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FURTHER EVIDENCE FOR SUSTAINED SYSTEMIC INFLAMMATION IN XENOGRAFT RECIPIENTS (SIXR)

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Abstract

Introduction—In pig-to-baboon heart/artery patch transplantation models, adequate costimulation blockade prevents a T cell response. After heart transplantation, coagulation dysfunction (thrombocytopenia, reduced fibrinogen, increased D-dimer) and inflammation (increased C-reactive protein [CRP]) develop. We evaluated whether coagulation dysfunction and/or inflammation can be detected following pig artery patch transplantation.

Methods—Baboons received heart (n=8) or artery patch (n=16) transplants from genetically-engineered pigs, and a costimulation blockade-based regimen. Heart grafts functioned for 15–130d. Artery recipients were euthanized after 28–84d. Platelet counts, fibrinogen, D-dimer, and CRP were measured.

Results—Thrombocytopenia and reduced fibrinogen developed only in recipients of hearts not expressing a coagulation-regulatory protein (n=4), but not in other heart or patch recipients. However, in heart recipients (n=8), there were sustained increases in D-dimer (<0.5–1.9ug/mL [p<0.01]), and CRP (0.26–2.2mg/dL [p<0.01]). In recipients of artery patches, there were also sustained increases in D-dimer (<0.5–1.4ug/mL [p<0.01]), and CRP (0.26–1.5mg/dL [p<0.001]). An IL-6R antagonist suppressed the increase in CRP, but not D-dimer.

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Conflict of interest

DA is an employee of Revivacor, Blacksburg, VA, USA. None of the other authors reports a conflict of interest.

Conclusion—The pig artery patch model has proved valuable for determining immunosuppressive regimens that prevent sensitization to pig antigens. This model also provides information on the sustained systemic inflammation seen in xenograft recipients (SIXR). An IL-6R antagonist may help suppress this response.

Keywords

Baboons; Coagulation; C-reactive protein; D-dimer; IL-6R blockade; Inflammation; Platelets; Pigs, genetically-engineered; Xenotransplantation

INTRODUCTION

Significant progress has been made in experimental xenotransplantation (xenoTx), largely through the availability of organs from genetically-modified pigs, but coagulation dysfunction and an inflammatory response to the graft remain barriers (1,2). Coagulation dysfunction, for which there are several contributing factors, is manifest by the development of thrombotic microangiopathy in the graft and/or consumptive coagulopathy in the recipient (3–9). Platelet-rich fibrin thrombi accumulate in the vasculature of the graft, and the recipient develops thrombocytopenia, fibrinogen depletion, and an increase in D-dimer (a fibrin degradation product). The inflammatory response is less well defined, but can be associated with the increased movement of leukocytes (especially granulocytes) from the blood into injured tissues (2). A sustained increase in C-reactive protein (CRP) is a marker of inflammation (10).

Because it is difficult to determine the effect of an immunosuppressive regimen on the immune response to a pig organ graft in a baboon (because thrombotic microangiopathy and/or consumptive coagulopathy often obscures this), we have developed a simple pig-to-baboon artery patch Tx model. This has provided us with valuable information on the efficacy of immunosuppressive regimens in preventing sensitization to pig antigens (11,12). We have investigated whether the patch model can also provide information on the coagulation dysfunction and/or inflammatory state that develops after pig organ Tx in nonhuman primates.

Although there were numerous variables in the experiments, e.g., differences in pig genetics, anti-inflammatory and immunosuppressive agents, we believe the data indicate that the patch model provides (i) relatively little information on coagulation dysfunction, but (ii) possibly valuable information on the inflammatory state that develops. This latter observation was surprising in view of the small size of the patch grafts, but we demonstrated its value in assessing the effect of an anti-inflammatory agent.

MATERIALS AND METHODS

Animals

Baboons (*Papio* species, n=24; Division of Animal Resources Oklahoma University Health Sciences Center, Oklahoma City, OK) weighing 6–10kg and of various AB blood group were recipients of pig heart (n=8) or artery (n=16) grafts.

Genetically-engineered pigs (Revivicor, Blacksburg, VA), weighing 7–30kg, all of nonA(O) blood group, served as sources of heart or carotid artery patch grafts (Table 1) (12–17). Four baboons received hearts from α 1,3-galactosyltransferase gene-knockout (GTKO) pigs expressing two human complement-regulatory proteins (CD46/CD55) (Group 1A), and four from GTKO/CD46 pigs expressing at least one human complement-regulatory and one human coagulation-regulatory protein (Group 1B). Carotid artery patches were taken from GTKO/CD46 pigs or GTKO/CD46 pigs expressing a human dominant-negative MHC class II transactivator (CIITA) as described by Hara et al (18), but none expressed a human coagulation-regulatory protein. Transgene expression was determined by flow cytometry and/or immunofluorescence/immunohistochemistry of pig aortic endothelial cells.

Cynomolgus monkeys (*Macaca fascicularis*, n=3, Alpha Genesis, Yamassee, SC) weighing 3–5kg were recipients of ABO-compatible heart allografts, as previously reported (19) (Table 1).

All animal care was in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Heterotopic heart or artery patch transplantation

Anesthesia, intravascular line placement in baboons and monkeys, and pig-to-baboon heterotopic heart Tx or alloTx has been described previously (3,5,19,20), as has pig carotid artery patch Tx in baboons (11).

Immunosuppressive and adjunctive therapy

For the xenoTx experiments, immunosuppressive therapy was based on costimulation blockade using either (i) anti-CD154mAb (Group 1A) or (ii) anti-CD40mAb (Group 1B) or (iii) CTLA4-Ig +/- anti-CD40mAb (2C10R4) (Group 2A and B) (12,16) (Table 1). In addition, two baboons with artery patch grafts (Group 2B) received IL-6R blockade with tocilizumab (Actemra, Genentech, South San Francisco, CA) (21) which, to our knowledge, has not been tested in a large animal model of xenotransplantation previously. For the alloTx experiments, immunosuppressive therapy was based on alemtuzumab (19).

Coagulation and inflammatory parameters

Whole blood and serum samples were obtained from recipients before and serially after Tx. Blood samples were tested for blood cell count, platelet count, coagulation parameters (fibrinogen and D-dimer), and CRP. In monkeys with heart allografts (Group 3), only CRP was measured.

Experimental groups

Baboons in Group 1 received heart grafts, either from GTKO pigs expressing two human complement-regulatory proteins (Group 1A, n=4) or from GTKO pigs expressing at least

one human complement-regulatory and one coagulation-regulatory proteins (Group 1B, n=4) (Table 1). Baboons in Group 2 received artery patch grafts from GTKO/CD46 or GTKO/CD46/CIITA (18) pigs and a regimen either without (Group 2A, n=14) or with (Group 2B, n=2) tocilizumab therapy (Table 1). Monkeys in Group 3 received heart allografts.

Statistical analysis

Statistical analysis was performed using social sciences software GraphPad Prism 5.0 Data are presented as mean and standard deviation (SD) for all variables. Significance of the difference between two groups was determined by paired Student t test or Mann-Whitney test. A p value of <0.05 was considered statistically significant.

RESULTS

Clinical course and graft survival

Baboons with heart grafts (Group 1) were euthanized for either graft failure (n=2) or for complications of immunosuppressive therapy (n=5) or indwelling vascular catheters (n=1) between 2 and 18 weeks after Tx (Table 1). Baboons with carotid artery patches (Group 2; n=16) were electively euthanized 28, 48, or 84 days after Tx (Table 1); initially, follow-up was planned for 28 days but, to ensure that the late development of elicited anti-pig antibodies was not being missed, follow-up was increased in some baboons to 48 or 84 days. Monkeys in Group 3 (n=3) survived for between 1 and 3 months (19).

Parameters of coagulation and inflammation

Platelets—In recipients of hearts in Group 1A, mean platelet count fell significantly by post-Tx day 14 ($p<0.05$), and did not recover (Figure 1A). In contrast, in recipients of hearts in Group 1B and of artery patches in Groups 2A and 2B, no sustained thrombocytopenia was observed during the period of follow-up. The platelet count in Group 1A on day 14 was significantly lower than in Group 1B ($p<0.05$). There was no difference between Groups 2A and 2B.

Fibrinogen—In heart recipients in Group 1A, after an initial temporary rise associated with the surgical procedure, mean fibrinogen fell significantly by post-Tx day 14 ($p<0.05$) (Figure 1B). In Group 1B and in artery patch recipients in Group 2A, the fibrinogen level was maintained in the normal range (Figure 1B). The fibrinogen level in Group 1A on day 14 was significantly lower than in Group 1B ($p<0.05$). It was also lower in Group 2B than in Group 2A (mean 171 vs 325 mg/dL), though, in view of the small number in Group 2B, the statistical significance was not calculated.

D-dimer—There was an immediate rise in D-dimer following both heart and artery patch Tx (Groups 1 and 2), which was sustained for the duration of the experiment in both groups (Figure 1C). The rise was greater in Group 1 than in Group 2.

C-reactive protein—In heart recipients, mean CRP increased significantly ($p<0.05$) in both Groups 1A and 1B (Figure 1D), although there was again some variation in Group 1B.

In artery patch recipients in Group 2A, mean CRP rose significantly by day 4 ($p<0.05$) and remained elevated throughout the post-Tx course (Figure 1D). In contrast, in Group 2B (treated with IL-6R blockade), there was no increase in CRP at any time (Figure 1D). The CRP in Group 2B on day 28 was substantially lower than in Group 2A (mean 0.3 vs 1.4mg/dL).

In monkey heart allograft recipients (Group 3), there was no substantial change in CRP levels, with follow-up for up to 3 months (Figure 2). Although a small initial rise (presumably associated with the surgical procedure) occurred, no sustained increase was documented, strongly suggesting that the observed rise in CRP seen in the xenoTx models was associated with an inflammatory response to the pig graft, even when this was a small artery patch.

Macroscopic and microscopic appearances of grafts at euthanasia—At euthanasia, in the heart xenografts some thrombi were seen in the left ventricle (as is commonly seen in the heterotopic model). In some cases, histopathology showed features of thrombotic microangiopathy (not shown), which have been described elsewhere (12). No thrombi were adherent to patch grafts, and on histological examination there were mild or insignificant cellular infiltrates and no features of vasculopathy (not shown) (16).

In summary, thrombocytopenia and reduced fibrinogen developed in baboons in which hearts not expressing a human coagulation-regulatory protein had been transplanted. In all heart and artery patch Tx recipients, D-dimer increased and remained elevated throughout the post-Tx course. CRP rose post-Tx and was sustained at an increased level in all heart and artery patch recipients except those treated with IL-6R blockade. No increase in CRP was seen after heart alloTx.

DISCUSSION

Variable degrees of thrombotic microangiopathy and/or consumptive coagulopathy resulting from coagulation pathway activation have been observed after pig-to-baboon organ xenoTx (22–24). Decreased platelet counts and fibrinogen levels (8,12) develop shortly after Tx, and markedly elevated D-dimer levels have been reported after pig heterotopic heart Tx in baboons (25). These changes are associated with the onset of a consumptive coagulopathy. We have also recorded a sustained increase in CRP, suggesting a prolonged inflammatory response.

The pig artery patch Tx model, which is associated with few complications, has proved valuable for determining the efficacy of an immunosuppressive regimen in preventing sensitization to pig antigens. The present study indicated that, although the graft is small ($2\times 1\text{cm}$), this model might also provide some important information on the inflammatory response, though relatively little on coagulation dysfunction. We suggest the presence of the pig vascular endothelium activates antibody, complement, and innate immune cells that generate the inflammatory response, which seems to persist as long as the graft is present (unless perhaps the porcine endothelial cells are replaced by recipient endothelial cells).

In the artery patch model, after the immediate increase associated with the surgical procedure, there were no significant changes in platelet count or in fibrinogen levels, but there was a sustained increase in D-dimer, which might be an indicator of an inflammatory response, though possibly might also indicate a degree of coagulation dysfunction (26). The increase in D-dimer did not appear to be due simply to the surgical procedure and/or anesthesia (as it has not been seen after operations for intravascular catheter insertion in baboons [not shown] or following heart alloTx in monkeys), and its maintenance for 28 days or longer suggests a persisting dysfunctional state accompanying the presence of the graft.

The increase in D-dimer was not as marked as that which occurred after pig heart Tx. This, together with the absence of thrombocytopenia and reduced fibrinogen, suggests that the small area of pig vascular endothelium that is exposed to the recipient blood after artery patch Tx stimulates a weaker coagulation/inflammatory response. It is possible that this is associated with the relative lengths of the surgical procedures, but it is more likely related to the extent of exposure to vascular endothelial cells of the graft, which is clearly much greater after heart Tx. Nevertheless, we suggest that the artery patch model may prove valuable in assessing the efficacy of novel genetically-engineered pigs, e.g., those expressing an anti-inflammatory transgene, or of new pharmacologic regimens directed towards preventing an inflammatory response. Investigation in this simple and less expensive model may prove a valuable preliminary to the more complex heart or kidney Tx models.

A good example of how this model might prove relevant is offered by comparison of the CRP response between Groups 2A and 2B. In Group 2A, there was a sustained (>80 days) rise in CRP (which again has not been seen after intravascular catheter insertion or heart alloTx). IL-6R blockade completely abrogated the increase in CRP, and this therapy may prove valuable in suppressing the inflammatory response after pig organ Tx. A recent study suggests it might also reduce the antibody response (27). As far as we are aware, this is the first report of IL-6R blockade in a model of xenoTx. The inflammatory response, which we increasingly believe plays an important role in failure of an organ xenograft, which we have termed systemic inflammation in xenograft recipients (SIXR), has recently been discussed (2). The state of systemic inflammation that develops after pig organ xenoTx might be associated with both adaptive and innate immune responses (2).

Inflammation is known to amplify activation of the coagulation system (28–31). Additionally, pro-coagulant proteins, e.g. thrombin, are considered as pro-inflammatory factors. An amplification loop may exist between inflammation and coagulation, leading to escalation of each other. Furthermore, a significant positive correlation between elevated plasma levels of CRP and D-dimer has been reported in patients with aortic dissections (32), deep vein thrombosis (33,34), or ischemic heart disease (35–37). The mechanism of this relationship is not yet completely understood.

We hypothesize that, in xenoTx, elevated CRP and D-dimers are associated with inflammation. Local fibrin formation and lysis are part of the inflammatory response, and fibrin degradation products, including D-dimer, have been shown to have diverse effects on inflammatory processes and acute phase responses (neutrophil and monocyte activation),

secretion of cytokines (e.g., IL-6), and hepatic synthesis of acute-phase proteins (fibrinogen and CRP) (38–41).

Although in only two baboons, our observation that an IL6-R antagonist prevented a CRP response to a pig artery patch suggests that this form of therapy might be beneficial in organ xenoTx by reducing the inflammatory response. Of interest in this respect is the observation that, even in the presence of sepsis, CRP remains at normal levels if the subject is receiving tocilizumab (42).

In conclusion, the present study indicates that, although the graft is small, the pig-to-baboon artery patch model provides information on the systemic inflammatory response after xenoTx. The maintenance of elevated D-dimer and CRP levels for 28–84 days suggests a persisting inflammatory state accompanying the presence of the graft. Therefore, further genetic modifications of donor pigs to express not only coagulation-regulatory but also inflammation-regulatory proteins may be necessary to prevent or control the systemic inflammation that develops in xenograft recipients (SIXR) (2). An alternative might be therapy with an IL6-R antagonist and/or antagonists of other relevant cytokines.

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Abbreviations

CRP	C-reactive protein
GTKO	α 1,3-galactosyltransferase gene-knockout
IL-6	interleukin-6
MCP-1	monocyte chemotactic protein-1
TNF-α	tumor necrosis factor-alpha
Tx	transplantation

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Figure 1.

Changes in (A) platelet count, (B) fibrinogen, (C) D-dimer, and (D) C-reactive protein after pig heart (n=8) or artery patch (n=14) transplantation in immunosuppressed baboons.

(A) In pig heart recipients in Group 1A, mean platelet count fell from $270 \times 10^3/\text{mm}^3$ pre-Tx to $122 \times 10^3/\text{mm}^3$ on post-Tx day 14 ($p < 0.05$), and did not recover. No thrombocytopenia was observed in recipients of hearts in Group 1B or of artery patches in Groups 2A and 2B.

(B) In heart recipients in Group 1A, mean fibrinogen fell from 224mg/dL pre-Tx to 144mg/dL on post-Tx day 14 ($p < 0.05$). In heart recipients in Group 1B and artery patch

recipients in Groups 2A and 2B, mean fibrinogen remained within the normal range (or higher) on day 28 (314mg/dL, 361mg/dL, and 202mg/dL, respectively).

(C) In heart recipients, mean D-dimer increased from <0.5ug/mL pre-Tx to >2.0ug/mL where it was maintained throughout the post-Tx course in Group 1A, although it was variable in Group 1B. In artery patch recipients, mean D-dimer increased from <0.5ug/mL pre-Tx to 1.4ug/mL on post-Tx day 48 ($p<0.01$) in Group 2A and to 1.2ug/mL on day 48 in Group 2B.

(D) In heart recipients in Group 1A, mean CRP increased from 0.24mg/dL pre-Tx to 3.0mg/dL on post-Tx day 14 ($p<0.05$). In heart recipients in Group 1B, mean CRP increased from 0.29mg/dL pre-Tx to 2.6mg/dL on post-Tx day 48 ($p<0.05$). In artery patch recipients not receiving IL-6R blockade (Group 2A), mean CRP increased from 0.26mg/dL pre-Tx to 1.4mg/dL on post-Tx day 48 ($p<0.01$), whereas in the two baboons that received an IL6-R antagonist (tocilizumab) (Group 2B) it did not increase significantly throughout the post-Tx course.

Figure 2.

CRP levels in baboons with pig artery patch grafts (a) not receiving IL-6R blockade (Group 2A; n=14), (b) receiving IL-6R blockade (Group 2B; n=2), and in monkeys with heart allografts (Group 3; n=3). An immediate rise after Tx and sustained CRP levels was observed in baboons not receiving IL-6R blockade, but there was no increase in CRP in baboons receiving IL-6R blockade or in monkeys with heart allografts.

Table 1

Experimental groups

GROUP	PIG	MAINTENANCE THERAPY	Survival (days)
(HEART)			
1A (n=4)	GTKO/hCRP ^(a)	Anti-CD154mAb-based	15,18,23,33
1B (n=4)	GTKO/hCRP/hCoagRP ^(b)	Anti-CD40mAb-based	42,52,99,130
(ARTERY PATCH)			
	GTKO/hCRP+/-CIITA ^(c)		
2A (n=14)		Belatacept +/- Anti-CD40mAb-based	28-84 *
2B (n=2)		Belatacept+/-Anti-CD40mAb-based+Tocilizumab	48 *
(ALLO HEART)			
3 (n=3)		Alemtuzumab-based	33,96,97

^(a) GTKO/hCRP = α 1,3-galactosyltransferase gene-knockout pigs transgenic for two human complement-regulatory proteins (CD46 and CD55).

^(b) GTKO/hCRP/hCoagRP = α 1,3-galactosyltransferase gene-knockout pigs transgenic for at least one human complement-regulatory protein (CD46 +/- CD55) and at least one human coagulation-regulatory protein (i.e., thrombomodulin +/- endothelial protein C receptor).

^(c) GTKO/hCRP+/-CIITA = α 1,3-galactosyltransferase gene-knockout pigs transgenic for one human complement-regulatory protein (CD46) and expressing a human dominant-negative MHC class II transactivator (CIITA) as described by Hara et al (18).

Anti-CD40 (2C10R4) and anti-CD154 mAb were kindly provided by Dr. Keith Reimann through the NHP Reagent Resource.

* Electively euthanized