATGATCC (forward) and GGCAGAAGTCAAGG-TACTGAG (reverse) oligonucleotides, generating a 580-bp fragment. Rat TNF-α (X66539) was amplified using AGGCACAAGGCATGATCC (forward) and CAGGACTCTGTC (reverse) oligonucleotides, generating a 580-bp fragment. Guinea pig PAI-1 was cloned using CATGGTGCAC (forward) and CACGTCACACTTCATGATGGA (reverse). To compare the levels of various mRNA, a semiquantitative RT-PCR strategy was used in which cytokine cDNA for each sample was compared to β-actin mRNA determined for the same sample, and concentrations of cDNA were adjusted to generate approximately equal β-actin PCR products.

Results

Development of Acute Vascular Rejection

To explore the sequence of events that might initiate the development of acute vascular rejection of organ grafts, we used a model system in which guinea pig hearts are transplanted into rats that have been treated with CVF for 24 hours before transplantation. We also used CVF because complement would otherwise cause hyperacute rejection 10 to 30 minutes after reperfusion. CVF activates complement in solution and in this way consumes most complement activity. While CVF has systemic effect on rats as early as 10 minutes following the injection, we detected no changes in recipients at the time of transplantation. We used this model because it is classic for acute vascular rejection and because accommodation, which might confound the analyses, has not been observed. In the absence of hyperacute rejection, acute vascular rejection ensues and destroys the graft within about 3 days. Cardiac xenografts were removed from recipients at various times after reperfusion, and examined for histological changes, deposition of fibrin, and expression of genes that have been linked to endothelial cell activation and inflammatory changes in acute vascular rejection. The development of acute vascular rejection was characterized by sequential thickening of endothelial cells, disruption of vascular barriers leading to edema and hemorrhage, leukocyte adhesion and infiltration, formation of fibrin thrombi (Figure 1A), and progressive deposition of fibrin in cardiac xenografts (Figure 2A). The development of acute vascular rejection was also associated with sequential changes in gene expression in the graft. As Figure 3 and Figure 4 show, expression of guinea pig IL-1α, IL-1β, TNF-α, IL-8, MCP-1, and PAI-1 mRNA were detected within 3 hours of reperfusion, whereas rat TNF-α was not detected until later (Figure 4), suggesting that the initial changes involved donor cells rather than influx of recipient leukocytes.

Contribution of Organ Harvesting and Reperfusion Injury to Initiation of Acute Vascular Rejection

Because the changes associated with acute vascular rejection could have been caused by organ harvesting and reperfusion injury, we examined isogenic transplants in the same way we had studied xenogenic transplants (Figure 1B). Guinea pig cardiac isografts in recipients not treated with CVF had some evidence of tissue injury, including influx of inflammatory cells and focal
deposition of fibrin, at 3 hours. These changes were much milder than those observed in xenografts and resolved spontaneously over 16 to 24 hours. Because activation of complement may play a role in reperfusion injury,45– 48 we next asked whether injury seen in the isografts was prevented by CVF. Recipients of isografts were treated with CVF at the same doses as those used in recipients of xenograft and the isografts were studied as described above. As shown in Figures 1B and 2B, tissue injury and fibrin deposition were nearly absent in isografts in the CVF-treated recipients. These results suggest that the contribution of organ harvesting and reperfusion injury accompanying organ transplants may be minimal to the pathogenesis of acute vascular rejection at least under conditions in which complement is inhibited.

While tissue changes induced by organ harvesting and reperfusion injury were minimal in isografts, it is possible that the transplant procedure might have induced expression of genes, contributing to the development of acute vascular rejection. To explore this possibility, we tested for expression of PAI-1, IL-1β, and MCP-1 genes by Northern blots in cardiac isografts. Three hours after transplantation of guinea pig hearts into guinea pigs not treated with CVF, PAI-1, IL-1β, and MCP-1 mRNA were expressed, but at low levels. However, when recipients were treated with CVF, IL-1β, and MCP-1 mRNA was hardly detectable (Figure 5). Sixteen hours after transplantation, isografts did not exhibit expression of any of these genes (Figure 5). Taken together, these results suggest that under the experimental conditions used, injury arising from harvesting and reperfusion contributed imperceptibly to the tissue changes of acute vascular rejection. At most, this injury may contribute a very low level of PAI-1 mRNA to the overall picture of events transpiring during the development of acute vascular rejection.

**A Pathway of Tissue Injury in Acute Vascular Rejection: Role of IL-1**

We next asked if initial changes manifested in vascular rejection reflect activity of IL-1. We explored the role of IL-1 because donor IL-1 mRNA was detected as early as 3 hours (Figure 3), and because we have shown that activation of small amounts of complement (as exists in CVF-treated recipients) on endothelial cells induces IL-1α, which serves as an autocrine factor inducing the other genes expressed by activated endothelial cells.37,44,49,50 Similarly, activated platelets express IL-1α on the surface, and platelet-associated IL-1 can induce the same changes as complement in endothelial cells.23
To assess the role of IL-1 in initiation of acute vascular rejection, we studied a series of guinea pig cardiac xenografts in rats treated with CVF and with an antagonist for the IL-1 receptor, IL-1ra. As Figure 1A shows, when rats were treated with IL-1ra by continuous infusion, changes in tissue morphology characteristic of early stages of acute vascular rejection did not occur. Instead, the tissues remained normal in appearance and lacked fibrin deposits until 48 hours after transplantation (Figure 2A). Treatment with IL-1ra generally diminished expression of cytokines especially, IL-1β (Figure 3). However, 16 hours after transplantation expression of guinea pig IL-1α and TNF-α was increased in hearts transplanted into rats treated with IL-1ra. This change, which was observed in repeated experiments, may reflect transient response following reperfusion. Consistent with the histological picture and inhibition of cytokines, IL-1ra treatment had a dramatic effect on expression of coagulant and inflammatory genes. At all time points studied up to 48 hours, expression of PAI-1, IL-8, and MCP-1 mRNA was dramatically lower in xenografts from rats treated with IL-1ra than in control xenografts (Figure 4). These results are consistent with the idea that acute vascular rejection is initiated by one or a few factors and that IL-1 is either central or is among few factors central to the early stages of acute vascular rejection. While treatment with IL-1ra was dramatically effective in preventing changes associated with ischemia-reperfusion injury and the early changes associated with acute vascular rejection, it did not entirely prevent the ultimate demise of the xenografts. Cardiac xenografts in recipients treated with IL-1ra ceased beating 101 ± 1.2 hours after transplantation, and the grafts at that time revealed changes associated with vascular rejection, albeit milder. The failure of the graft in the presence of IL-1ra may reflect the incomplete inhibitory effects of IL-1ra delivered in this model system, the involvement of other factors like TNF-α in the later stages of tissue injury, or the failure of accommodation to occur in this model system.

**Discussion**

Characterized by focal ischemia, endothelial swelling and activation, intravascular coagulation and inflammatory changes, acute vascular rejection is a complex problem in clinical and experimental organ transplantation. When it occurs in clinical organ transplants, acute vascular rejection characteristically resists conventional approaches to treatment of rejection and may lead to loss of the graft.1-5 Because acute vascular rejection can hinder the success of clinical allotransplantation and because it blocks the clinical application of xenotransplantation, there has been much interest in understanding its pathogenesis and devising effective therapies to prevent or treat it. Multiple factors including anti-donor antibodies, complement, ischemia-reperfusion injury, leukocytes, and platelets have been thought to be capable of initiat-
Role of organ harvesting in the induction of coagulant and inflammatory genes in cardiac transplants. Guinea pig hearts were transplanted into guinea pigs (isograft) or into rats (xenograft). Rats were treated with CVF, and guinea pig recipients were either untreated or were treated with CVF. Guinea pig hearts were harvested at indicated times. Total RNA from tissues was then isolated and analyzed by Northern blots for expression of PAI-1, MCP-1, and IL-1β, using indicated guinea pig probes. While the expression of PAI-1, MCP-1, and IL-1β genes persists in the xenografts, the expression occurs in isografts but then it ceases. Un, untransplanted guinea pig hearts; Iso, isograft; Xen, xenograft.

Although IL-1ra dramatically altered the early events in acute vascular rejection and delayed somewhat the loss of organs, it did not prevent the eventual failure of cardiac xenografts. Failure of the grafts in the presence of IL-1ra could suggest that other factors, like TNF-α, the mRNA of which was detected at all time points examined (Figure 3), supervened to cause demise of the graft. It is important to point out that, at later time points, while IL-1ra inhibits expression of IL-1α, IL-1β, and rat TNF-α in the graft, IL-1ra does not inhibit expression of guinea pig TNF-α in the grafts. Another explanation for the limited effect of IL-1ra is species-specificity of IL-1ra and the short half-life in rats may preclude full efficacy in this system. It is also possible that the grafts in animals treated with IL-1ra failed for reasons not related to acute vascular rejection, because tissues were relatively preserved in these grafts.

The severity of acute vascular rejection and absence of accommodation in this system may result from species specificity of IL-1α. While IL-1 causes injury, it also can...
protect the graft. For example, while large doses of IL-1 given to experimental animals cause death,\(^{38,59}\) pretreatment of hearts with low doses of IL-1 protects hearts against ischemia/reperfusion injury.\(^{60,61}\) Thus, rat IL-1α expressed on platelets or secreted by rat macrophages may not effectively interact with guinea pig IL-1 receptors to induce protective mechanisms in the transplant. IL-1 plays a role in protection in homologous systems by inducing inducible nitric oxide synthase\(^62\) or hemoxyge-

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