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Protection Against Hyperacute Xenograft Rejection of Transgenic Rat Hearts Expressing Human Decay Accelerating Factor (DAF) Transplanted into Primates

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Abstract

Background: Production of transgenic pigs for multiple transgenes is part of a potential strategy to prevent immunological events involved in xenograft rejection. Use of a genetically engineerable rodent as a donor in primates could allow testing in vivo of the effects of different transgenes on controling xenograft rejection. As a first step in the development of a donor containing multiple transgenes, transgenic rats for human decay-accelerating factor (DAF) were used as heart donors to test their resistence against complement (C)-mediated rejection by non-human primates.

Materials and Methods: Transgenic rats were generated by using a construct containing the human DAF cDNA under the transcriptional control of the endothelial cell (EC)-specific human ICAM-2 promoter. DAF expression was evaluated by immunohistology and by FACS analysis of purified ECs. Resistance of transgenic hearts against C-mediated damage was evaluated by ex vivo perfusion with human serum and by transplantation into cynomolgus monkeys.

Results: Immunohistological analysis of DAF expression in several organs from two transgenic lines showed uniform expression on the endothelium of all blood vessels. ECs purified from transgenic hearts showed 50% DAF expression compared to human ECs and >70% reduction of C-dependent cell lysis compared to control rat ECs. Hemizygous transgenic hearts perfused with human serum showed normal function for >60 min vs. 11.2 ± 1.7 min in controls. Hemi- or homozygous transgenic hearts transplanted into cynomolgus monkeys showed longer survival (15.2 ± 7 min and >4.5 hr, respectively) than controls (5.5 ± 1.4 min). In contrast to hyperacutely rejected control hearts, rejected homozygous DAF hearts showed signs of acute vascular rejection (AVR) characterized by edema, hemorrhage, and an intense PMN infiltration.

Conclusions: We demonstrate that endothelial-specific DAF expression increased heart transplant survival in a rat-to-primate model of xenotransplantation. This will aid in the analysis of AVR and of new genes that may inhibit this form of rejection, thus helping to define strategies for the production of transgenic pigs.

Introduction

The use of porcine organs for clinical transplantation is an approach to overcoming the shortage of human organs. The first obstacle to xenotransplantation is the recipient's innate immune response which results in xenograft hyperacute rejection (HAR) (for review see refs. 1). Rejection of transplants performed between discordant species, such as pig to primates, involves the binding of preformed xenogeneic natural antibodies (XNA) directed to Galα1-3Gal epitopes on the endothelial cell (EC) surface (2,3). Interaction between XNA and their targets promotes complement (C) activation, leading to graft endothelium injury and activation (4–7). Prevention of xenograft HAR has been successfuly achieved by either inhibition of XNA binding through depletion of XNA or reduction of Galα1-3Gal expression on ECs (8,9), or by blockade of complement activation (10). Transgenic pigs expressing human complement regulatory proteins (CRPs), including decay-accelerating factor (DAF) and/or CD59, have been produced to avoid complement-mediated graft damage (11–13). Dramatic improvement of xenograft survival has been obtained using these transgenic transplants in the pig-to-primate model of xenotransplantation (14–16). These studies demonstrated that human DAF and/or CD59 expression on graft ECs, in association with antibody depletion and/or immunosuppressive regimen, efficiently overcome HAR. However, optimal transgene expression levels as well as its optimal tissue distribution remained to be determined.

Since xenograft HAR can be circumvented by the use of transgenic animals for CRPs, humoral and cell-mediated processes involved in delayed xenograft rejection or AVR may be further controlled by the production of transgenic animals expressing other transgenes acting on these processes in addition to CRPs. To this end, prior to the generation of transgenic pigs, production of transgenic small laboratory animals is needed. These animals would not only serve as tools for analysis of DNA construction for transgenesis but more importantly would help us investigate in vivo the biological relevance of transgene expression on xenograft rejection. In addition, models bearing close resemblance to the pig-to-primate one may be particularly useful. In this regard, although transgenic mice have been used to define the expression of DNA construction (17,18) and their organs perfused ex vivo, their reduced size make it nearly impossible to graft their organs in primates.

We previously established an in vivo experimental model for xenotransplantation using the rat as organ donor and an Old World primate (cynomolgus) as recipient. In this rat-to-primate combination, HAR occurred 5 min after vascular anastomosis, with features mimicking HAR (19) and EC injury (7) observed in the pig-to-primate model. In addition, we demonstrated that, in vitro, human DAF and CD59 efficiently protect transfected xenogeneic rat ECs against cell lysis mediated by nonhuman primate sera (20). In the present study, we investigated the ability of human DAF expressed by the endothelium to delay HAR by producing transgenic rats for the human DAF. Endothelium-specific transgene expression was analyzed by immunohistochemistry on tissue sections and quantitated in vitro by isolation of cardiac ECs followed by flow cytometry. Ex vivo perfusion of transgenic hearts with human serum demonstrated the ability of human DAF expression to prevent XNA and C-mediated organ damage. Correlation between the DAF expression level on graft ECs and graft survival time was documented following cardiac heterotopic transplantation using hemizygous and homozygous transgenic rats as donors and unmodified cynomolgus as recipients. Histopathological features of acute vascular rejection (AVR) of DAF-expressing cardiac xenograft were characterized by polymorphonuclear leukocytes (PMN) infiltration, intersticial edema, and focal myocardium necrosis.

Materials and Methods

Generation of Transgenic Rats

The plasmid expression vector phICAM2DAF (Fig. 1A) contains a 1.9 kb *Eco*RI fragment of the human CD55 cDNA, 334 pb of the promoter region of the human intercellular adhesion molecule 2 (ICAM2) gene (nucleotides −292 to +44), a universal intron, and the SV40 polyA, as previously described (21). The 3.12 kb *Kpn*I-*Sac*I

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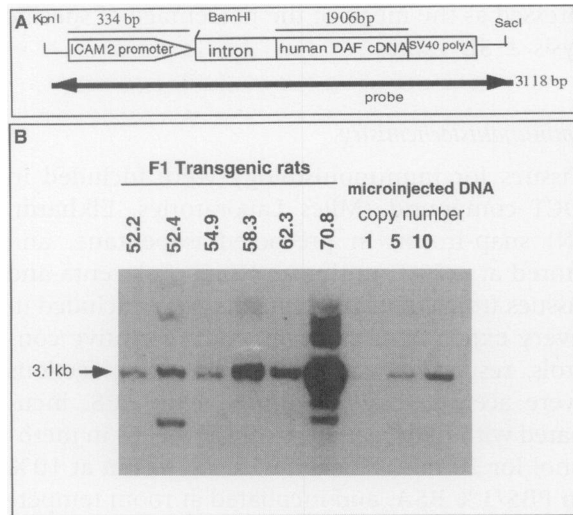

Fig. 1. Analysis of transgene integration in transgenic rats. (A) Design of ICAM2DAF cDNA construct used for microinjection (not drawn to scale). (B) Southern blot from transgenic rats ($DAF^{+/-}$). Genomic DNA (10 μg) was digested by *Bam*HI, separated through 1% agarose gel electrophoresis, and blotted. Known copy numbers of the microinjected fragment diluted into genomic DNA from a nontransgenic rat were used as controls. Blot was hybridized with a $^{\alpha32}$P-dCTP labeled cDNA probe encoding the microinjected cDNA sequences (3.1 kb). *Bam*HI releases a 3.1 kb fragment (arrow).

fragment was excised from phICAM2DAF and isolated by electrophoresis through a 1% agarose gel, electroeluted, purified through an Elutip column (Schleicher & Schull, Keene, NH), and diluted to a concentration of 2 ng/ml in 5 mM Tris-HCl and 0.1 mM EDTA, pH 7.4. Fertilized rat eggs were recovered from superovulated Sprague-Dawley (SD) females mated with SD males (Charles River, Saint-Aubin-Les-Elbeuf, France). DNA solution (2 ng/μl) was microinjected into the male pronucleus and transferred into both oviducts of day 0 pseudopregnant SD females as previously described (22).

Identification of the transgenic founder animals and their progeny was determined by polymerase chain reaction (PCR) analysis of genomic DNA obtained from tail biopsies digested with proteinase K in a total volume of 450 μl for 16 hr at 56°C and then confirmed by Southern blots. PCR analysis was performed on 2 μl of the DNA solution diluted to 1/20 with oligonucleotides specific for human DAF (sense: 5'GAC GCT AGT AAT CAT GGG CT3'; antisense: 5'TAG GAA AGG AAT CAC TCT CA3') for 30 cycles of amplification (94°C for 30 sec; 50°C for 30 sec; 72°C for 30 sec). For Southern blot analysis, 10 μg of DNA was digested by *Bam*HI, fractionated on 1% agarose gel, and alkali blotted. Hybridization was carried out with a $^{32\alpha}$P-dCTP-radiolabeled 3.12 kb *Kpn*I-*Sac*I fragment of the phICAM2DAF construct used for microinjection as a probe (probe showed in Fig. 1A). Determination of transgene copy number in genomic DNA from progeny and discrimination between hemizygous and homozygous rats was evaluated by probing a Southern blot of *Bam*HI-digested tail DNA and known quantities of the microinjected fragment diluted in genomic DNA, followed by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

EC Isolation and Culture

ECs were isolated from heart and lung. Briefly, tissues collected on transgenic or nontransgenic animals were cut in small pieces and then incubated with 1.8 U/ml Dispase II (Boehringer Mannheim, Mannheim, Germany), 0.5 mg/ml DNase I (Boehringer Mannheim) and 1% fetal calf serum (FCS) (Gibco BRL, Grand Island, NY) in Hank's buffered salt solution (HBSS) (Gibco BRL) for 4 hr at 37°C. Biopsies were then incubated overnight in 2 IU/ml collagenase B (Boehringer Mannheim) at 4°C. Cell suspension was washed three times in HBSS medium before being plated on gelatin-coated tissue culture dishes (Nunc, Napervile, IL). DAF-expressing porcine endothelial cells were isolated from transgenic pig aortas by collagenase digestion (1 U/ml; Boehringer Mannheim). Human umbilical vein endothelial cells (HUVEC) were prepared as previously described (23). Purity of EC preparations was checked by the uptake of Dil-Ac-LDL as previously described (20). Endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose (Gibco BRL) and supplemented with 20% serum [fetal calf serum (FCS) (Gibco BRL) and human serum for rat ECs and HUVECs, respectively], 5 IU/ml heparin, 200 μg/ml Endothelial Cell Growth Supplement (Collaborative Biomedical Research, Becton Dickinson, San Jose, CA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Cells were analyzed between passages 1 and 4.

FACS Analysis

Endothelial cells (1–2 × 10^5 cells/sample) were suspended with trypsin-EDTA (Gibco BRL),

washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% NaN_3, and then incubated on ice for 30 min with a saturating concentration of the relevant monoclonal antibody (MAb). This step was followed by three washes in cold 1% BSA/0.1% NaN_3/PBS. Staining with a FITC-labeled-F(ab')2 fragment of either goat anti-mouse, sheep anti-rabbit, or mouse anti-human immunoglobulin G (IgG) or IgM (Jackson Lab., West Grove, PA) was performed at 4°C for 30 min. Three washes in 1% BSA/0.1% NaN_3/PBS were performed before fixing the cells in 1% paraformaldehyde in PBS. The following antibodies were used in this study: rabbit polyclonal serum anti-human von Willebrand factor (vWf) (Dako, Trappes, France), anti-human DAF (1H4) MAb (CRTS, Nantes, France), and anti-rat ICAM-1 MAb (Seikagaku America, Rockville, MD). For analysis of human Ig binding on rat ECs, cells were incubated with purified human anti-Galα1-3Gal antibodies (10 μg/ml, 100 μl/ 2×10^5 cells) for 30 min at 4°C, washed, and then incubated with specific FITC-labeled goat F(ab')2 anti-human IgM (Fcμ) or IgG (Fcγ) (Jackson Laboratories) antibodies.

Analysis of complement activation was investigated following incubation of rat ECs with human serum (dilution 1:10) in DMEM medium for 30 min at 37°C. Immunostaining was performed using FITC-labeled anti-human factor B, C3, C4 MAb (Atlantic Antibodies, Stillwater, MN) and an anti-human C5b-9 polyclonal serum as first antibodies revealed using FITC-labeled goat anti-rabbit IgG or IgM (Jackson Lab). Fluorescence was measured on 10,000 cells per sample using a FACScalibur (Becton Dickinson). Data are depicted in histograms plotting mean fluorescence intensity (MFI) on a log scale (x-axis) versus cell number (y-axis).

Complement-dependent Cytotoxicity Assay

Cells were labeled with ^{51}Cr (50 μCi/1 $\times 10^6$ cells) for 1 hr at 37°C. Protection of DAF-expressing cells was assessed by incubating 51Cr-labeled rat ECs (2×10^4 cells) with cynomolgus primate serum (as a source of xenogeneic antibodies and complement) (100 μl, dilution 1:4 to 1:16) for 4 hr at 37°C. Chromium release was measured in supernatants, and the percentage of cell lysis was calculated by the following formula: (experimental cpm − cpm medium only)/(100% lysis cpm − cpm medium only). All experiments were done in triplicate, and the results are expressed as the mean of the percentage of specific lysis ± SD values.

Immunohistochemistry

Tissues for immunohistology were included in OCT compound (Miles Laboratories, Elkhardt, IN) snap-frozen in precooled isopentane, and stored at −70°C until use. Human placenta and tissues from nontransgenic rats were included in every experiment as positive and negative controls, respectively. Frozen 5-μm tissue sections were acetone fixed, hydrated with PBS, incubated with hydrogen peroxide at 0.15% in methanol for 20 min, blocked with rat serum at 10% in PBS/1% BSA, and incubated at room temperature for 60 min with MAb at 10 μg/ml. Tissue sections were then incubated with biotin-conjugated horse anti-mouse IgG absorbed with rat serum proteins (Vector, Burlingame, CA) followed by horseradish peroxidase streptavidin (Vector) and developed with very intense purple (VIP kit, Vector). Slides were then counter stained with hematoxilin and mounted with glycerol. The following antibodies were used in this study: anti-rabbit vWf, anti-human DAF (1H4), and purified human anti-Galα1-3Gal antibody. Negative controls were performed using an isotype-matched irrelevant MAb (3G8). Semiquantitative analysis of DAF expression on endothelium was obtained by visual evaluation of the intensity of specific staining, which was ranked from 0 to 4 (0, no staining; 1, low intensity; 2, medium intensity; 3, high intensity; 4, very high intensity). Cardiac xenografts were analyzed for complement and xenogeneic antibody binding by immunofluorescence. Cynomolgus Ig binding was revealed through FITC-labeled goat F(ab')2 anti-human IgM (Fcμ) or IgG (Fcγ) (Jackson Laboratories) antibodies.

Ex Vivo Perfusion of Transgenic Hearts with Human Serum

Rats were anesthetized with ether and the hearts were removed rapidly. A 22-gauge in-dwelling cannula (Vasocan, Braun Melsungen, Germany) was introduced into the aorta and secured with a ligature. Using a syringe, the hearts were perfused immediately with oxygenated perfusate and subsequently connected to the Langendorff circuit. The circuit was oxygenated by continuous gassing with a mixture of 95% O_2 to 5% CO_2. The perfusate was kept at 37°C by continuous heated water. The largest reservoir was

filled with a solution containing 30% Haemaccel (Behring, Marburg, Germany) in Krebs-Henseleit buffer (in mM: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.66, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 5.55, sodium pyruvate 2.0). The small reservoir was filled with 15% human serum (a pool of 20 human type-A plasma) diluted with the solution from the large reservoir. Before serum perfusion, hearts were allowed to stabilize by perfusing them with the Krebs-Henseleit-Haemaccel solution for 10 min. The hearts were monitored continuously for heart rate, apex amplitude, and flow by means of multichannel registration. Duration of heart function was defined as the time between the start of serum perfusion and complete cessation of heart beat. Decomplementation was performed by heating the serum for 1 hr at 56°C. Hemizygous rats ($n = 5$) from the transgenic line 52.2 were tested and compared to wild-type (WT) rats ($n = 5$).

Cardiac Xenografts into Primates

Transgenic and WT Sprague-Dawley rats of 300–400 g body weight were used as donors and cynomolgus monkeys of 6–9 kg body weight were used as recipients. Heterotopic heart xenotransplantation was performed as previously described (19). To compare graft survival of heart from hemizygous and homozygous transgenics, one of each organ was concomitantly grafted onto the same recipient in each femoral vessel. Xenograft survival was determined by visual examination and palpation of xenogeneic heart beating. Histological analysis of rejected xenografts was performed on paraffin-embedded sections (5 μm) stained with hematoxilin-eosin.

Statistical Analysis

All data (mean ± SD) were analyzed using the Student's t-test, with $p < 0.05$ being the level of significance.

Results

Production of Transgenic Rats for Human DAF and Transgene Integration Analysis

A total of 754 eggs was microinjected and subsequently reimplanted into 37 pseudopregnant foster mothers, 27 of whom gave birth to 145 rats. Transgene integration was first detected by PCR in six founder animals and then confirmed by Southern blot performed on genomic DNA. Figure 1 shows the presence of a human DAF sequence in a major restriction fragment of 3.1 kb in these transgenic lines as determined by hybridizing Southern blots, using the ICAM2DAF cDNA construct as a probe. The hybridization pattern revealed that multiple copy numbers of transgenes, in a head-to-tail orientation, had been integrated in all of these animals within one integration site, with the exception of line 70.8. Evaluation was done by comparing the size and intensity of hybridized fragment with known amounts of the microinjected fragment. High copy numbers (10 to 100) were observed in transgenic rats 58.3, 62.3, and 70.8. The transgene was transmitted to about 50% of the progeny for founders 52.2, 52.4, 62.3, and 70.8 (37%, 37%, 50%, and 50%, respectively). In contrast, a low transmission rate of the transgene to the offspring was obtained with founders 54.9 (8%) and 58.3 (<3%), which suggests that the germ-line in these founders was mosaic for DAF integration.

Tissue Expression of Human DAF in Transgenic Rats

Expression of human DAF was investigated in several organs from transgenic rats, including heart, kidney, liver, and lung, and on lymphoid organs (thymus, spleen). Cryosections of tissues were analyzed by immunohistology with an anti-human DAF MAb and compared to tissues from nontransgenic control rats. Consistent levels of DAF were detectable in tissues on progeny from lines 52.2 and 62.3. A low level of DAF was observed on line 52.4 while no expression was obtained for the lines 54.9, 58.3, and 70.8. For both transgenic lines (52.2 and 62.3), ECs expressing the transgene were detected in all analyzed tissues, including heart, kidney, lung, and liver. In these organs, DAF expression was restricted to both vascular (large and medium size vessels) and capillar endothelium as determined by comparison with the pattern of staining obtained with an antibody directed to vWf (data not shown). All tissues analyzed from rat 52.2 showed higher expression of human DAF than those in corresponding organs from line 62.3. The nontransgenic (WT) control rat showed no staining of the tissues analyzed. Specific stainings for human DAF obtained in heart and kidney from an hemizygous transgenic rat (line 52.2) are presented in Figure 2. Confirmation of endothelium-specific DAF expression in transgenic

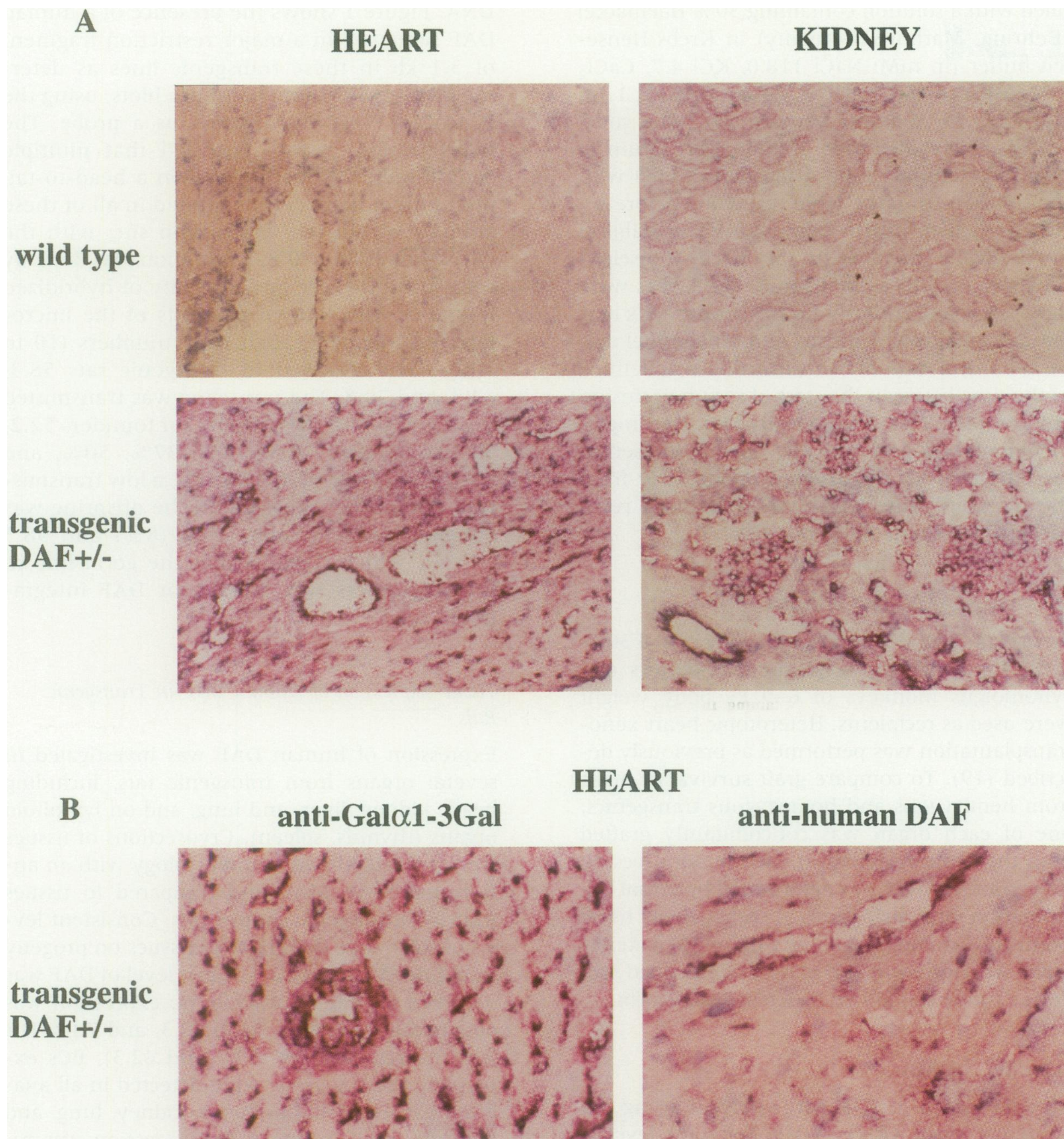

Fig. 2. Expression of human DAF in transgenic rat tissues. Frozen sections (5 μm) from transgenic (hemizygous $DAF^{+/-}$ rat from line 52.2) or wild-type (WT) rat tissues were fixed in acetone for 10 min at room temperature, air dried, and stained using an indirect immunoperoxidase technique. (A) Immunostaining of serial tissue sections from kidney or heart was performed with an anti-human DAF MAb as first antibody (magnification, ×200). Negative controls were performed using an isotype-matched irrelevant MAb (data not shown). (B) Immunostaining of $DAF^{+/-}$ transgenic rat heart with human anti-Galα1-3Gal antibodies or an anti-human DAF MAb as first antibody followed by staining with a peroxidase-labeled anti-human or anti-mouse Ig, respectively (magnification, ×400).

heart was achieved by comparison with the pattern of staining obtained with purified human anti-Galα1-3Gal (7). Relative levels of DAF expression on cells from these tissues were compared and are summarized on Figure 3. Expression was seen in a very small fraction of spleen

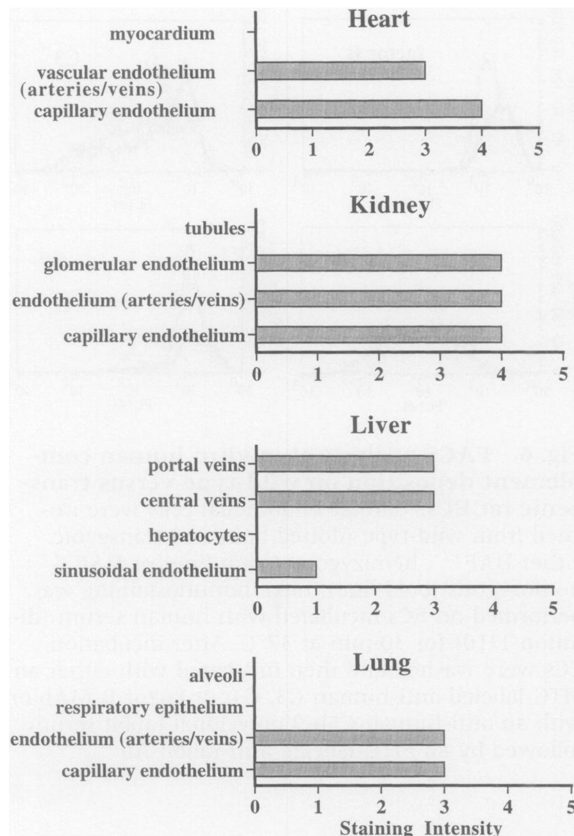

Fig. 3. Semiquantitative analysis of DAF endothelial expression in tissues from transgenic line 52.2. DAF expression in ECs from different tissues was determined by immunochemistry on cryosections as described in Materials and Methods. Results are expressed according to the intensity of specific staining (0, no staining; 1, low intensity; 2, medium; 3, high; 4, very high).

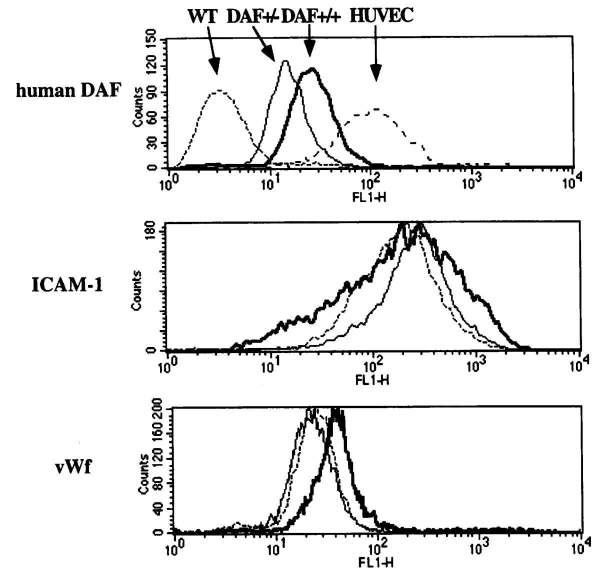

Fig. 4. FACS analysis of human DAF expression by ECs from hemizygous and homozygous transgenic rats. Endothelial cells were isolated from heart of wild-type (WT) and transgenic rats, either hemizygous ($DAF^{+/-}$) or homozygous ($DAF^{+/+}$). Immunostaining was performed using an anti-human DAF MAb to compare levels of DAF expression on ECs. An anti-rat ICAM-1 MAb and anti-human von Willebrand factor (vWf) antibody were used to assess the purity of EC preparations. Fluorescence was measured on 10,000 cells/sample using a FACScalibur. Data are depicted in histograms plotting mean fluorescence intensity (MFI) on a log scale (x-axis) versus cell number (y-axis).

cells in the white pulp. Similarly, a small fraction of medular thymic cells with dendritic prolongations showed expression of the transgene. High levels of expression were also detected in a fraction of granulocytes (7–10%) after indirect immunofluorescence labeling of leukocytes, red cells, and platelets followed by flow cytometry analysis (data not shown).

Levels of Human DAF Expressed by ECs Isolated from Transgenic Rats

Endothelial cells from heart and lung were isolated from hemizygous ($DAF^{+/-}$) and homozygous ($DAF^{+/+}$) rats derived from founder 52.2 and from a nontransgenic rat as control. Endothelial cell populations were over 95% pure as assessed through an anti-rat ICAM-1 and anti-vWF antibodies. Cell surface expression of human DAF was investigated by flow cytometry following staining with an anti-DAF MAb. Figure 4 shows that the level of human DAF expressed on cardiac ECs from homozygous ($DAF^{+/+}$) rat was about 50% the level of DAF on human ECs (HUVEC). As expected, DAF expression was higher in homozygous ($DAF^{+/+}$) transgenic rats than in hemizygous ($DAF^{-/+}$) rats. Similar results were obtained with ECs isolated from lung (data not shown).

Protection of DAF-expressing Rat ECs Against Primate Antibodies and C-Mediated Cell Lysis

The ability of human DAF to confer in vitro protection of rat ECs against primate xenogeneic (anti-rat) antibodies and complement-mediated cell lysis was investigated by incubating ECs isolated from transgenic rats with primate (cynomolgus) sera. Xenoreactivity of human serum for WT or transgenic rats EC was previously determined by flow cytometry analysis.

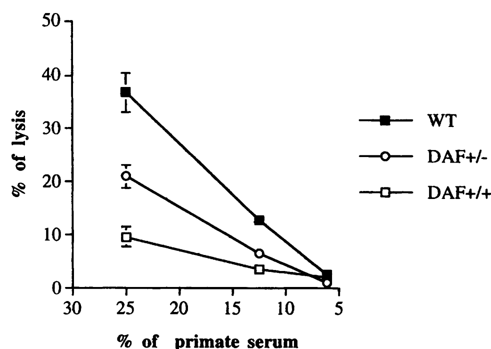

Fig. 5. In vitro protection of ECs isolated from transgenic rats against primate xenogeneic antibodies and complement-mediated cell lysis. Cardiac ECs from wild-type (WT) and transgenic, either hemizygous ($DAF^{+/-}$) or homozygous ($DAF^{+/+}$), rats were labeled with Cr^{51} and incubated for 4 hr at 37°C with serial dilutions (from 25% to 6.12%, 50 μl/well) of cynomolgus serum. Cell lysis was determined by measuring Cr^{51} release in culture supernatant as described in Materials and Methods. All experiments were done in triplicate, and the results are expressed as the mean of percent specific lysis ± SD values. Results are representative of three independent experiments.

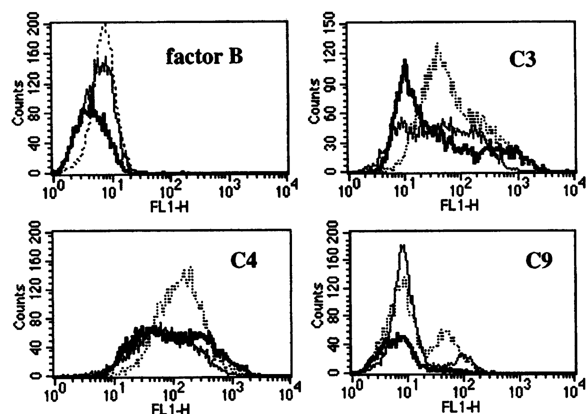

Fig. 6. FACS analysis of in vitro human complement deposition on wild-type versus transgenic rat ECs. Cardiac endothelial cells were isolated from wild-type (dotted line) and transgenic, either $DAF^{+/-}$ hemizygous (plain line) or $DAF^{+/+}$ homozygous (bold line), rats. Immunostaining was performed on ECs incubated with human serum (dilution 1/10) for 30 min at 37°C. After incubation, ECs were washed and then incubated with either an FITC-labeled anti-human C3, C4, or factor B MAb or with an anti-human C5b-9 polyclonal rabbit serum followed by an FITC-labeled anti-rabbit Ab.

Incubation of transgenic (hemizygous or homozygous) rat ECs with purified human anti-Galα1-3Gal antibodies showed binding of IgG and IgM XNA that was similar to that of ECs from WT nontransgenic rats (data not shown). Furthermore, ECs from either transgenic or control rats exhibited similar levels of Galα1-3Gal expression as measured by the binding of FITC-labeled *Bandera simplicifolia* lectin (data not shown). In vitro protection of DAF-expressing rat ECs against antibodies and complement-mediated cell cytotoxicity was measured by incubating rat ECs with serial dilutions of primate (cynomolgus) serum. As shown in Figure 5, primate serum induced a dose-dependent lysis of nontransgenic ECs for serum dilutions ranging between 6% and 25%. Compared to WT rat ECs, ECs isolated from transgenic rats were protected according to the level of human DAF expressed on the cell surface. When ECs were incubated in the presence of 25% serum, protection against cell lysis was 74 ± 6% ($p < 0.05$) and 43 ± 4% ($p < 0.05$) for homozygous and hemizygous, respectively, compared to WT rat ECs (9.6 ± 0.3%, 20.9 ± 0.1%, and 36.8 ± 0.1% of cell lysis for homozygous, hemizygous, and WT, respectively).

Flow cytometry analysis of complement activation on ECs, following incubation with human serum, confirmed the ability of DAF expression to prevent C3 deposition on ECs since lower amounts of human C3 were detected on transgenic ECs than on WT ECs (Fig. 6). An overall reduction in C9 was also observed on rat ECs from homozygous transgenic rats whereas no effect of DAF expression was observed for C4 and factor B deposition.

Ex Vivo Perfusion

The functional ability of hearts from hemizygous $DAF^{+/-}$ rats to prevent complement activation was investigated by ex vivo organ perfusion with 15% human serum. Cardiac function, assessed by cardiac frequence, was monitored over a 60-min perfusion time (Fig. 7). Our data showed that for DAF-expressing transgenic rats ($n = 5$), heart rate was not affected by serum perfusion and remained stable throughout the experiment. Similarly, perfusion of WT rat hearts with heat-inactivated serum ($n = 5$) showed normal cardiac function (data not shown). In contrast, cardiac function failed rapidly after perfusion of WT rat hearts with XNA and complement-containing human serum and stoped at 11.2 ± 1.7 min ($n = 5$).

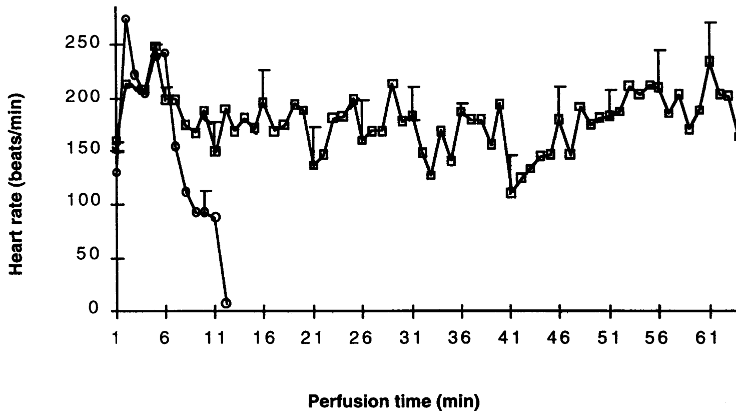

Fig. 7. Cardiac function of transgenic rat hearts during ex vivo perfusion with human serum. Hearts from wild-type (circles) ($n = 5$) or DAF$^{+/-}$ hemizygous transgenic (squares) ($n = 5$) rats were perfused with 15% human serum for 60 min using a Langendorff apparatus. Heart rate (y-axis) was monitored every min (x-axis) throughout the experiment. Every point of analysis corresponds to the mean value of five experiments and SD is shown for values taken every 5 min.

Xenotransplantation of DAF^{+} Transgenic Rat Hearts into Primates

Transplantation of transgenic hearts was performed on cynomolgus to assess whether the expression levels of human DAF on ECs were sufficient to prevent HAR. We previously demonstrated that in this discordant combination, cardiac xenografts were hyperacutely rejected in 5.5 ± 1.4 min following binding of primate XNA and activation of complement through the classical pathway (19). To overcome a potential variability in xenoreactivity of primate recipients, hemizygous and homozygous transgenic hearts were grafted in each femoral vessel from the same recipient. Survival times of transplanted transgenic hearts are reported in Table 1. These results show that levels of DAF expression were correlated in vivo with the degree of protection against XNA and complement-mediated HAR. Nevertheless, protection for homozygous hearts compared to that for hemizygous hearts was higher than expected from in vitro experiments (Figs. 5 and 6). Indeed, for hearts from hemizygous rats, rejection was delayed from 5 min to 15.5 min ($n = 4$) ($p < 0.05$ compared to WT hearts), whereas cardiac xenograft survival was up to >4.5 hr for homozygous rats ($n = 2$) ($p < 0.05$ compared to WT or hemizygous transgenic hearts). Because the time of rejection could only be assessed on anesthetized primates, only a minimal estimation can be given here.

Histology of DAF$^{+/-}$ rejected hearts, collected at the time of rejection, showed no significant evidence of edema or hemorrage but showed microthrombosis, as previously described for nontransgenic rats (19). Nevertheless, differences were observed among tissue samples according to survival time. Indeed, a transplant that survived for 28 min exhibited a significant PMN infiltration in the interstitium and binding

Table 1. Survival time of heterotopic cardiac xenografts of transgenic rat hearts (hemizygous DAF$^{+/-}$ or homozygous DAF$^{+/+}$) transplanted into cynomolgus recipients

	Rat donor		
	Wild type	**Hemizygous (DAF$^{+/-}$)**	**Homozygous (DAF$^{+/+}$)**
Survival time	5.5 ± 1.4 min	15.2 ± 7.4 min*	>1.5 hr**[a]
		(10, 11, 12, 28 min)	>4.5 hr**[a]
	($n = 10$)	($n = 4$)	($n = 2$)

Rat hearts were grafted on femoral vessels bilaterally for hemizygous and homozygous rats. Wild-type heart survival has been previously reported (19).

[a]Analyses of the last time points for each graft showing beating hearts (next analysis at 24 hr showed rejection).

*$p < 0.05$ for transplants from hemizygous DAF rats versus wild-type rats.

**$p < 0.05$ for transplants from homozygous DAF rats versus wild-type rats or hemizygous DAF rats.

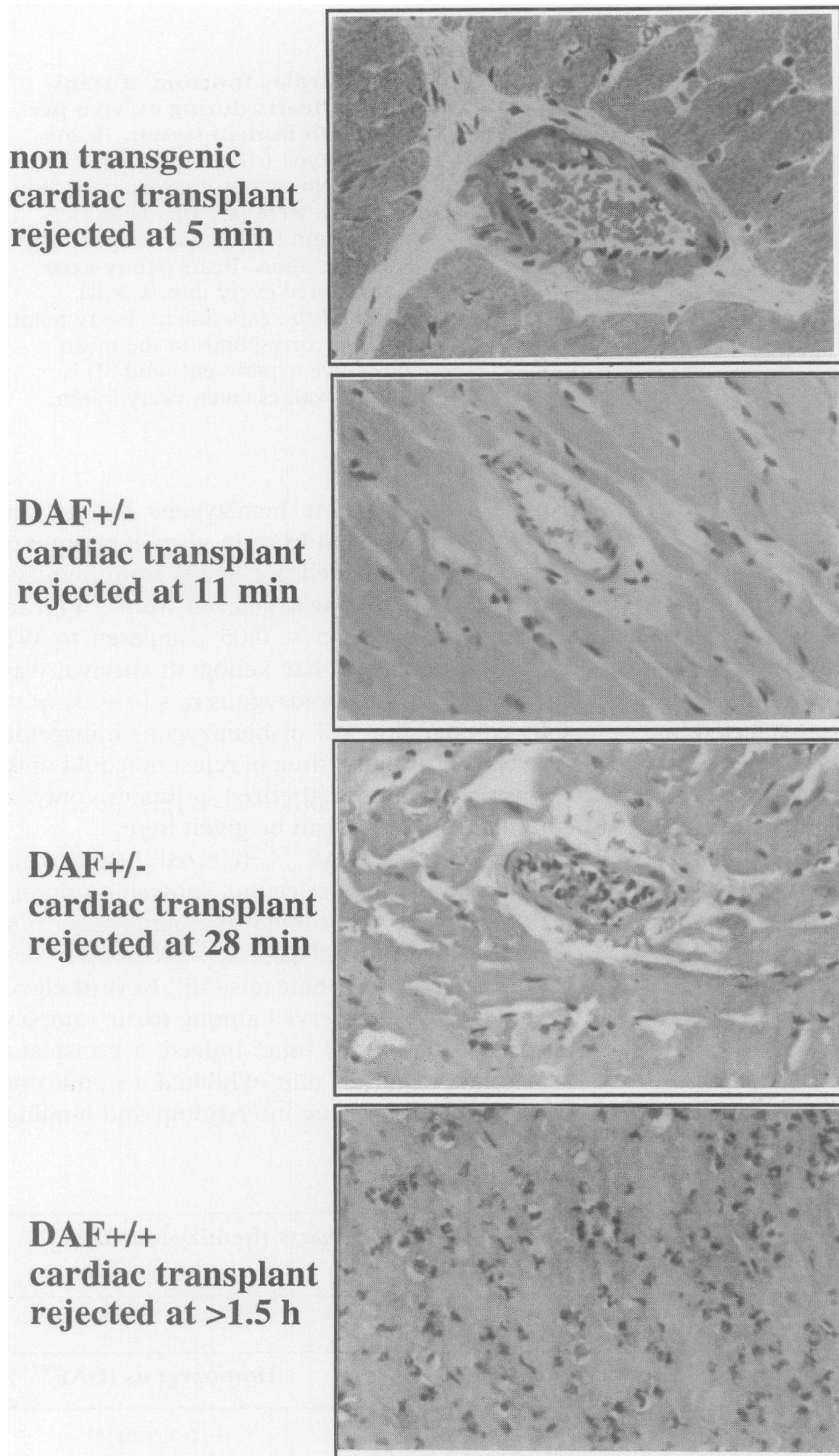

Fig. 8. Histological analysis of DAF-expressing cardiac xenografts. Hearts were collected at the following times of rejection: 5 min for wild type, and 11 min and 28 min for $DAF^{+/-}$ transgenic rats, and after rejection for $DAF^{+/+}$ transgenic rats. Tissue sections were counterstained with hematoxylin-eosin (magnification, ×400).

to the endothelium of blood vessels compared to cardiac xenograft rejected at 11 min (Fig. 8). Biopsies from DAF homozygous heart transplants also showed an abundant leukocyte infiltration that was mostly composed of PMN, but also include a small fraction of monocytes. Vascular damage, focal necrotic lesions of myocardium, and interstitial hemorrhage were also observed.

Immunohistochemical analysis revealed deposition along endothelial surfaces of recipient IgG, IgM, and complement components of the

Table 2. Immunohistochemical analysis of cardiac xenografts for human Ig and complement deposition

Rats	Human DAF	vWf	Human IgG	Human IgM	C3	C4	C9	Factor B
Ungrafted wild type ($n = 3$)	−	++	−	−	−	−	−	−
Wild type ($n = 3$)	−	+++	++	+	++	+/++	++	−
$DAF^{+/-}$ transgenic ($n = 3$)	+++	+++	++	+	+	+	−/+	−
$DAF^{+/+}$ transgenic ($n = 2$)	++++	+++	++	+	+	+	+/−	−

Immunostaining of xenograft cryosections was performed as described in Materials and Methods. Results are expressed as a mean of experiments performed on two ($DAF^{+/+}$ transgenics) or three (ungrafted, wild-type, and $DAF^{+/-}$ transgenic) animals. −, no staining; +, low intensity; ++, medium intensity; +++, high intensity; ++++, very high intensity.

classical but not the alternative pathway (Table 2). Less C3 and C9 fragment deposition was observed on transgenic tissues than on that of rejected organs from WT rats.

Discussion

Genetically engineered animals offer a unique opportunity for using donor animal organs for clinical transplantation. Transgenic pigs, which express molecules that could control immunological events associated with xenograft rejection, could be the first suitable source of these transplants. Transgenic mice are useful models for the analysis of promoter tissue specificity and transgene expression levels (24,25). However, there are no mouse models available for the study of transplantation of vascularized organs into primates. Investigation of transgenic or knockout mouse models (although restricted to ex vivo perfusion experiments) can be informative for several aspects of transgene function, but they do not allow analysis of the broad range of humoral and cellular interactions between graft and recipient that operate in vivo (25,26). Therefore, we have postulated that production of transgenic rats, which can be used for xenotransplantation into primates (19), could provide a useful in vivo model to test the benefits of genetic engineering interventions in xenotransplantation (22). The first step in validating this model was to demonstrate that endothelium-directed expression of human DAF in rat organs can delay xenograft HAR.

Transgene distribution in transgenic organs is a major concern for xenotransplantation. For membrane-bound proteins such as CRPs, transgenes should be expressed at least on ECs (the first target of XNA binding and complement activation), since few other cell types in the rat (and also the pig) express the Galα1-3Gal epitope (7). Our data show that endothelium-restricted transgene expression can be obtained in transgenic rats by using part of the human ICAM-2 gene promoter, and that tissue distribution of transgene expression in transgenic rats is almost identical to transgene expression in lines of transgenic mice (18).

The ability of human DAF to overcome primate XNA and complement-mediated rat cell lysis was demonstrated in vitro on isolated transgenic rat ECs. The data generated from this study provided in vivo confirmation of our previous in vitro experiments performed on transfected rat ECs, which demonstrated the ability of human DAF and CD59 to protect rat ECs from primate complement-mediated damage (20). Cardiac-derived ECs from rats homozygous for DAF expressed around 50% of the DAF levels observed in HUVECs. However, compared with other human cells, HUVECs express very high levels of DAF (3×10^5 molecules per cell compared with 8×10^4 molecules per cell for human leukocytes) (27,28). DAF levels on human ECs derived from organs have not yet been reported.

Because ECs from transgenic rats homozygous for DAF were more resistant to primate serum-mediated damage than ECs from rats hemizygous for DAF, in vitro inhibition of com-

plement-dependent EC lysis and complement fragment deposition was said to be correlated with DAF levels. In ex vivo perfusion experiments, hemizygous DAF hearts were very efficiently protected from complement-mediated damage from 15% human serum, but showed only limited survival in vivo. Although a statistically significant improvement of graft survival time for hemizygous rats was observed (5 min for WT rats versus 15 min for hemizygous DAF rats), this has little relevance in vivo. The difference in in vivo versus ex vivo survival time is probably due to higher XNA and complement concentrations in vivo and the contribution of recipient blood cells absent from the ex vivo experiments. In contrast, the prolongation of xenograft survival to a minimal period of several hours (>1.5 hr to 4.5 hr) in homozygous DAF rats indicates that this level of transgene expression is sufficient to delay HAR in the absence of XNA depletion or immunosuppression of the recipient.

In some previous reports on the generation of transgenic animals for CRPs, levels of transgene expression have been quantified on ECs. Diamond et al. showed that expression of CD59 on aortic ECs from transgenic pigs reached 20% of the levels of HUVEC, conferred 50% protection against complement-mediated EC lysis in vitro, and compared with the controls, slightly prolonged xenograft survival (0.5 hr and 1 hr versus 2.2 hr and 3 hr, respectively) (29). Byrne et al. showed that aortic ECs expressed CD59 and DAF levels that were 67% and 45% of HUVEC levels, respectively, and had a 3- to 5-fold increased resistance against complement-mediated lysis in vitro (14). Xenografts were shown to have variable survival periods compared with controls (range 6 hr to 5 days versus 0.5 hr to 1 hr, respectively), and survival could be prolonged by depletion of xenoreactive antibodies (16). Importantly, in these transplantation trials, recipients also received extensive immunosuppressive and anti-coagulant treatment (14,16,29). Taken together these studies suggest that although moderate levels of DAF transgene expression (20–40% of HUVEC levels) are able to confer protection against HAR in conditions in which complement or antibodies are limited (in vitro or ex vivo perfusion), a minimal threshold of protection in vivo requires DAF levels that are at least close to 50% of HUVEC levels. In agreement with this hypothesis, Cozzi et al. reported that transgenic hearts, which expressed DAF at levels several-fold higher than HUVECs (13), showed effective protection during ex vivo perfusion with human blood, and xenograft survival was considerably prolonged (5.1 days versus 1.6 days in controls) in the absence of any other treatment (15). However, these transgenic organs, even in immunosuppressed recipients, have been almost invariably rejected by AVR (30,31).

Nontransgenic rat hearts transplanted into primates have been rejected with a histological pattern of HAR showing endothelial cell damage, platelet microthrombi, and little PMN vascular accumulation (19). In contrast, rat hearts transplanted into primates treated with cobra venom factor (19) or DAF transgenic hearts that have survived transplantation for at least 28 min have been rejected, with a histological pattern of AVR showing extensive thrombosis, leukocyte accumulation (mainly PMN) in the vessels and tissues, edema, and hemorrhage. Furthermore, increased levels of DAF in transplanted organs (from homozygous and hemizygous DAF transgenic rats) result in longer periods of graft survival that correlate with progressive PMN infiltration, hemorrhage, and focal myocardial necrosis. Similarly, AVR is the most common result of xenotransplantation between discordant species (guinea pig-to-rat or pig-to-primate) after XNA depletion, complement inactivation of recipients, or transplantation of CRP transgenic organs (4,10,12,14–16,32,33). Although AVR in these models share common pathological features (hemorrhage, edema, and presence of leukocytes), they also show differences in the type of infiltrating leukocytes. In transplant combinations involving rats as recipients, mononuclear cell infiltrates predominate (33,34), whereas combinations involving primates as recipients (especially when the primates do not receive immunosuppressive drugs) show leukocyte infiltrates with mononuclear cells and a high proportion of PMN (4,15).

Taken together, our results demonstrate that a moderate level of human DAF expression on rat endothelium prolongs xenograft survival beyond HAR and that the PMN infiltration, hemorrhage, and focal myocardial necrosis associated with AVR in pig-to-primate transplants are also present in rat-to-primate transplants. It is likely that higher levels of DAF expression are required to further improve xenograft survival, or that other factors in addition to complement (such as XNA binding to ECs and mechanisms such as coagulation and EC activation) must be controlled to prevent xenograft AVR.

In conclusion, xenotransplantation of transgenic organs expressing CRPs, from either pig or

rat, prevents HAR but not the development of AVR, even when immunosuppressed primates are used as recipients (14–16,30,31). Therefore, there is an urgent need to express new transgenes to protect xenografts and decrease the side effects of immunosuppression. These transgenes should probably be aimed at decreasing Galα1-3Gal expression or inhibiting coagulation and tissue infiltration by leukocytes. The development of transgenic rats represents a more rapid alternative than the production of transgenic pigs for testing the usefulness of new strategies and allows an easier analysis of mechanism of action. In this regard, the backcross of transgenic rats expressing DAF to prevent HAR, and the backcross of rats expressing Fas ligand in the endothelium (35) that could inhibit PMN adherence to endothelium (36) may facilitate analysis of the role of early leukocyte infiltration in AVR.

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