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Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection

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ABSTRACT The serious shortage of human organs available for transplantation has engendered a heightened interest in the use of animal organs (xenografts) for transplantation. However, the major barrier to successful discordant xenogeneic organ transplantation is the phenomenon of hyperacute rejection. Hyperacute rejection results from the deposition of high-titer preformed antibodies that activate serum complement on the luminal surface of the vascular endothelium, leading to vessel occlusion and graft failure within minutes to hours. Although endogenous membrane-associated complement inhibitors normally protect endothelial cells from autologous complement, they are species restricted and thus confer limited resistance to activated xenogeneic complement. To address the pathogenesis of hyperacute rejection in xenotransplantation, transgenic mice and a transgenic pig were engineered to express the human terminal complement inhibitor hCD59. High-level cell surface expression of hCD59 was achieved in a variety of murine and porcine cell types, most importantly on both large vessel and capillary endothelium. hCD59-expressing porcine cells were significantly resistant to challenge with high-titer anti-porcine antibody and human complement. These experiments demonstrate a strategy for developing a pig-to-primate xenogeneic transplantation model to test whether the expression of a human complement inhibitor in transgenic pigs could render xenogeneic organs resistant to hyperacute rejection.

The lack of effective therapies aimed at eliminating antibody- and complement-mediated hyperacute rejection presents a major barrier to the successful transplantation of discordant animal organs into human recipients (1–6) and has precluded the development of animal models aimed at evaluating the *in vivo* cellular immune response to discordant xenografts. Old World primates, including humans, have high levels of pre-existing circulating natural antibodies that predominantly recognize carbohydrate determinants expressed on the surface of xenogeneic cells from discordant species (2–6). Recent evidence indicates that most of these antibodies react with the carbohydrate epitope, Gal(α 1-3)Gal (7), an epitope absent from Old World primates because of a lack of the functional α -1,3-galactosyltransferase enzyme (8). Therefore, after transplantation of a vascularized xenogeneic donor organ into a primate recipient, the massive inflammatory response that ensues from natural antibody activation of the classical complement cascade leads to activation and destruction of the vascular endothelial cells and ultimately of the donor organ within minutes to hours after revascularization (2–6). Endogenously expressed membrane-associated

complement regulatory proteins normally protect endothelial cells from autologous complement. However, the activity of these complement inhibitors is species restricted. This property makes them relatively ineffective at inhibiting xenogeneic serum complement (9, 10). The demonstration that a human complement inhibitor could protect a xenogeneic cell from human complement-mediated lysis showed that it was possible to inhibit human anti-porcine hyperacute rejection in *in vitro* models (11).

The strategy used to address the pathogenesis of hyperacute rejection in the porcine-to-primate xenotransplantation model was to produce transgenic swine expressing high levels of the human terminal complement inhibitor hCD59. hCD59 is an 18- to 20-kDa glycosyl-phosphatidylinositol-anchored cell surface glycoprotein that is expressed in a variety of tissues of both hematopoietic and nonhematopoietic lineage and functions to inhibit formation of the membrane attack complex by binding to membrane C5b-8 and C5b-9 (9, 10). Stable expression of hCD59 on xenogeneic cells *in vitro* protected the cells from human complement-mediated cell lysis (12–14) and the level of protection was directly proportional to the number of molecules of hCD59 expressed on the surface of the xenogeneic cell (14). Importantly, hCD59-expressing porcine aortic endothelial cells were resistant not only to cell lysis but also to complement-mediated formation of a procoagulant surface when challenged with either human or baboon serum (15). Taken together, these results indicated that high-level expression of hCD59 could provide porcine tissue with significant protection from human serum complement in a xenotransplantation setting. Therefore, hCD59 was chosen as a candidate molecule for production of transgenic swine resistant to human complement. In this report, we demonstrate the successful production of a transgenic pig expressing high levels of hCD59 that protect the pig cells from human complement-mediated cell lysis.

MATERIALS AND METHODS

H2K^b-hCD59 DNA Construct, Purification, and Microinjection. A hCD59 cDNA was directionally cloned into exon 1 of the murine H2K^b-gene 12 nucleotides downstream of the transcriptional start site. Briefly, the hCD59 cDNA fragment was excised from a hCD59-pcDNAI-Amp (pcDNAI-Amp; Invitrogen) expression plasmid by digestion with *Hind*III, followed by enzymatically filling in the 5' 4-nucleotide overhang with T4 DNA polymerase and dNTPs. Subsequently,

Abbreviations: MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; mAb, monoclonal antibody; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; hrTNF- α , human recombinant tumor necrosis factor α ; IFN, interferon.

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the DNA was digested with *Not I* at the 3' end of the multiple cloning site of the vector to yield a 452-bp cDNA fragment. The 9.0-kbp *EcoRI* *H2K^b* genomic restriction fragment (16) cloned into pGEM7Z (Promega) was digested with *Nru I* and *Not I*, resulting in the removal of 51 nucleotides from the *H2K^b* gene including the ATG start codon. The hCD59 cDNA was then directionally ligated into the *H2K^b* gene in the pGEM7Z vector.

Purification of the *H2K^b*-hCD59 DNA for embryo injection was accomplished by digesting the plasmid with *Xho I* to remove the vector sequences followed by agarose gel electrophoresis, electroelution, and Elutip purification (Schleicher & Schull). Transgenic mice were produced by pronuclear microinjection of murine ova as described (17). Ten of 60 offspring were identified as transgenic founder animals by DNA slot blot hybridization (18) (data not shown). Transgenic swine were generated by porcine embryo injection (19). A total of 18 piglets were analyzed by DNA slot blot analysis of genomic DNA (18). One founder animal, *H2K^b*-hCD59 153-2, contained 10–20 copies of the *H2K^b*-hCD59 DNA. Two additional founder animals, *H2K^b*-hCD59 152-1 and *H2K^b*-hCD59 152-2, contained ≈ 1 copy of the *H2K^b*-hCD59 DNA and exhibited no expression or very low and inconsistent levels of expression in peripheral blood mononuclear cells (PBMCs) (data not shown). These animals were not analyzed further.

Cell Culture, Immunofluorescence, and Immunohistochemistry. PBMCs from transgenic and negative littermate control pigs were purified from whole blood by Ficoll gradient centrifugation (ref. 20, pp. 7.1.1–7.1.2). Adherent monocytic mononuclear cells were cultured in Dulbecco's modified Eagle's medium/15% fetal bovine serum. PBMCs from transgenic mice and negative littermate control animals were purified from whole blood by ACK lysis (Biofluids, Rockville, MD). Indirect immunofluorescence of porcine PBMCs was performed with the anti-hCD59 mouse monoclonal antibody (mAb) MEM-43 (Biodesign International, Kennebunkport, ME) and with the anti-swine leukocyte antigen (SLA) class I mAb PT85A (VMRD, Pullman, WA). Indirect immunofluorescence of murine PBMCs was performed with polyclonal antisera specific for hCD59 (P. Sims, Blood Research Institute, Milwaukee). Goat anti-rabbit IgG (polyclonal sera; Zymed) or goat anti-mouse IgG (monoclonal sera; Zymed) fluorescein isothiocyanate (FITC)-conjugated antisera were used to detect specific antibody binding to the cell surface. Cell surface expression was then measured by flow cytometry on a Becton Dickinson FACSsort.

The cytokine inducibility of *H2K^b*-hCD59 and the endogenous porcine SLA class I molecule was tested on adherent peripheral blood monocytes. Briefly, porcine cytokine-conditioned medium supernatants were produced from control pig PBMCs. PBMCs harvested from a control pig were stimulated with phytohemagglutinin (PHA; 5 μ g/ml) for 48 h. PHA-conditioned media were collected and treated with 10 mM methyl α -mannoside and filter sterilized. Human recombinant tumor necrosis factor α (hrTNF- α ; Collaborative Biomedical Products, Bedford, MA) was used at 500 units/ml. Adherent peripheral blood monocytes were then treated with medium alone, 50% PHA-conditioned medium (diluted 1:1 with complete medium), 50% PHA-conditioned medium/hrTNF- α , or hrTNF- α for 24 h. Cytokine-induced expression of hCD59 and SLA class I was detected by immunofluorescence and fluorescence-activated cell sorter analysis as described above.

Immunohistochemistry was performed on fresh frozen sections embedded in Tissue-Tek OCT compound (Miles). Tissue sections (5–10 μ m) were processed as described (ref. 20, pp. 5.8.1–5.8.2). Sections that were double stained were processed simultaneously with the mouse anti-hCD59 mAb, MEM-43 (20 μ g/ml), and the anti-type IV collagen rabbit

polyclonal antiserum (21) (1:50 dilution). Fluorochrome-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antisera were used to detect specific antibody interactions with the hCD59 antigen (goat anti-mouse rhodamine; AMAC, Westbrook, ME) and type IV collagen antigen (goat anti-rabbit FITC; Zymed).

Complement-Mediated Dye Release Assays. PBMCs or peripheral blood adherent cells were labeled with the intracellular dye Calcein AM (Molecular Probes). The cells were subsequently incubated with anti-porcine blood cell IgG (2 mg/ml) (Intercell Technologies, Hopewell, NJ) followed by incubation in increasing concentrations of human whole serum (Sigma) at 37°C for 30 min. Dye released from the cells was determined by flow cytometry on a Becton Dickinson FACSsort. The C5b-9-specific dye release was calculated as percentage of total, correcting for nonspecific dye release and background fluorescence measured on identically matched controls without the addition of serum. Antibody blocking experiments were performed by the complement-mediated dye release assay as described above with the following exceptions. The cells were incubated in 20% C8-deficient serum (C8d; Quidel, San Diego) at 37°C for 30 min after anti-porcine blood cell antibody activation. The cells were then incubated with hCD59 polyclonal antiserum (100 μ g/ml) or anti-SLA class I antiserum PT85A (100 μ g/ml). Purified human C8 (Quidel) and C9 (Quidel) complement components were then added in increasing concentrations and incubated at 37°C for 30 min. Dye released from the cells was detected by flow cytometry on a Becton Dickinson FACSsort as described above.

RESULTS

Transgenic Expression. To achieve expression of the transgene-encoded hCD59 we engineered a murine major histocompatibility complex (MHC) class I gene, *H2K^b* (16), to control the expression of a hCD59 cDNA, *H2K^b*-hCD59 (Fig. 1). The MHC class I gene is ubiquitously expressed on most somatic cells and, most importantly, is a predominant endothelial cell surface antigen (22, 24). In addition, the MHC class I promoter contains cis-acting regulatory elements that bind cytokine-inducible trans-acting factors, resulting in up-regulation of the class I gene upon stimulation with interferon (IFN)- α/β , IFN- γ , and TNF- α (22–25). A hCD59 cDNA was cloned into exon I of *H2K^b* and results in a transcript that initiates at the *H2K^b* transcriptional start site and proceeds through both the cDNA insert and the entire transcriptional unit of the *H2K^b* gene. Translation initiates at the ATG codon of the inserted cDNA and terminates at the cDNA stop codon. The rest of the *H2K^b* gene remains untranslated and functions only in RNA processing, providing the cDNA with a genomic structure that contains all the regulatory elements required for *H2K^b* expression (22–25).

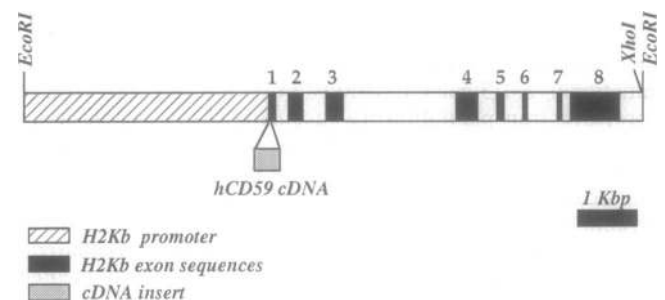


FIG. 1. *H2K^b* genomic cassette. A linear representation of the hybrid gene construct detailing the exon-intron structure of *H2K^b* and the insertion of the hCD59 cDNA into exon 1.

The efficacy of the $H2K^b$ -hCD59 genomic expression construct in directing cell surface expression of hCD59 in various tissues was tested in transgenic mice and pigs. Initial analysis demonstrated that the $H2K^b$ -hCD59 genomic construct directed the expression of hCD59 on the surface of PBMCs in several founder transgenic mice and transgenic pig 153-2 (Fig. 2A and B, respectively). Importantly, expression of hCD59 on the surface of the porcine mononuclear cells paralleled that of SLA class I (Fig. 2B). The comparable expression of hCD59 to SLA class I indicated that the murine

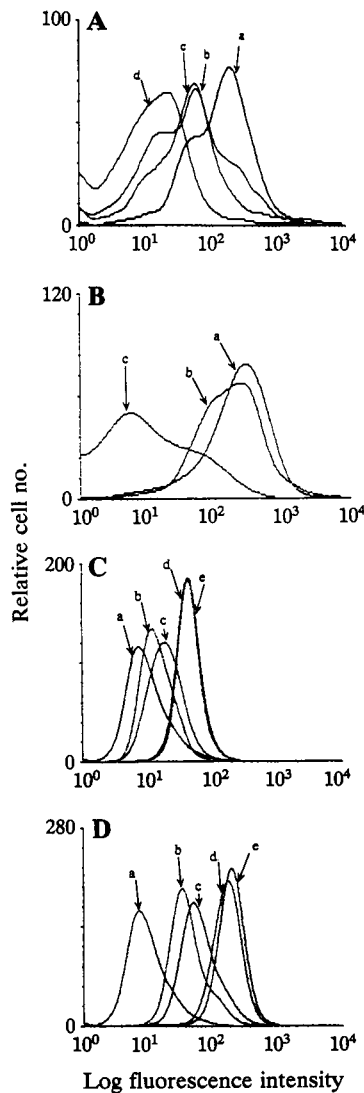


FIG. 2. Cell surface expression of hCD59 in transgenic mice and a transgenic pig. (A) Expression of hCD59 on murine PBMCs detected in transgenic mice $H2K^b$ -CD59-11 (curve a), $H2K^b$ -CD59-23 (curve b), $H2K^b$ -CD59-21 (curve c), and a negative littermate control (curve d). (B) Cell surface expression of hCD59 and SLA class I detected on porcine PBMCs. Curve a, hCD59 expression in transgenic pig $H2K^b$ -hCD59 153-2; curve b, SLA class I expression in transgenic pig $H2K^b$ -hCD59 153-2; curve c, negative littermate control PBMCs incubated with the hCD59 mAb. (C) Cytokine-induced cell surface expression of hCD59 on cultured adherent PMBCs from pig $H2K^b$ -hCD59 153-2; goat-anti-mouse FITC control antisera (curve a); hCD59 expression on uninduced cells (curve b); hrTNF- α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF- α (curve e). (D) Cytokine-induced cell surface expression of SLA class I on cultured adherent PMBCs from pig $H2K^b$ -hCD59 153-2; goat-anti-mouse FITC control antiserum (curve a); uninduced cells (curve b); hrTNF- α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF- α (curve e).

$H2K^b$ -hCD59 chimeric gene was constitutively regulated, similar to the endogenous porcine SLA class I molecules. To establish whether the $H2K^b$ -hCD59 chimeric gene exhibited cytokine inducibility comparable to the endogenous SLA class I gene, we cultured adherent monocytic PBMCs. Interestingly, after prolonged culture, these monocytes had downregulated cell surface expression of both SLA class I as well as the hCD59 transgene-encoded protein (compare Fig. 2B, curve b, to Fig. 2D, curve b for class I and Fig. 2B, curve a, to Fig. 2C, curve b, for hCD59). Treatment of the transgenic porcine cells with PHA-induced cytokine-conditioned medium, with hrTNF- α , or with a combination of the treatments resulted in an increase in hCD59 expression (Fig. 2C) as well as an increase in SLA class I expression (Fig. 2D).

We next examined hCD59 expression on the endothelium of vascularized organs. Immunohistochemical analyses were performed on fresh-frozen tissue sections derived from hCD59 transgenic mice and pigs as well as from nontransgenic littermates. Phase-contrast micrographs illustrating the structure of mouse myocardium are shown in Fig. 3A and D. Tissue sections from three founder mice were analyzed for hCD59 expression. Mouse hearts were incubated with anti-collagen type IV polyclonal antisera to detect basement membrane structures underlying the endocardium as well as intramyocardial capillary endothelia (21). Fig. 3B and E, respectively, confirmed equivalent collagen staining in the negative littermate control and a representative hCD59 transgenic mouse, $H2K^b$ -hCD59-8. In contrast, staining with a mAb specific for hCD59 revealed intense cell surface expression on endothelial cells in the heart of transgenic mouse $H2K^b$ -hCD59-8 (Fig. 3F) and an absence of hCD59 expression in the negative littermate control (Fig. 3C). Fig. 3F dramatically highlights the expression of hCD59 on vascular structures and clearly shows high-level expression of hCD59 on the endocardium in the ventricular chamber. Abundant hCD59 was also detected on capillary vessels within the myocardium (Fig. 3F). All three founder transgenic mice analyzed revealed hCD59 staining on the endocardium and capillary endothelium. To evaluate vascularized structures in the transgenic pig without having to sacrifice the founder animal, tail sections were prepared and analyzed by immunohistochemistry as described for the mice. Phase-contrast micrographs illustrate the morphological structure of a tail artery from a negative control pig (Fig. 4A) and a tail artery

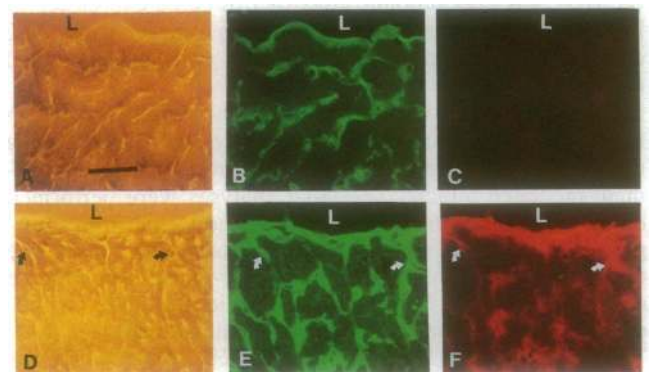


FIG. 3. Double-label immunofluorescence microscopy of hCD59 and type IV collagen on murine heart tissue from a $H2K^b$ -hCD59 transgenic mouse and a negative littermate control. Phase-contrast micrographs of murine ventricular myocardium (A and D). L, lumen of the left ventricle lined by endothelial cells. (B and E) Immunofluorescence micrographs detecting type IV collagen (fluorescein) of the same myocardial sections illustrating basement membrane structures underlying the endocardium. Immunofluorescence micrographs (rhodamine) of the same myocardial sections detecting hCD59 in a negative littermate control (C) and $H2K^b$ -hCD59-8 (F). ($\times 400$). (Bar = 25 μ m.)

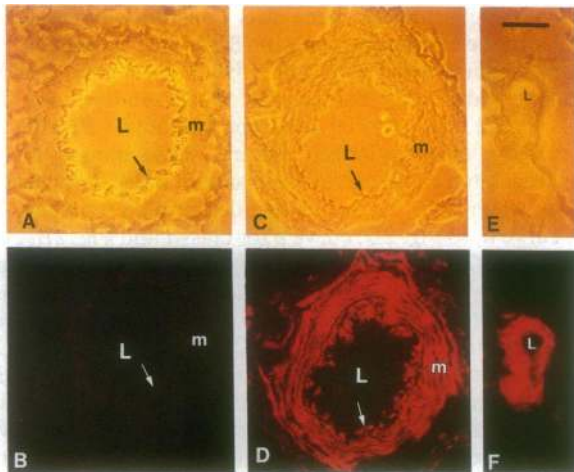


FIG. 4. Immunofluorescence microscopy of hCD59 on swine tail sections from pig $H2K^b$ -hCD59 153-2 and a negative littermate control. (A) Phase-contrast micrograph of a dermal artery from the negative littermate illustrating the lumen (L), the endothelial cell layer (arrow), and the tunica media (m). (B) Immunofluorescence micrograph (rhodamine) of the same section pictured in A, illustrating the lumen, the endothelial cell layer, and the tunica media. (C) Phase-contrast micrograph of a dermal artery from pig $H2K^b$ -hCD59 153-2, illustrating the lumen, the endothelial cell layer, and the tunica media. (D) Immunofluorescence micrograph (rhodamine) of the same section pictured in C, illustrating the lumen, the endothelial cell layer, and the medial smooth muscle cells (m). (E) Phase-contrast micrograph of a dermal microvessel from pig $H2K^b$ -hCD59 153-2, illustrating the lumen and the vessel wall. (F) Immunofluorescence micrograph (rhodamine) of the same section pictured in E, illustrating the lumen, and an abundance of hCD59 expression. ($\times 400$). (Bar = 25 μm .)

and small vessel from the transgenic founder pig 153-2 (Fig. 4 C and E, respectively). High-level hCD59 expression was observed on a variety of tissue and cell types, including fibroblasts, epithelial cells, vascular endothelial cells, and smooth muscle cells within the tail section of the transgenic pig (Fig. 4 D and F) but not in the negative littermate (Fig. 4B). Not all tissue in the transgenic pig tail section revealed hCD59 staining; however, tissues such as striated muscle are known to express very low levels of the class I antigen and therefore would not be expected to express the class I-regulated hCD59 transgene (24). These analyses confirmed that the $H2K^b$ -hCD59 genomic construct directed expression of hCD59 to a variety of cells and tissues in transgenic pig 153-2 and, most importantly, to the surface of vascular endothelial cells.

Complement Resistance. To determine whether the high levels of transgene expression observed on the transgenic pig cells conferred significant protection from human complement-mediated attack, functional analyses were performed on hCD59-expressing porcine PBMCs collected from transgenic pig 153-2 and a nontransgenic littermate control. The data clearly demonstrated that hCD59-expressing porcine cells, but not cells from a nontransgenic littermate, significantly resisted human complement-mediated lysis (Fig. 5A). The percentage dye released from hCD59 protected cell was ≈ 5 -fold less when compared with the amount of dye released from negative littermate control cells. To confirm that the protection observed in PBMCs was due specifically to hCD59 expression, antibody blocking experiments were performed. As shown in Fig. 5B, the anti-hCD59 polyclonal antisera blocked the hCD59-mediated protection, resulting in an increased susceptibility of the porcine cells to human complement-mediated cell lysis. In contrast, the control antibody had no effect.

To evaluate whether the degree of protection of porcine cells from human complement attack was a function of the

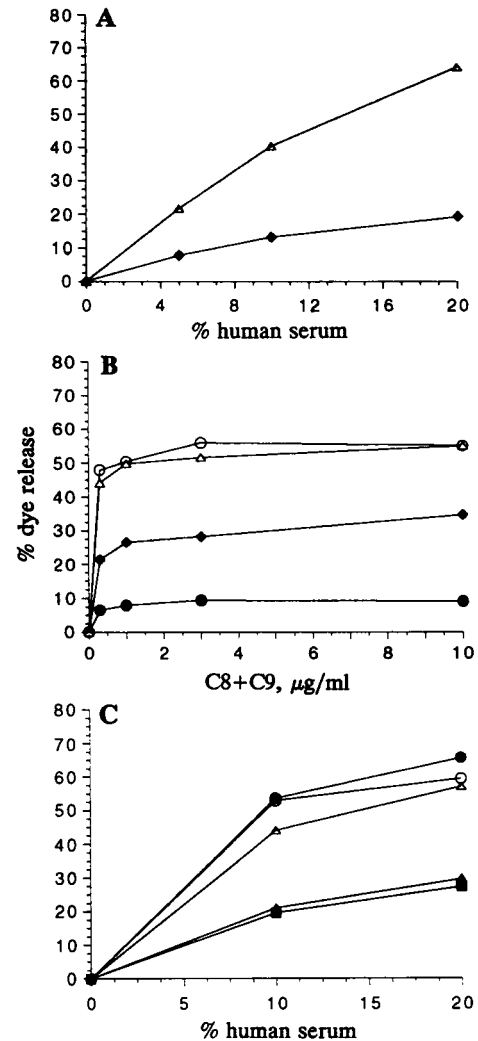


FIG. 5. Complement-mediated dye release assays on porcine PBMCs and cultured peripheral blood adherent cells. (A) Dye release assay performed on porcine PBMCs (\blacklozenge), transgenic pig $H2K^b$ -hCD59 153-2; (Δ), negative littermate control. (B) Dye release assay performed on PBMCs from transgenic pig $H2K^b$ -hCD59 153-2 incubated in the presence of anti-hCD59 polyclonal antiserum (\blacklozenge); control class I antibody PT85A (\bullet); negative littermate control PBMCs incubated in the presence of anti-hCD59 polyclonal antiserum (Δ); control class I antibody PT85A (\circ). (C) Complement-mediated dye release assays on porcine peripheral blood adherent cells from pig $H2K^b$ -hCD59 153-2; uninduced cells (Δ), PHA supernatants (\blacksquare), PHA supernatants + hrTNF- α (\blacktriangle), hrTNF- α (\bullet), and control negative littermate cultured peripheral blood adherent cells (\circ).

level of hCD59 expressed on the cell surface, experiments were performed on the cultured monocyte lines derived from the $H2K^b$ -hCD59 153-2 transgenic pig, which showed increased cell surface expression in response to cytokine treatments (see Fig. 2). Significantly, these monocytes demonstrated increased susceptibility to human complement-mediated lysis, consistent with the loss of hCD59 expression (Fig. 5C). As previously shown, culture of these cells in the presence of cytokines known to induce the MHC class I promoter—i.e., IFN- γ and TNF- α —upregulated hCD59 expression (Fig. 2C). Importantly, upregulating hCD59 expression restored their complement-resistant phenotype (Fig. 5C). These results confirm that the level of transgene expression correlates with cellular protection and also highlight the potential utility of the inducible $H2K^b$ promoter in the setting of a cytokine-mediated inflammatory response.

DISCUSSION

Expression of human complement inhibitor hCD59 was established in transgenic mice and in a transgenic pig utilizing the murine MHC class I gene as a genomic expression cassette. The proteins encoded by the MHC class I genes from human (HLA), mouse (MHC), and swine (SLA) are expressed in most somatic cell types including the vascular endothelium (22, 24, 26). Therefore, a MHC class I promoter should direct high-level transgene expression in the endothelial cells of vascularized organs. The additional advantage to this genomic expression strategy is that the class I promoter has the capacity to upregulate hCD59 expression in response to the inflammatory cytokines IFN- γ and TNF- α (22, 24, 25).

We have approached the problem of complement-mediated hyperacute rejection during pig-to-primate xenotransplantation by engineering the xenogeneic donor tissue with human complement inhibitor hCD59. The analyses of hCD59 in *H2K^b*-hCD59 transgenic mice and transgenic pig 153-2 demonstrated that the *H2K^b*-hCD59 genomic construct regulated the expression of hCD59 in the context of a transgenic genome. Cell surface expression of hCD59 was detected in a variety of cells and tissues, including the vascular endothelium. The assays used to determine the protective effects of hCD59 expressed on the transgenic cells were performed with human whole serum, which contains serum complement components, as well as high-titer natural antibodies (W.L.F. and S.A.R., unpublished data). In addition, anti-porcine lymphocyte antiserum was used to enhance the activation of the classical complement pathway on the surface of the target cell. Our data demonstrated that the level of hCD59 expressed on the cell surface protected the xenogeneic cell even in the presence of additional complement-activating antibodies.

The utility of blocking complement as a method to prevent hyperacute rejection in pig-to-primate xenotransplantation was demonstrated by using cobra venom factor (CVF) and recombinant soluble complement receptor type 1 (sCR1) (refs. 27 and 28, respectively). A significant delay of complement-mediated hyperacute rejection in pig-to-primate heterotopic cardiac xenotransplantation was observed with the administration of CVF for two consecutive days before transplantation (27) or with a single intravenous bolus of sCR1 before xenograft reperfusion (28). The advantage of developing a transgenic donor animal expressing a human complement inhibitor is to provide the donor tissue with an endogenously expressed membrane-bound inhibitor and therefore does not rely on repeated administration of pharmacological agents.

The successful engineering of transgenic swine expressing a human complement inhibitor, and the demonstration that cells from these animals were significantly protected from human complement attack, suggests that this strategy may represent a useful component of an overall approach to discordant xenotransplantation. This transgenic approach will hopefully make porcine-to-primate transplantation models feasible that will allow the cellular aspects of discordant xenograft rejection to be evaluated. In addition, the production of porcine organs resistant to hyperacute rejection may open therapeutic windows for organ transplantation into humans, particularly when this technology is coupled with advances in cellular immunosuppressive regimens.

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