Clin Exp Immunol 1995; **102**:589–595

Functional activity of the membrane-asssociated complement inhibitor CD59 in a pig-to-human *in vitro* model for hyperacute xenograft rejection

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(Accepted for publication 2 June 1995)

SUMMARY

Hyperacute rejection triggered by activation of the recipient's complement system represents the major barrier to successful xenotransplantation. Transfer of human membrane-associated complement regulators to donor organs has been suggested as one strategy to interfere with complement-mediated hyperacute xenograft rejection. Pigs are discussed as potential organ donors. We therefore investigated a putative protective function of the membrane-bound complement inhibitor CD59 in a pig-to-human *in vitro* model of hyperacute xenograft rejection. Aortic porcine endothelial cells were transfected with human CD59 cDNA. Expression of human CD59 was demonstrated by cytofluorimetric and RNA analysis. Removal of CD59 from the cell surface by phosphatidylinositol-specific phospholipase C (PI-PLC) demonstrated its production as a glycosyl phosphatidylinositol (GPI)-anchored protein. Functional activity of the transfected CD59 was tested by a lactate dehydrogenase (LDH) release assay for complement-mediated lysis. Porcine endothelial cells expressing human CD59 were significantly protected from lysis by human serum complement compared with $CD59^-$ cells. The protective effect was abolished by preincubating the cells with anti-CD59 antibodies or PI-PLC. We calculated by Scatchard analysis that the established $CD59^+$ cell line expressed a CD59 level comparable to that of human endothelial cells. Our results recommend the production of pigs transgenic for CD59.

Keywords CD59 complement porcine endothelial cells hyperacute graft rejection xenotransplantation

INTRODUCTION

Xenotransplantation is considered a possible strategy to overcome the worldwide shortage of organs for transplantation. Attention has turned to the use of porcine donor organs which are of acceptable size, similar anatomy and physiology and, in addition, of unlimited availability [1]. The major obstacle to xenotransplantation of vascularized organs in discordant species combinations such as pig to human is the development of hyperacute rejection that occurs within a period of minutes to a few hours [2,3]. Only when these early rejections are overcome can strategies for a long-time survival of the xenogenic organ be developed.

Activation of the recipient's complement system is a critical factor in the pathogenesis of hyperacute rejection [3–5]. Three mechanisms are discussed: (i) the specific reaction of natural antibodies of the organ recipient with antigens of the donor organ, resulting in activation of the classical pathway of complement [1,3,6]; (ii) the direct activation of the alternative pathway of complement by cells of the donor organ [4]; and (iii) the inability of complement regulatory proteins of the donor organ to inhibit the host's complement system [7].

In attempts to prevent hyperacute rejection in xenografts, various substances, such as cobra venom factor [4], anti-complement drugs [8] and soluble complement receptor type 1 (sCR1; [9]), have been applied to interfere with the activation of complement in the host (reviewed in [10]), with varying success in prolonging xenograft survival. An alternative approach to protecting the graft from complement attack of the recipient would be to supply the xenograft with the recipient's complement inhibitory membrane proteins. This hypothesis was first tested for the C3-regulator decay accelerating factor (DAF, CD55; [11]) in an *in vitro* model of hyperacute xenograft rejection consisting of porcine endothelial cells as a target, and human serum as a source of natural antibodies and complement [12]. Purified DAF that had been incorporated into the membranes of porcine endothelial cells by its glycosyl phosphatidylinositol (GPI) moiety was shown to protect these cells efficiently from lysis by human complement in a dose-dependent manner. Recently, transgenic pigs expressing human DAF in different tissues have been produced [13,14].

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Functional active DAF could be demonstrated on blood mononuclear cells [13]. DAF is only one member of a family of membrane-bound proteins that inhibit cells lysis by homologous complement. These regulators act synergistically by different modes on the suppression of complement attack. Membrane cofactor protein (MCP, CD46; [15,16]) and CR1 interfere, like DAF, with C3 activation. C8-binding protein (C8bp; [17,18]) and CD59 [19,20] function as effective inhibitors of the membrane-attack complex. The well characterized membrane-associated complement inhibitory proteins MCP, DAF and CD59 are currently transferred to a variety of animal cells and/or tissues by different techniques to evaluate their efficacy in the protection of animal donor organs against human complement attack [13,14,21–31].

In the present study we investigated the potential utility of human CD59, a 18–25-kD glycoprotein expressed in almost all human tissues, in preventing hyperacute rejection in a pig-to-human *in vitro* model. For this purpose we transfected porcine aortic endothelial cells with CD59 cDNA and investigated the sensitivity of the CD59 transfectants to human serum complement.

MATERIALS AND METHODS

Cell culture

Endothelial cells were isolated from porcine aorta by incubation with Dispase (grade II, 2·4 U/ml; Boehringer, Mannheim, Germany) for 30 min at 37°C and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS), glutamine, HEPES and antibiotics. Culture vessels precoated with 0·2% gelatine in PBS were used.

Endothelial cells were recognized by their typical cobblestone-like morphology and the expression of the endothelial cell marker von Willebrand factor (vWF; polyclonal rabbit antiserum directed against human vWF; Dako, Hamburg, Germany; 98% positive cells). Culture medium used for transfectants contained in addition 0·6 mg G418/ml (Gibco). In an experiment cells between passages 3 and 10 were used. Human umbilical vein endothelial cells (HUVEC) kindly provided by Dr J. Grulich-Henn (Heidelberg) were grown in DMEM supplemented with 20% FCS, glutamine, HEPES, antibiotics and 2·5 ng/ml basic fibroblast growth factor. Cells between passages 5 and 10 were used.

Transfection

Polybrene-mediated transfection [32,33] of pig endothelial cells was performed with 10 μg plasmid-DNA. CD59 cDNA [34] subcloned into SalI/BamHI digested expression vector pHβapr-1-neo [35] was used. A control transfection with vector DNA alone was carried out in parallel. Transfectants were selected in G418 containing culture medium. Positive transfection was monitored by cytofluorometry using the anti-CD59 MoAb MEM-43.

Anti-CD59 antibodies

The MoAb YTH 53.1 (rat IgG2b) kindly provided by Professor H. Waldmann (Cambridge, UK) is described in [36]. The MoAb MEM-43 (mouse IgG2a) was purchased from Cell Systems (Remagen, Germany). Both antibodies were shown to be unable to induce cell lysis performing the assay for complement-dependent lysis with a serum of an agammaglobulinaemic patient with known cytolytic activity which did not contain any detectable immunoglobulins [37]; the extent of cytotoxicity observed after preincubating the cells with the anti-CD59 antibodies did not exceed the level of non-specific lysis.

Immunofluorescence studies

Cells were detached from the tissue culture flask by scraping into PBS. By trypan blue staining a viability of 95% was seen. After centrifugation, 5×10^6 cells/ml were resuspended in FACS buffer (PBS 1% bovine serum albumin (BSA) 0·1% sodium azide). Cell suspension (100 μl) was stained with 5 μg/ml anti-CD59 antibody (MEM-43; mouse IgG2a) for 30 min on ice. Non-immune mouse IgG2a (Sigma, Munich, Germany) served as control. The second antibody (FITC-labelled goat anti-mouse IgG, 28 μg/ml; Dianova, Hamburg, Germany) was applied after two washes with FACS buffer. After 30 min on ice the cells were washed again twice, resuspended in PBS 1% paraformaldehyde, and analysed in a FACScan (Becton Dickinson, Mountain View, CA).

Phosphatidylinositol-specific phospholipase C treatment

Porcine endothelial cells expressing CD59 were grown to confluence in 25-ml culture flasks, washed twice with Hanks' balanced salt solution (HBSS; Gibco) and incubated with phosphatidylinositol-specific phospholipase C (PI-PLC; 1 U/ml in HBSS; Boehringer) at 37°C for 1 h. HUVEC constitutively expressing human GPI-linked CD59 and Chinese hamster ovary (CHO) transfectants expressing transmembrane-anchored CD59 (Heckl-Östreicher *et al.*, manuscript in preparation) were used as controls. Removal of CD59 was tested by immunofluorescence as described above.

Northern blot analysis

Total cellular RNA was isolated using the acid guanidinium thiocyanate phenol-chloroform extraction method [38]. Total RNA (10 μg) was separated on a formaldehyde-containing 1% agarose gel and blotted to nylon membranes (Hybond-N; Amersham Buchler, Braunschweig, Germany). RNA transfer and hybridization of the blot with a ^{32}P-labelled CD59 cDNA probe [34] was performed by standard techniques [39].

Quantification of cell-surface CD59

Specific binding of ^{125}I-labelled MoAb MEM-43 (Immunodiagnostik, Bensheim, Germany) was utilized to quantify the level of CD59 expression. Cells were grown to confluence in 24-well tissue culture plates (Nunc, Wiesbaden, Germany), washed twice in HBSS, and were incubated for 30 min at 4°C with several dilutions of the ^{125}I-labelled anti-CD59 antibody in HBSS/1% BSA. Each antibody dilution was applied in duplicates with or without the addition of a 50-fold molar excess of unlabelled antibody to correct for non-specific binding. After removal of the unbound antibody, the cells were washed carefully with ice-cold HBSS. Cell-associated antibody was eluted with 1 N NaOH. Glacial acid was used for neutralization. Radioactivity was determined in both the cell-free supernatant and the cell lysate. The cell number used for each experiment was determined by trypan blue staining of cells harvested by trypsinization from four wells of the 24-well culture plate. The number of CD59 molecules/cell was calculated by Scatchard analysis.

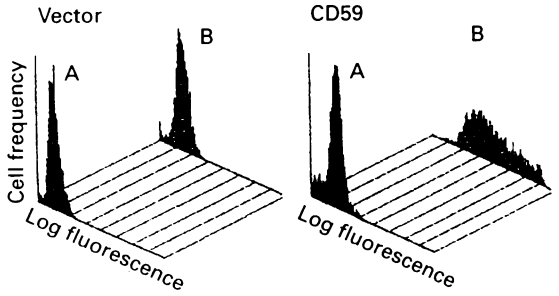

Fig. 1. Cytofluorimetric analysis of transfected aortic porcine endothelial cells. The fluorescence profiles of cells transfected with expression plasmid (vector) and CD59 cDNA (CD59) are compared. (A) Cells stained with non-immune mouse IgG as control. (B) Staining with the anti-CD59 MoAb MEM-43.

Assay for complement-dependent lysis

Cells (1×10^4) were grown overnight in flat-bottomed 96-well microtitre plates (Nunc). After washing with warm HBSS, cells were incubated for 2 h at 37°C with 100 μl of human serum (NHS) diluted in HBSS/1% BSA. Three different human serum pools with comparable cytotoxic activity were used. Control for non-specific lysis included incubation with heat-inactivated NHS (56°C, 1 h). The complement lysis assay was done in triplicates. In experiments performed to abolish CD59 function the cells were preincubated with 100 μg/ml of the anti-CD59 MoAbs MEM-43 and YTH 53.1 (shown to be unable to induce cell lysis, see Materials and Methods) or 1 U/ml PI-PLC for 1 h at 37°C. Cell lysis was quantified by measuring the release of lactate dehydrogenase (LDH) using a colorimetric assay (Cytotox 96 Non-Radioactive Cytotoxicity Assay; Serva, Heidelberg, Germany) according to the manufacturer's protocol. Quantitative analysis was performed using an ELISA reader at 492 nm. The amount of LDH present in NHS was subtracted from the calculated means of the corresponding experimental values. Cells incubated with buffer alone allowed correction for spontaneous LDH release. Cells lysed with Triton X-100 represented the maximal possible LDH release. The percentage of cytotoxicity was calculated according to the following formula:

$$\text{Per cent lysis} = \frac{\text{experimental LDH release} - \text{spontaneous LDH release}}{\text{maximum} - \text{spontaneous LDH release}} \times 100$$

For determination of specific lysis the value obtained with inactivated serum was subtracted from the corresponding experimental value with native NHS.

RESULTS

Expression of recombinant CD59 on porcine endothelial cells

Porcine endothelial cells were transfected with an expression vector containing CD59 cDNA. The stable transfectants were analysed after 2 weeks for CD59 expression by cytofluorometry. CD59-transfected cells showed strong reactivity with the anti-CD59 MoAb MEM-43, whereas control cells transfected with expression vector alone gave only slight background staining (Fig. 1). The fluorescence profile of the CD59-transfected porcine endothelial cells revealed a widespread distribution pattern representing a heterogeneous cell population with regard to CD59 expression.

In order to test whether the CD59 molecule expressed by the transfectants is, as in human cells, linked to the membrane by means of a GPI anchor, the cells were treated with PI-PLC, an enzyme known to release GPI-anchored molecules from the cell surface. PI-PLC treatment of CD59 transfectants as well as of HUVEC (positive control) caused a decrease in the amount of surface staining for CD59, indicated by a shift of the mean fluorescence intensity to that of the negative controls (Fig. 2a,b). In contrast, PI-PLC treatment of CHO transfectants expressing a transmembrane-anchored CD59 protein (Heckl-Östreicher *et al.*, manuscript in preparation) did not result in the release of CD59 from the cell surface (Fig. 2c). It can therefore be assumed that the CD59 expressed by porcine endothelial cell transfectants is attached to the membrane via a GPI anchor.

RNA analysis

Porcine endothelial cells and cells transfected with expression plasmids were then analysed for CD59-specific RNA expression by Northern blot analysis (Fig. 3). RNA of human CD59$^+$ cells (HUVEC), run on the same gel, revealed three bands of about 2·0, 1·4 and 0·8 kb (Fig. 3) as described [40]. No hybridization signals were obtained with RNA of non-transfected and vector-transfected porcine endothelial cells. Pig cells that had been transfected with CD59 showed one major band that comigrated with the 0·8-kb band observed with RNA derived from HUVEC. This message is known to encode the functional protein, whereas

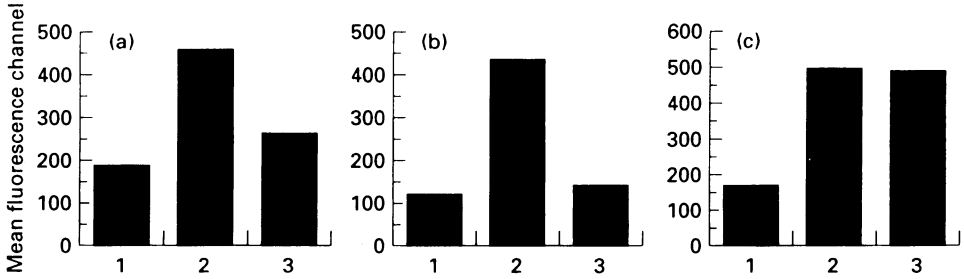

Fig. 2. Effect of phosphatidylinositol-specific phospholipase C (PI-PLC) treatment on CD59-expressing porcine endothelial cells and control cells. FACS analysis after staining with non-immune mouse IgG (1), anti-CD59 antibody MEM-43 of untreated cells (2) and after PI-PLC treatment of the cells (3). (a) CD59$^+$ porcine endothelial cells. (b) Human umbilical vein endothelial cells (HUVEC) (positive control). (c) Chinese hamster ovary (CHO) cells expressing a transmembrane-anchored CD59 (negative control).

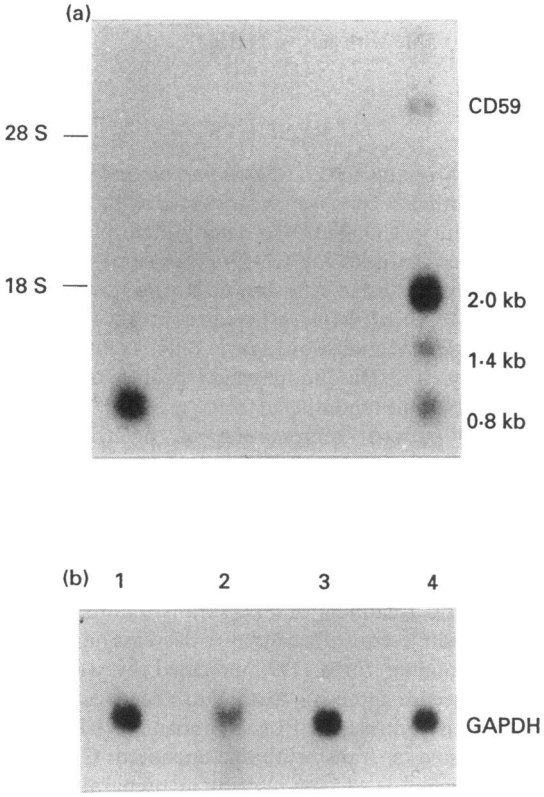

Fig. 3. Northern blot analysis of total cellular RNA using a CD59 probe (a). (1) CD59-transfected. (2) Non-transfected. (3) Vector-transfected porcine endothelial cells. (4) Human umbilical vein endothelial cells (HUVEC). Hybridization of the RNAs to a GAPDH probe (ATCC 57091) is shown in (b).

the higher molecular mass bands represent RNA species with different non-translated 3′ ends [41].

Determination of the number of CD59 molecules on CD59⁺ transfectants

Binding of ^{125}I-labelled anti-CD59 MoAb MEM-43 to pig cells transfected with CD59 or vector was measured to determine the amount of CD59 expressed on the cell surface. Radioactivity bound by the control transfectants did not exceed the level of non-specific binding (data not shown), whereas specific binding of ^{125}I-labelled anti-CD59 antibody increased as a function of the antibody concentration, and can be presented in a saturation curve (Fig. 4). The maximum number of CD59 molecules/cells and the affinity constant were calculated according to Scatchard from the specific binding data. It was determined from three independent experiments (data not shown) that the CD59 transfectants expressed on the average 2×10^5 CD59 molecules per cell. For HUVEC we calculated a number of $3{\cdot}4 \times 10^5$ CD59 molecules/cell (data not shown), which corresponds to published results [40]. In all binding experiments performed with the ^{125}I-labelled MoAb MEM-43, approximately the same K_D value of about 8×10^{-8} M was obtained.

Effect of human CD59 expressed by porcine endothelial cells on complement-mediated lysis

To determine the functional activity of the recombinant human

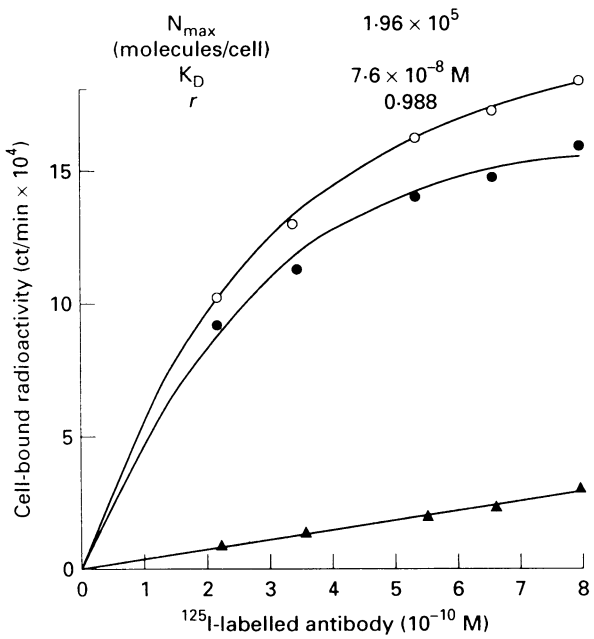

Fig. 4. Determination of the level of human CD59 expressed by transfected porcine endothelial cells. The binding of ^{125}I-labelled anti-CD59 antibody MEM-43 is measured. ○, Total radioactivity bound; ●, specific binding (total binding − non-specific binding); ▲, non-specific binding determined in the presence of a 50-fold molar excess of unlabelled antibody. Values for N_{max} and K_D were calculated from the specific binding according to Scatchard.

CD59 molecule, non-transfected and transfected porcine endothelial cells were incubated with NHS as a source of natural antibodies and complement. After 2 h, cell lysis was quantified [42] by measuring the release of LDH in the supernatant. NHS (10%) caused 25–54% specific lysis of non-transfected and vector-transfected cells, and in the presence of 20% NHS, specific lysis was increased to 40–96%. Incubation with heat-inactivated serum only slightly

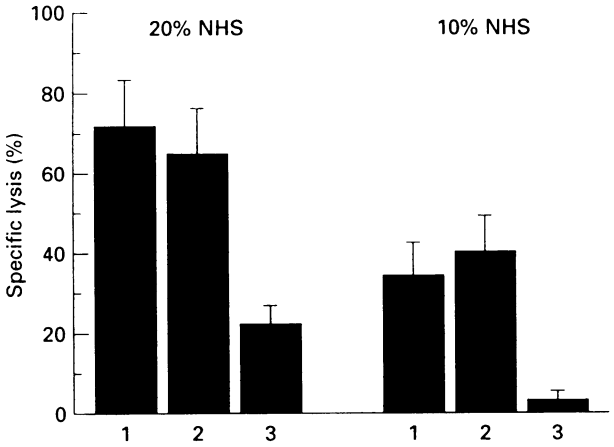

Fig. 5. Complement-mediated lysis of porcine endothelial cells by 20% and 10% human serum. Results are expressed as means ± s.e.m. of triplicate determinations of one representative experiment. (1) Non-transfected. (2) Vector-transfected. (3) huCD59-transfected cells.

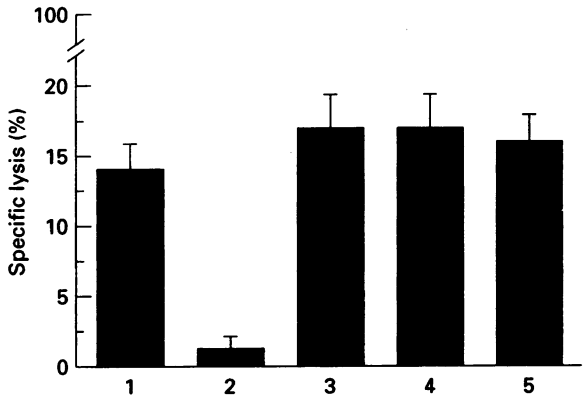

Fig. 6. Complement-mediated lysis of porcine endothelial cells expressing human CD59 after preincubation with anti-CD59 MoAbs or phosphatidylinositol-specific phospholipase C (PI-PLC). Controls include $CD59^-$ (1) and untreated $CD59^+$ (2) porcine endothelial cells incubated with 10% human serum. Preincubation of the cells with the MoAbs MEM-43 (3) and YTH 53.1 (4) or PI-PLC (5) abolished the protective effect.

increased the level of spontaneously released LDH. Figure 5 shows the results of one representative experiment performed with 10% and 20% NHS. With both serum concentrations a significant reduction in lysis of CD59-expressing porcine endothelial cells was seen, demonstrating an inhibition of cytotoxicity of 90% and 70%, respectively. An increase of the serum concentration to 40% reduced the protective effect to 50% (data not shown). From these data we conclude that human CD59 is expressed in its functionally active form. The protective effect was abolished after removal of CD59 by PI-PLC treatment of the CD59 transfectants as well as by addition of the anti-CD59 antibodies MEM-43 and YTH 53.1 (Fig. 6), which for their part were unable to induce cell lysis (as described in Materials and Methods).

DISCUSSION

Hyperacute graft rejection mediated by activation of the recipient's complement system [1–5] is a major problem of xenotransplantation. Approaches to its prevention include the inhibition of complement by anti-complement drugs, as well as by soluble and membrane-bound complement regulators. In the present study we investigated a possible protective function of the human membrane-associated complement regulator CD59. We have chosen a pig-to-human *in vitro* model for hyperacute rejection since pigs are in discussion as the most suitable organ donors for humans [1]. As the target for complement attack we used endothelial cells, since this cell type is primarily involved in hyperacute xenograft rejection. Aortic porcine endothelial cells were transfected with CD59 cDNA. Expression of the human CD59 molecule was demonstrated by cytofluorimetric and RNA analysis. Removal of the CD59 protein from the cell surface by PI-PLC revealed that the CD59 protein expressed by porcine endothelial cells is, as in human cells, attached to the membrane by a GPI anchor [43]. Functional activity of the CD59 protein on porcine endothelial cells was verified by a significantly reduced susceptibility of the CD59 transfectants to lysis by human serum as a source of natural antibodies and complement. With regard to the controversial discussion on species-restricted function of membrane-bound complement inhibitors [44], our data speak against a combined protective effect of human CD59 and the corresponding porcine regulatory protein. PI-PLC treatment of the CD59 transfectants, which is known to release human CD59 as well as the porcine CD59 analogue [45], did not increase the susceptibility to complement-mediated lysis to a higher extent than pretreatment of the cells with anti-human CD59 MoAbs that do not cross-react with the pig analogue. In addition, PI-PLC treatment of non-transfected porcine endothelial cells did not lead to increased susceptibility to human serum.

Our data contradict the findings of Miyagawa *et al.* [46], who was not able to demonstrate a protection from complement-mediated lysis in the established porcine endothelial cell lines expressing human CD59. Unfortunately, the authors did not determine the numbers of CD59 molecules expressed by the pig cells. Therefore, it is not possible to correlate their functional data with the level of human CD59 expressed by their transfectants. In our study calculating the CD59 level by Scatchard analysis we have shown that if the number of expressed human CD59 molecules approaches the level of that expressed on human endothelial cells, approximately $2–3\times10^5$ molecules/cell, significant protection can be achieved. Preliminary data obtained in experiments with a porcine endothelial cell line expressing a low level of CD59 indicate that a certain threshold level of CD59 is required to achieve significant protection (data not shown). We are currently isolating subclones of our CD59-expressing porcine endothelial cell line to be able to relate the protective effect to the number of CD59 molecules expressed on the cell surface.

Recently, a protective function of human CD59 on neonatal porcine aortic endothelial cells also has been demonstrated by Kennedy *et al.* [31]. However, in their assay for complement-dependent lysis cells were incubated with saturating amounts of a rabbit antibody directed against porcine aortic endothelial cells before the addition of human complement. We focused on the physiological situation in xenotransplantation, where complement-mediated rejection is induced by human anti-endothelium antibodies that bind to the transplanted organ.

Human endothelial cells, such as HUVEC, are completely protected from lysis by homologous complement not only by CD59, but also by the complement inhibitors MCP and DAF [40]. Blocking studies with $F(ab)_2$ anti-complement regulatory antibodies, as well as removal of GPI-anchored proteins by PI-PLC, revealed a prominent synergistic inhibitory function for DAF and CD59 on HUVEC [40]. Experiments with double transfection of cDNAs encoding two different complement regulatory proteins to xenogeneic endothelial cells also revealed a synergistic protective effect of membrane-associated complement inhibitors. Synergism was demonstrated for DAF and CD59 on rat [47] and bovine [27] endothelial cells, as well as for DAF and MCP on pig endothelial cells [28].

As an *in vivo* application of these studies to interfere with hyperacute xenograft rejection, transgenic animals, especially mice and pigs, expressing membrane-bound complement regulators in various tissues were produced recently [13,14,21,22,24,29,48–50]. So far, functional data on possible protection against human complement-mediated tissue destruction have not been reported.

Our findings suggest human CD59 as another candidate molecule, possibly in combination with DAF and MCP, for engineering transgenic pigs which then could become valuable organ donors in xenotransplantation. To engineer donor organs that are well protected from complement attack of the recipient, however, it will be necessary to optimize the level of the complement inhibitors expressed on the cell surface. Expression vectors used for the production of transgenic animals should direct an expression level for each of the proteins which is comparable with or, better, exceeds that of human endothelial cells.

ACKNOWLEDGMENTS

The monoclonal antibody YTH 53.1 was kindly provided by Professor H. Waldmann (Department of Pathology, Addenbrooke's Hospital, University of Cambridge, UK), and Dr S. Meri (Department of Bacteriology and Immunology, University of Helsinki, Finland). The authors thank Dr J. Grulich-Henn and Ulrike Hügel (Department of Pediatrics, University of Heidelberg, Germany) for advice isolating aortic endothelial cells and performing Scatchard analysis. We would also like to thank Silvia Ellwanger for technical assistance.

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