Published in final edited form as:

Int J Surg. 2020 November ; 83: 184–188. doi:10.1016/j.ijssu.2020.09.034.

Does expression of a human complement-regulatory protein on xenograft cells protect them from systemic complement activation? Experimental research

Abhijit Jagdale, MD^{1,*}, Huy Quoc Nguyen, BS, MS^{1,*}, Juan Li, MD^{1,2}, KaLia Burnette, BSE, ME¹, David Ayares, PhD³, David K.C. Cooper, MD, PhD, FRCS¹, Hidetaka Hara, MD, PhD¹

⁽¹⁾Xenotransplantation Program, Department of Surgery, University of Alabama at Birmingham, Birmingham, AL, USA

⁽²⁾Second Affiliated Hospital, University of South China, Hengyang City, Hunan, China

⁽³⁾Revivacor, Blacksburg, VA, USA

Abstract

Background: There are many causes of systemic complement activation, which may have detrimental effects on a pig xenograft. Transgenic expression of one or more human complement-regulatory proteins (hCRPs), e.g., hCD46, provides some protection to the xenograft, but it is not known whether it protects the xenograft from the effects of systemic complement activation. We used wild-type (WT) pig aortic endothelial cells (pAECs) to activate complement, and determined whether the expression of hCD46 on α 1,3galactosyltransferase gene- knockout (GTKO) pAECs protected them from injury.

Methods: CFSE-labeled and non-labeled pAECs from a WT, a GTKO, or a GTKO/hCD46 pig were separately incubated with heat-inactivated pooled human serum in vitro. Antibody pre-

Address for correspondence: Hidetaka Hara MD, PhD, Xenotransplantation Program, Department of Surgery, University of Alabama at Birmingham, Lyons Harrison Research Building Rm752, 701 19th Street South, Birmingham, AL 35233, USA, Telephone: 205-934-2690; Fax: 205-996-1043, harahjp@icloud.com.

*Both authors contributed equally to this manuscript

Author contribution and authorship

Abhijit Jagdale participated in the performance of the research, statistical analysis and in the writing of the paper. Huy Quoc Nguyen participated in the performance of the research, statistical analysis and in the review of the paper. Juan Li and KaLia Burnette participated in the performance of the research and in the review of the paper. David Ayares provided the genetically engineered pigs, and reviewed the paper. David K.C. Cooper participated in research design and in writing the paper. Hidetaka Hara participated in research design, the performance of the research, statistical analysis and writing the paper. Hidetaka Hara is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure of conflict of interest

David Ayares is an employee of Revivacor, Blacksburg, VA. The other authors declare no conflicts of interest.

Ethical statement

All animal care was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council (8th edition, revised 2011), and was conducted in an AAALAC-accredited facility. Protocols were approved by the University of Pittsburgh (IACUC#13082323) and University of Alabama at Birmingham (IACUC#20673) Institutional Animal Care and Use Committees (IACUC).

bonded CFSE-labeled and non-labeled pAECs were mixed, and then incubated with rabbit complement. The complement-dependent cytotoxicity was measured by flow cytometry.

Results: There was significantly less lysis of GTKO/CD46 pAECs (6%) by 50% human serum compared to that of WT (91%, $p<0.001$) or GTKO (32%, $p<0.01$) pAECs. The lysis of GTKO pAECs was significantly increased when mixed with WT pAECs ($p<0.05$). In contrast, there was no significant change in cytotoxicity of GTKO/CD46 pAECs when mixed with WT pAECs.

Conclusions: The expression of hCD46 protected pAECs from systemic complement activation

Keywords

CD46; complement; complement-regulatory protein; pig; xenotransplantation

1. Introduction

The transplantation of organs from genetically-engineered pigs in combination with novel immunosuppressive therapy is associated with prolonged xenograft survival in nonhuman primates [1–4]. An important genetic manipulation is the expression of a human complement-regulatory protein (hCRP), e.g., hCD46, on the cells of the graft to protect against recipient complement injury following anti-pig antibody binding to the graft. However, it is not known whether a complication, e.g., infection, that activated complement systemically might be detrimental to the graft.

All primates have natural antibodies, especially IgM, against $\alpha 1,3$ -galactosyltransferase gene-knockout (GTKO) pig cells [5–7] and, after perfusion of the graft, these bind to the pig vascular endothelial cells, leading to complement activation that can be detrimental to the xenograft [8]. The protective mechanism of hCRPs has been reviewed [9]. Besides antibody-mediated complement activation, many factors, e.g., bacteremia, sepsis [10], ischemia-reperfusion injury [11,12], major surgery [13,14] and pretransplant lymphocyte ablation therapy [15,16], can activate the complement cascade.

Little is known whether, in the presence of anti-donor pig antibodies, the expression of an hCRP in the xenograft would be fully protective against systemic complement activation (e.g., during a systemic infection). We therefore investigated whether expression of hCD46 on GTKO pig aortic endothelial cells (pAECs) protect against complement activation (initiated by the presence of wild-type (WT) pig cells (in the presence of anti-pig antibodies) *in vitro*.

2. Materials and Methods

2.1 Source of pooled human serum

Pooled human serum (pooled from 50-150 donors) was purchased from Innovative Research (Novi, MI). Decomplementation of serum was carried out by heat-inactivation for 30min at 56°C, and sera were stored at –80°C until use.

2.2 Sources of pig cells

pAECs from a WT pig (Prestige Farms, West Point, MS) and from GTKO and GTKO/hCD46 pigs (Revivicor, Blacksburg, VA) [3 pigs from each type] were isolated and cultured, as previously described [5]. The current study has been reported in accordance with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines [17].

2.3 Complement-dependent cytotoxicity (CDC) assay

pAECs (3×10^6) were suspended in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA), followed by labeling with carboxyfluorescein diacetate succinimidyl esterase (CFSE) (Molecular Probes, Eugene, OR) at a final concentration of $0.3 \mu\text{M}$. CFSE-unlabeled pAECs were also incubated with same volume of DMSO as a control.

CFSE-labeled and unlabeled pAECs from WT, GTKO, and GTKO/CD46 pigs (0.5×10^5 pAECs/50 μL) in CDC medium (composed of RPMI 1640 culture medium [Invitrogen], 10% fetal bovine serum [FBS] [Sigma -Aldrich, St. Louis, MO], 1% HEPES buffer [Invitrogen], and 100 IU/mL penicillin-100 $\mu\text{g}/\text{mL}$ streptomycin [Invitrogen]) were incubated with heat-inactivated pooled human serum at various concentrations for 30min at 4°C , and then washed with PBS. CFSE-labeled and unlabeled pAECs from the same pig (each 0.5×10^5 pAECs/50 μL CDC medium) were mixed into 5mL round-bottom polystyrene tubes with cap (Corning, Tewksbury, MA) (total 1×10^5 pAECs/100 μL), followed by incubation with 20% rabbit complement (Sigma, St. Louis, MO) for 1h at 37°C . The cells were then washed and resuspended in staining buffer (PBS containing 1% bovine serum albumin [Invitrogen] and 0.1% sodium azide [Sigma]).

In separate experiments, two different types of AECs (from WT, GTKO, or GTKO/hCD46 pigs) (0.5×10^5 of each pAEC/50 μL CDC medium), which had been preincubated with pooled human serum, were mixed together, followed by incubation with 20% rabbit complement for 1h at 37°C . After washing, the cells were suspended in staining buffer, and then propidium iodide (PI) (BD, San Jose, CA) (1:25 concentration) was added. The cells were incubated for 20min at 4°C in the dark, followed by washing with staining buffer. CDC (PI-positive) of pAECs was measured by LSR II flow cytometry (BD) and analyzed by FlowJo software (Treestar, Ashland, OR).

Percentage (%) cytotoxicity was determined as follows; % cytotoxicity = $([A - C] / [B - C]) \times 100\%$, where A represents dead cells (target cells incubated with pooled human serum and rabbit complement), B is the maximal dead cells (target cells lysed with 70% alcohol), and C is the minimal dead cells (target cells incubated with rabbit complement only).

2.4 Statistical analysis

Data are presented as mean and standard deviation (SD) for all variables. The statistical significance of differences was determined by Student's t-test or non-parametric Kruskal-Wallis tests followed by Dunn's multiple comparisons test, as appropriate, using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). A p value of <0.05 was considered to be statistically significant.

3. Results

3.1 Comparison of lysis between CFSE-labeled and CFSE-unlabeled pAECs in mixed CDC assays

To distinguish the lysis of different cells of the same phenotype during a CDC assay, labeling with CFSE was used. The viable and dead (PI-positive) cells in both CFSE-labeled and unlabeled pAECs were easily detected by flow cytometry after CDC assay (Figure1A). There was no significant difference in CDC between CFSE-labeled and CFSE-unlabeled pAECs of the same phenotype (Figure1B).

3.2 Protection from CDC by a combination of GTKO and hCD46 expression

Significantly less lysis of GTKO/hCD46 pAECs (6%) by 50% human serum was found compared to lysis of WT (91%, $p<0.001$) or GTKO (32%, $p<0.01$) pAECs (Figure1C), confirming that expression of a hCRP on pAECs protects against antibody- and complement-mediated cell lysis [5].

3.3 WT pAEC-mediated complement activation did not increase lysis of GTKO/CD46 pAECs

To distinguish the lysis of cells of same or different phenotypes during a CDC assay, labeling with CFSE was used. The viable and dead (PI-positive) cells in both CFSE-labeled and unlabeled pAECs were easily detected by flow cytometry after CDC assay (Figure2A).

Lysis of WT pAECs, when mixed with an equal number of WT pAECs (91%) or GTKO (95%) or GTKO/CD46 (96%) pAECs was not significantly changed (Figure2B). In contrast, lysis of GTKO pAECs (which was 23% when mixed with an equal number of GTKO pAECs), was significantly increased when mixed with WT pAECs (36%, $p<0.05$). No significant difference in lysis was observed when GTKO pAECs were mixed with an equal number of GTKO/CD46 pAECs (21%). There was no significant change in the lysis of GTKO/CD46 pAECs (which was 5% when mixed with an equal number of GTKO/CD46 pAECs), when mixed with WT (11%) or GTKO (8%) pAECs.

These results indicate that the expression of hCD46 on GTKO pAECs protects the cells from the increased CDC induced by the presence of WT pAECs.

4. Discussion

Human or nonhuman primate natural preformed antibodies bind to pig xenoantigens, resulting in activation of the classical complement pathway [9,18]. Many pig-to-nonhuman primate xenotransplantation models have shown the efficacy of expression of hCD46 or another hCRP on pig cells in preventing hyperacute rejection and/or early graft failure [19–22].

Expression of hCD46 on pig cells inhibits cytotoxicity by human serum [5,23]. CD46 is an intrinsic complement regulator and (with complement receptor-1 [CD35], factor H, and C4b-binding protein) acts as a cofactor for factor I-mediated lysis of C4b and C3b [24]. CD46 cleaves C4b and C3b in both the classical and alternate complement pathways [9,23,25].

As anticipated, there was significantly less killing of GTKO/hCD46 pAECs than of WT and GTKO pAECs when exposed to human serum and rabbit complement. This was associated with decreased antibody binding to GTKO/hCD46 pAECs (due to the absence of Gal expression) and the protective effect of hCD46 [5]. When human serum bound WT and GTKO pAECs were mixed and then exposed to rabbit complement, there was increased killing of the GTKO pAECs. This suggests that the close proximity of WT pAECs increased the effect of activated complement on the GTKO pAECs (i.e., a ‘ripple effect’ of complement activation). This ripple effect could be prevented by the expression of hCD46 on GTKO pAECs.

We presumed that GTKO/hCD46 pAECs were exposed to the classical complement pathway activated by (i) the expression of Gal and nonGal carbohydrates on WT pAECs, and (ii) the expression of nonGal carbohydrates on the GTKO/hCD46 pAECs. In addition, activation of the alternate pathway may have occurred through spontaneous hydrolysis of C3 [26]. These data indicated that, even when there is systemic complement activation (initiated by the presence of the WT pAECs), GTKO/hCD46 pAECs remained protected.

One of the limitations of the current study is that our conclusions are drawn from an in vitro study. Ideally, an in vivo study needs to be carried out to confirm our finding. This would entail the transplantation of a kidney (or other organ) from a pig that expressed one or more hCRPs, followed by the induction of a systemic infection or other ‘insult’ in the recipient baboon. This would be a major undertaking. However, we already have evidence from our previous in vivo studies that, when a systemic infection develops in a baboon with a pig kidney graft expressing a hCRP (and therefore is subjected to systemic complement activation), the organ is not rejected, even though the baboon may have to be euthanized for uncontrollable infection [2, 27]. This provides some confirmation that the results of our present in vitro study translate in vivo.

In conclusion, we have demonstrated that a transgenically-expressed hCRP on GTKO pig cells contributes to protection of a xenograft from systemic complement activation, and further indicates the benefit (if not the absolute necessity) of hCRP expression in pig organs in future clinical xenotransplantation.

Acknowledgements

Work on xenotransplantation at the University of Alabama at Birmingham is supported in part by NIH NIAID U19 grant AI090959, and in part by a grant to UAB from United Therapeutics, Silver Spring, MD, USA.

Abbreviations

CDC	complement-dependent cytotoxicity
CFSE	carboxyfluorescein diacetate succinimidyl esterase
GTKO	α 1,3-galactosyltransferase gene-knockout
GTKO/hCD46	α 1,3-galactosyltransferase gene-knockout pig transgenic for the human complement-regulatory protein, CD46

hCRP	human complement regulatory protein
pAECs	pig aortic endothelial cells
WT	wild-type

References

- [1]. Mohiuddin MM, Singh AK, Corcoran PC, et al., Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat. Commun* 7 (2016) 11138 Doi: 10.1038/ncomms11138 [PubMed: 27045379]
- [2]. Iwase H, Hara H, Ezzelarab M, et al., Immunological and physiological observations in baboons with life-supporting genetically engineered pig kidney grafts. *Xenotransplantation*. 24 (2017). Doi: 10.1111/xen.12293
- [3]. Langin M, Mayr T, Reichart B, et al., Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature*. 564 (2018) 430–433. Doi: 10.1038/s41586-018-0765-z [PubMed: 30518863]
- [4]. Adams AB, Kim SC, Martens GR, et al., Xenoantigen Deletion and Chemical Immunosuppression Can Prolong Renal Xenograft Survival. *Ann. Surg* 268 (2018) 564–573. Doi: 10.1097/SLA.0000000000002977 [PubMed: 30048323]
- [5]. Hara H, Long C, Lin YJ, et al., In vitro investigation of pig cells for resistance to human antibody-mediated rejection. *Transpl. Int* 21 (2008) 1163–1174. Doi: 10.1111/j.1432-2277.2008.00736.X [PubMed: 18764834]
- [6]. Byrne GW, McGregor C, Breimer ME. Recent investigations into pig antigen and anti-pig antibody expression. *Int. J. Surg* 23 (2015) 223–228. Doi: 10.1016/j.ijsu.2015.07.724 [PubMed: 26306769]
- [7]. Estrada J, Martens G, Li P, et al., Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. *Xenotransplantation*. 22 (2015) 194–202. Doi: 10.1111/xen.12161 [PubMed: 25728481]
- [8]. Chen G, Qian H, Starzl T, et al., Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. *Nat. Med* 11 (2005) 1295–1298. Doi: 10.1038/nm1330 [PubMed: 16311604]
- [9]. Zhou H, Hara H, Cooper DKC. The complex functioning of the complement system in xenotransplantation. *Xenotransplantation*. 26 (2019) e12517 Doi: 10.1111/xen.12517 [PubMed: 31033064]
- [10]. Helling H, Stephan B, Pindur G. Coagulation and complement system in critically ill patients. *Clin. Hemorheol. Microcirc* 61 (2015) 185–193. Doi: 10.3233/CH-151993 [PubMed: 26410872]
- [11]. Amsterdam EA, Stahl GL, Pan HL, et al., Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs. *Am. J. Physiol* 268 (1995) 448–457. Doi: 1152/ajpheart.1995.268.1.h448
- [12]. Straatsburg IH, Boermeester MA, Wolbink GJ, et al., Complement activation induced by ischemia-reperfusion in humans: a study in patients undergoing partial hepatectomy. *J. Hepatol* 32 (2000) 783–791. Doi: 10.1016/S0168-8278(00)80247-0 [PubMed: 10845665]
- [13]. Tarnok A, Hambsch J, Emmrich F, et al., Complement activation, cytokines, and adhesion molecules in children undergoing cardiac surgery with or without cardiopulmonary bypass. *Pediatr. Cardiol* 20 (1999) 113–125. Doi: 10.1007/s002469900417 [PubMed: 9986887]
- [14]. Kvarnstrom A, Sokolov A, Swartling T, et al., Alternative pathway activation of complement in laparoscopic and open rectal surgery. *Scand. J. Immunol* 76 (2012) 49–53. Doi: 10.1111/j.1365-3083.2012.02702.x [PubMed: 22486843]
- [15]. Genestier L, Fournel S, Flacher M, O. et al., Induction of Fas (Apo-1, CD95)-mediated apoptosis of activated lymphocytes by polyclonal antithymocyte globulins. *Blood*. 91 (1998) 2360–2368. [PubMed: 9516135]

- [16]. Cumpelik A, Gerossier E, Jin J, et al., Mechanism of platelet activation and hypercoagulability by antithymocyte globulins (ATG). *Am. J. Transplant* 15 (2015) 2588–2601. Doi: 10.1111/ajt.13316 [PubMed: 25966640]
- [17]. Kilkeny C, Browne WJ, Cuthill IC, et al., Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS. Biol* 8 (2010) e1000412. [PubMed: 20613859]
- [18]. Pierson RN 3rd. Antibody-mediated xenograft injury: mechanisms and protective strategies. *Transpl. Immunol* 21 (2009) 65–69. Doi: 10.1016/j.trim.2009.03.008 [PubMed: 19376229]
- [19]. Fodor WL, Williams BL, Matis LA, et al., Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. *Proc. Natl. Acad. Sci* 91 (1994) 11153–11157. Doi: 10.1073/pnas.91.23.11153 [PubMed: 7526391]
- [20]. Cozzi E, White DJ. The generation of transgenic pigs as potential organ donors for humans. *Nat. Med* 1 (1995) 964–966. Doi: 10.1038/nm0995-964 [PubMed: 7585226]
- [21]. Azimzadeh AM, Kelishadi SS, Ezzelarab MB, et al., Early graft failure of GalTKO pig organs in baboons is reduced by expression of a human complement pathway-regulatory protein. *Xenotransplantation*. 22 (2015) 310–316. Doi: 10.1111/xen.12176 [PubMed: 26174749]
- [22]. McGregor CG, Ricci D, Miyagi N, et al., Human cd55 expression blocks hyperacute rejection and restricts complement activation in gal knockout cardiac xenografts. *Transplantation*. 93 (2012) 686–692. Doi: 10.1097/TP.0b013e3182472850 [PubMed: 22391577]
- [23]. Oglesby TJ, Allen CJ, Liszewski MK, et al., Membrane cofactor protein (CD46) protects cells from complement-mediated attack by an intrinsic mechanism. *J. Exp. Med* 175 (1992) 1547–1551. Doi: 10.1084/jem.175.6.1547 [PubMed: 1588280]
- [24]. Liszewski MK, Post TW, Atkinson JP. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu. Rev. Immunol* 9 (1991) 431–455. Doi: 10.1146/annurev.iy.09.040191.002243 [PubMed: 1910685]
- [25]. Barilla-LaBarca ML, Liszewski MK, Lambris JD, D. et al., Role of membrane cofactor protein (CD46) in regulation of C4b and C3b deposited on cells. *J. Immunol* 168 (2002) 6298–6304. Doi: 10.4049/jimmunol.168.12.6298 [PubMed: 12055245]
- [26]. Romanella M, Aminian A, Adam WR, et al., Involvement of both the classical and alternate pathways of complement in an ex vivo model of xenograft rejection. *Transplantation*. 63 (1997) 1021–1025. Doi: 10.1097/00007890-199704150-00020 [PubMed: 9112358]
- [27]. Iwase H, Liu H, Wijkstrom M et al. Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. *Xenotransplantation*. 22 (2015) 302–309. Doi: 10.1111/xen.12174. [PubMed: 26130164]

Highlights

- The complement dependent cytotoxicity (CDC) of α 1,3-galactosyltransferase gene-knockout (GTKO) pig aortic endothelial cells (pAECs) expressing hCD46 (GTKO/hCD46 pAECs) by human serum was significantly less than wild type (WT) and GTKO pAECs.
- The expression of human CD46 on GTKO pAECs protect against complement activation initiated by the presence of WT pig cells (in the presence of anti-pig antibodies) in vitro.
- Transgenic expression of hCD46 on the GTKO pAECs protects the xenograft from systemic complement activation.
- Protection of the xenograft by transgenic expression of the hCRP suggests indispensable role of hCRP in xenotransplantation.

Figure 1: Complement-dependent cytotoxicity of CFSE-labeled and unlabeled cells from pigs of the same phenotypes

To distinguish target cells of the same phenotype in the complement-dependent cytotoxicity assay, some pAECs were labeled with CFSE. **(A)** Representative flow cytometry dot plot. Human serum-bound CFSE-labeled and unlabeled pAECs (of the same phenotype) were mixed, followed by the addition of rabbit complement. Staining with propidium iodide (PI) was performed to distinguish between vital and dead cells. FSC and SSC were determined to identify pAECs (first and second from the left). Target cells (CFSE-positive and -negative) were gated (middle), and examined for cell death based on the uptake of PI (first and second from the right). **(B)** After exposure to human serum (at various concentrations [50%, 25%, 12.5% and 6.25%]), CFSE-labeled and unlabeled pAECs (from the same pig) were mixed and incubated with 20% rabbit complement. There was no difference in lysis between CFSE-positive and -negative cells ($n=3$). **(C)** Deletion of Gal (GTKO) and the additional expression of a hCRP (GTKO/CD46) was associated with a significant reduction in lysis of pAECs ($n=4$). (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. = not significant).

Figure 2: Complement-dependent cytotoxicity of CFSE-labeled and unlabeled cells from pigs of same and different phenotypes

To distinguish target cells of same and different phenotypes in the complement-dependent cytotoxicity assay, pAECs of one phenotype were labeled with CFSE. **(A)** Representative flow cytometry dot plot. CFSE-positive GTKO and CFSE-negative WT pAECs (left); CFSE-positive GTKO and CFSE-negative GTKO pAECs (middle); CFSE-positive GTKO and CFSE-negative GTKO/CD46 pAECs (right). pAECs were gated and examined for cell death based on the uptake of PI. **(B)** After exposure to human serum (at 50% serum concentration), CFSE-labeled and unlabeled pAECs mixed with pAECs from the same or a different phenotype were incubated with rabbit complement. There was a significant increase in the lysis of GTKO pAECs when mixed with WT pAECs. ($n=4$), but not of GTKO/hCD46 pAECs when mixed with WT pAECs ($n=4$). (* $p<0.05$; ** $p<0.01$; n.s. = not significant).