

Survival of pig-to-rhesus corneal xenografts prolonged by prior donor bone marrow transplantation

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Abstract. The aim of the present study was to explore the survival of pig-rhesus corneal xenografts following donor bone marrow transplantation (BMT). Wuzhishan pigs were used as donors and rhesus monkeys as recipients for corneal xenotransplantation. Twelve rhesus monkeys were divided into two groups. Group 1 received intravenous injection of cyclophosphamide (CP) followed by pig bone marrow cell transplantation, while group 2 was used as a control and only received intravenous CP injection. All xenografts were evaluated using a slit-lamp microscope. The immunological status of the recipients following transplantation, including the formation of chimerism, mixed lymphocyte reaction (MLR) and immunoglobulin and complement in the serum, was analyzed. Two rhesus monkeys in each group were sacrificed for corneal histopathology examination. The mean survival time was 36.0 ± 4.7 days in group 1 and 17.7 ± 3.2 days in group 2. The mean chimerism percentage in group 1 at week 1 was $5.20 \pm 1.02\%$, but decreased with time and was $<1\%$ after week 3. MLR demonstrated that immune reactivity to donor spleen cells in group 1 was decreased following surgery. Immunoglobulin and complement levels in the serum revealed a decreasing trend. Histopathological examination demonstrated that the corneal xenografts in group 1 had minimal inflammatory cell infiltration and no eosinophil infiltration. Survival of corneal xenografts may be prolonged by prior BMT, suggesting that immune reactivity to donors is suppressed, and is highly dependent on chimerism formation.

Introduction

In the majority of developing countries, a severe shortage of donor corneas has limited performance of keratoplasty.

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Xenografts may provide a solution to this supply-demand disparity. In view of the ethical issues and impracticalities associated with the use of nonhuman primates, interest has focused on other species, in particular the pig, as a suitable organ donor species for humans. In addition to organ size and physiological similarities to humans, pigs are highly inbred, making them particularly amenable to genetic modifications that may improve their ability to function as organ donors to humans (1).

A barrier to successful xenograft is hyperacute rejection. In the case of the cornea, distinctive features exist that partially exempt this tissue from this type of pathogenesis (2). Previous studies (3) demonstrated that corneal xenografts from WZS pigs were not hyperacutely rejected in the rhesus monkey model. Immune rejection occurred approximately 15 days following penetrating corneal xenotransplantation. The endothelium may be a main target for rejection, due to evidence of cellular or chronic graft rejection in this membrane region (3).

Induction of donor-specific immunological tolerance would avoid graft rejection and side-effects associated with chronic, non-specific immunosuppressive therapy. Establishment of mixed chimerism (donor/host) by bone marrow transplantation (BMT) is a reliable method to induce donor-specific tolerance (4-6). Mixed chimerism refers to a state in which allogeneic cells coexist with recipient cells. Together in the thymus, cells delete host- and donor-reactive T cells, resulting in a peripheral T-cell repertoire tolerant towards donor and host (7). Mixed hematopoietic chimeras exhibit donor-specific transplantation tolerance and decreased immunocompetence (8-12).

Lan *et al* demonstrated that mixed hematopoietic chimerism mediates deletion of donor antigen-reactive human thymocytes in the thymus and induces human T-cell tolerance to porcine xenografts (1). WZS pigs are suitable donor alternatives in corneal xenotransplantation, due to high rates of inbreeding and high homology between WZSP SLA and human HLA. However, it remains unclear whether mixed chimerism, induced by donor BMT, is a solution to the prevention of pig-rhesus xenograft rejection in this model. To address this issue, we used the WZS pig-rhesus xenotransplantation model to explore xenograft survival following donor BMT.

Materials and methods

Animals. All animals in this study were used in accordance with the ARVO Resolution on Use of Animals in Research.

WZS pigs (inbred for 14 generations; 3-4 months old; weight, 15-20 kg) were purchased from the Institute of Animal Sciences (Chinese Academy of Agricultural Sciences, Beijing, China). Rhesus monkeys (aged 3-5 years old) were purchased from the Academy of Military Medical Sciences (Beijing, China). WZS pigs were used as donors and rhesus monkeys as recipients. Only the right eye of each rhesus monkey received the corneal xenotransplantation.

Donor preparation

Pig bone marrow cell preparation. Bone marrow cells were harvested from the ilium bone of WZS pigs as previously described (6,7,13). Marrow was filtered through a sterile nylon mesh and red cells were lysed with Tris-NH₄Cl. Bone marrow cells were resuspended to produce a concentration of 1x10⁸ cells/ml (8-10,14,15).

Pig cornea preparation. WZS pigs were sacrificed and eyeballs were enucleated and washed in saline (9). Eyeballs were immersed in sterile saline solution containing 2000 U/ml tobramycin and streptomycin for 20 min. The cornea was excised with Vannas scissors and preserved in Optisol solution at 4°C for 3-5 days.

Groups

Group 1. Prior to bone marrow transplantation, 6 rhesus monkeys received daily intravenous injection of cyclophosphamide (CP; 15 mg/kg) for 2 days (16,17). At day 3, recipients received bone marrow cells by intravenous injection (2.5x10⁸ cells/kg). In addition, between days 5-7 and 11-13, monkeys were injected intravenously with CP at the same dosage described above. At day 14, orthotopic penetrating corneal xenotransplantation was performed.

Group 2. Six rhesus monkeys received intravenous injection of CP in the same way as group 1. No BMT was performed. Orthotopic penetrating corneal xenotransplantation was also performed at day 14.

Orthotopic penetrating corneal xenotransplantation.

Rhesus monkeys were anesthetized with an intramuscular injection of 10 mg/kg ketamine and an intravenous injection of 25 mg/kg amobarbital. Donor grafts were excised by a 6.0 mm trephine and the graft bed was prepared using a 5.75 mm trephine on the right eye. The donor graft was placed on the recipient's bed and sutured with 8 interrupted 10-0 nylon sutures (Ethicon, Somerville, NJ, USA). The anterior chamber was restored at the end of surgery with sterilized saline buffer. Antibiotic and atropine ointment were applied and eyelids were closed with 5-0 nylon tarsorrhaphy. Eyelids remained closed for 48 h and were then opened for clinical evaluation.

Assessment of xenograft survival. Following surgery, corneal xenografts were evaluated by slit-lamp microscope every other day for 2 weeks, then twice a week until the end of observation. Graft opacity, edema and neovascularization were evaluated using a modified scoring system as described previously (3). A rejection index (RI) was based on the sum of the grades for opacity, edema and neovascularization (Table I). Grafts with scores ≥ 6 were considered to be rejected.

Table I. Grades for opacity, edema and neovascularization.

Grades	Criteria
Opacity	
0	Clear
1	Slight haze
2	Increased haze but iris structure still clear
3	Advanced haze with difficult view of iris structure
4	Severe opacity without view of chamber structure
Edema	
0	No stromal or epithelial edema
1	Slight stromal edema
2	Diffuse stromal edema
3	Diffuse stromal edema with epithelial microcystic edema
4	Bullous keratopathy
Neovascularization	
0	No vessels
1	Vessels appearing in the peripheral corneal bed
2	Vessels appearing in the graft periphery
3	Vessels appearing in the graft middle-periphery
4	Vessels extending to the graft center

Flow cytometry assay. Prior to surgery and at postoperative weeks 1, 2 and 4, peripheral blood samples from the rhesus monkeys were obtained. Red blood cells were depleted from the samples by Tris-NH₄Cl and a mouse anti-pig MHC-I monoclonal antibody was used to monitor the recovery of pig cell populations in the monkeys by flow cytometry (Serotec, Oxford, UK). FITC-conjugated goat anti-mouse IgG was set as the negative control (Southern Biotech, Birmingham, AL, USA).

Histopathological analysis. At postoperative week 4, 2 rhesus monkeys/group were sacrificed. Corneas were embedded in paraffin, sliced, stained with hematoxylin and eosin and examined under a light microscope (Olympus, Tokyo, Japan).

Mixed lymphocytes reaction (MLR) assay. Prior to surgery and at postoperative weeks 1, 2 and 4, peripheral blood of the rhesus monkeys was taken and lymphocytes were used as reaction cells. RPMI-1640 culture medium was added to the cells to prepare single-cell solutions at a concentration of 5x10⁶ cells/ml. Cells were cultured in 96-well flat-bottom tissue culture plates (200 μ l/well) and stimulated with 200 μ l concanavalin A (ConA; 5 μ g/ml) or xenogeneic donor spleen cells (radiated by cobalt-60 for 30 min, at a concentration of 5x10⁶ cells/ml). Cultures were performed in triplicate, including a blank RPMI-1640 control group in the absence of mitogen. Following 72 h incubation at 37°C, with 5% CO₂ in a humidified incubator, the cultures were pulsed with 10 μ l methyl-thiazolyl-tetrazolium (5 mg/ml). OD (optical density) values were measured 5 h later, at a wavelength of 570 nm by an MRX Microplate reader (Synateck Laboratories, Chantilly, VA, USA) (18).

Immunoglobulin and complement assay (19). Time points and cell numbers were as described above. Sera were separated by centrifugation at 3,000 rpm for 3 min and suspensions were used to detect concentration of immunoglobulin and complement with a Nephelometer (Beckman Coulter, Brea, CA, USA) (19).

Statistical analysis. Data were presented as the mean \pm SD and evaluated using a two-tailed Mann-Whitney U-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Incidence and timing of graft rejection. All 12 rhesus monkeys remained alive and healthy during the follow-up period. Group 1, which had been injected with CP and bone marrow cells, showed vomiting, inappetence and wilted condition, returning to normal within 48 h. Group 2, which received intravenous injection with CP only, had no symptoms of sickness.

Mean survival time (MST) of the xenografts was 36.0 ± 4.7 days in group 1 and 17.7 ± 3.2 days in group 2 (Table II). A statistically significant difference was identified between groups 1 and 2 ($P < 0.01$).

In group 1, corneal xenografts remained almost transparent during postoperative week 1, although ciliary congestion injection was noted. At postoperative day 20, xenografts remained transparent. At postoperative day 40, xenografts revealed slight diffuse inflammatory infiltration and stromal edema, but no exudative membrane was observed in the anterior chamber and no new-forming vessels appeared (Fig. 1A and B). Infiltration and edema in the xenografts was intensified at postoperative day 90, however, no neovascularization and exudative membrane were observed in the anterior chamber and no scarring was noted.

In group 2, corneal xenografts revealed mild edema and exudative membrane was observed in the anterior chamber. No infiltration in the stroma was observed during the initial postsurgical period. At postoperative day 15, corneal edema increased, stromal infiltration was observed and neovascularization was noted in the peripheral corneal bed. At postoperative day 20, xenografts demonstrated characteristics of immune rejection with loss of graft clarity. Increased edema and new-forming vessels were noted on the recipients' bed. At postoperative day 40, xenografts were filled with new-forming vessels and revealed complete opacity. The pupil could not be viewed (Fig. 1C and D). All grafts developed scarring 3 months following surgery.

Following corneal xenotransplantation surgery, index of opacity, edema and neovascularization increased with time. However, at all time points, the index of group 1 was lower than that of group 2 (Fig. 2).

Chimerism formation. Rhesus monkeys that received CP and pig bone marrow cell transplantation formed mixed chimerism. In postoperative week 1, the mean chimerism percentage was $5.20 \pm 1.02\%$. At postoperative week 2, the mean chimerism percentage decreased to $1.20 \pm 0.03\%$, decreasing further at postoperative week 4 to $< 1\%$ (Fig. 3). However, in the non-BMT group, no chimerism was detected. These results

Table II. Corneal graft survival following xenotransplantation.

Groups	n	Survival time (d)	MST (d)	Median (d)
1	6	32, 42, 40, 34, 38, 30	36.0 ± 4.7^a	36
2	6	12, 18, 16, 20, 20, 20	17.7 ± 3.2	19

^a $P < 0.01$, compared with group 2. MST, mean survival time; d, days.

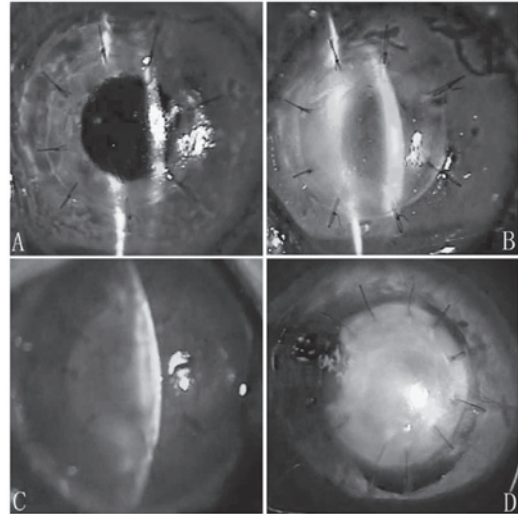


Figure 1. Clinical evaluation of pig-to-rhesus monkey corneal xenografts by slit-lamp microscopy (magnification, $\times 25$). Postoperative day 20, (A) xenograft was clear in group 1, (C) significant opacity and edema of the xenograft in group 2. Post-operative day 40, (B) stroma of the xenograft was moderately cloudy and edematous in group 1, (D) stroma of the xenograft was severely cloudy with copious neovascularization, thus the anterior chamber could not be observed in group 2.

indicate that CP administered in combination with BMT produces a transient mixed chimerism across a fully xenogeneic barrier in the host.

Histopathological staining. In group 1, low levels of inflammatory cells at the intersection between the graft and bed were observed. The epithelium and endothelium were intact. No eosinophil infiltration was noted in the grafts (Fig. 4A and C). However, in group 2, there was more inflammatory cell infiltration in the stroma of the xenograft and scattered eosinophil infiltration was observed (Fig. 4B and D).

MLR. Prior to CP injection, no significant difference was found between the lymphocyte reaction ability of the rhesus recipients in groups 1 and 2 to ConA and donor lymphocyte antigen ($P > 0.05$). Following CP and BMT, lymphocyte reaction ability was identified as significantly lower in group 1 compared with group 2. The lowest value of 0.90 ± 0.15 was obtained at postoperative week 2 and was identified as significantly reduced compared to group 2 ($P < 0.05$; Table III).

Immunoglobulin and complement levels in the serum. Concentration of IgA at postoperative week 2 was 100.85 ± 65.74 mg/dl in group 1 and 126.30 ± 61.17 mg/dl in group 2. A statistically significant difference was found

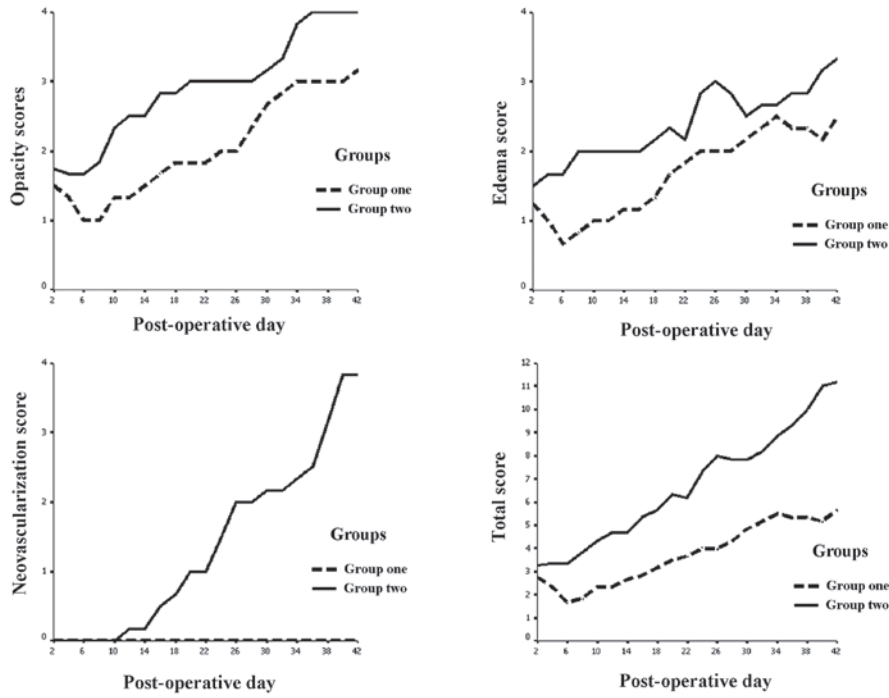


Figure 2. Curve of corneal grafts score changes at defined times following xenotransplantation. Group 1 received CP injection and BMT, while group 2 received CP only. BMT, bone marrow transplantation; CP, cyclophosphamide.

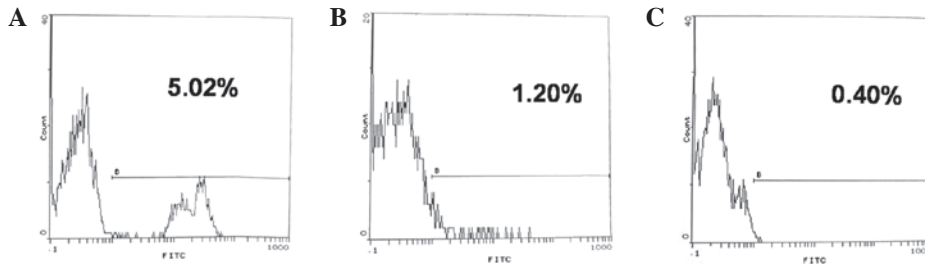


Figure 3 Flow cytometry plots to show chimerism in group 1. Percentage of pig cells detected by monoclonal mouse anti-pig MHC-I (A) postoperative week 1 (B) postoperative week 2; (C) postoperative week 4.

($P < 0.05$). Concentration of complement C3 at postoperative week 2 was 123.75 ± 13.89 mg/dl in group 1, compared with 160.50 ± 28.41 mg/dl in group 2, which was identified as a statistically significant difference ($P < 0.05$).

Discussion

Xenografts from pigs may provide a potential solution to the severe shortage of donor cornea in developing countries, including China. However, xenogeneic corneas are subject to vigorous immune rejection, and the non-specific immunosuppression that is required to overcome rejection negatively affects recipients with dangerously low immune systems (3). Therefore, it is highly desirable to eliminate immune response to xenografts, through the induction of immune tolerance. Mixed chimerism has been proven to be a powerful and reliable approach for tolerance induction across allogeneic and closely related xenogeneic barriers (20,21).

In 1986, Cobbold and Waldmann (22) demonstrated that fully MHC-mismatched marrow engraftment and specific tolerance was achieved, by pretreating recipients

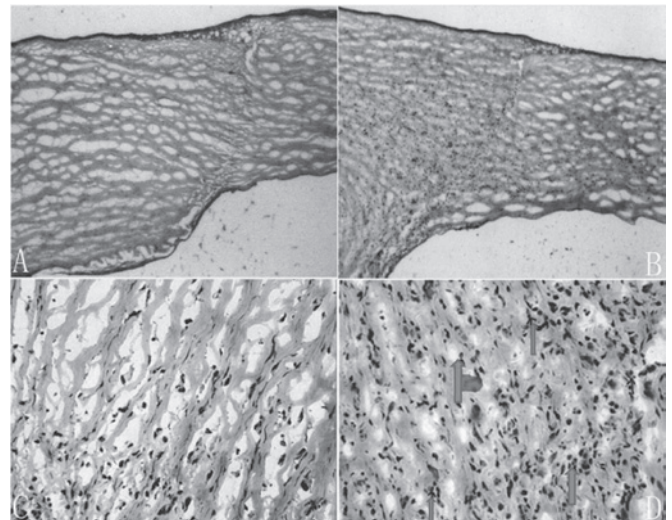


Figure 4. Histopathology of xenografts at postoperative week 4 (stained with hematoxylin and eosin). Intersection part between graft and bed (x40), (A) group 1, low levels of infiltrating inflammatory cells, (B) large number of infiltrating inflammatory cells. Xenograft (x400), (C) no acidophil cells, (D) scattered acidophil cells (shown as black arrows).

Table III. Comparison of lymphocyte proliferation capacity following BMT (n=4).

Period	Group 1			Group 2		
	Negative control	ConA	Donor lymphocyte	Negative control	ConA	Donor lymphocyte
Before CP injection	0.02±0.00	1.22±0.27	1.18±0.27	0.01±0.00	1.22±0.25	1.17±0.14
Postoperative week 1	0.02±0.01	0.98±0.27	0.91±0.43 ^a	0.02±0.01	1.12±0.38	1.10±0.16
Postoperative week 2	0.02±0.01	0.95±0.48	0.90±0.15 ^a	0.01±0.00	1.24±0.42	1.23±0.21
Postoperative week 3	0.01±0.00	1.13±0.32	1.06±0.24 ^a	0.02±0.00	1.19±0.27	1.19±0.38

^aP<0.05, compared with group 2. BMT, bone marrow transplantation; CP, cyclophosphamide.

with depleting doses of anti-CD4 and anti-CD8 monoclonal antibodies combined with a sublethal dose (6 Gy) of total body irradiation (TBI) (9). In a variety of non-myeloablative BMT preparative regimens, CP was administered instead of TBI (17). With this protocol, long-lasting mixed chimerism was induced in a series of HLA-matched donor BMT recipients and in a small series of HLA-mismatched BMT recipients (15). The mixed chimerism approach has been successfully extended to xenogeneic models.

Mixed hematopoietic chimerism is associated with tolerance to skin, islet cell, cardiac grafts and lung allograft. However, donor-specific tolerance to corneal xenografts has never been reported. Allogeneic graft rejection rarely occurs in clinical BMT patients, therefore, BMT is highly favored for use in high-risk patients, particularly those with immune system dysfunction. A previous study demonstrated that steroid treatment alone prolonged xenograft mean survival time (23). However, steroid treatment is associated with reduced innate and acquired immune response, including hyperacute immune response. Therefore, CP was utilized to reduce activities of host lymphocyte cells and donor hematopoietic cells with the aim to weaken donor-versus-host disease, while retaining weak stimulation of donor cells.

Our data demonstrate that the donor chimerism was detectable at postoperative week 1. Following this, donor chimerism decreased and was not detectable 1 month after BMT. This was correlated with xenograft survival at approximately 1 month. We conclude that induction of prior mixed chimerism existence prolongs corneal xenograft survival and xenograft survival was correlated with the formation of chimerism in the recipient. A reduction in MLR and lower levels of immunoglobulin and complement following surgery also demonstrates that recipients were in lower immunocompetence. CP treatment had no effect on graft survival, consistent with previous data. CP has been revealed to suppress lymphocyte cells reaction but not innate immune cells. We hypothesize that innate immune cell-mediated xenograft rejection is the main cause of graft failure.

Histopathology of the xenografts revealed a reduction of inflammation cell infiltration in group 1 and no eosinophil infiltration in the xenografts was observed. Observation of eosinophil cells is characteristic of xenotransplantation rejection and it has been demonstrated by Tanaka *et al* that eosinophil-dependent xenograft rejection bears similarities

to immune elimination of parasites (24). However, this does not suggest that eosinophil cells dominate the main population of cells involved in corneal xenotransplantation. Lymphocytes, including CD4⁺ cells, antigen-presenting cells and neutrophils, remain the main population involved in corneal xenotransplantation. Absence of eosinophil infiltration in group 1 suggest that xenograft rejection is inhibited by BMT.

In the MLR assay, a decreased response in group 1 to donor lymphatic cells was observed, indicative of specific immunosuppression. However, response to ConA was indicative of non-specific immunosuppression. These MLR results require further clarification, and may be explained by poorly controlled CP doses. Further studies should utilize a third party mitogen to elucidate the suppression response. There was reduction of IgA levels in group 1, however, levels of IgG and IgM were not identified as significantly different between the two groups (data not shown). These results suggest that humoral immunity is also involved in corneal xenotransplantation despite playing a minor role in the immune response.

In general, preconditioning with CP and conventional BMT induced immune suppression, prolonging corneal graft survival in pig-monkey xenotransplantation. At present, chimerism only exists in the recipient for a short time period. To sustain this presence, future analysis may utilize suppressants, including CsA, rapamycin and antibodies against T cells or costimulator cytokines (CTLA4-Ag, CD40L). Staphylococcus enterotoxin B is a superantigen used in a previous study (?) to induce immune tolerance in a high risk rat corneal transplantation procedure. This tolerogen may prove useful for induction of long-term chimerism formation. Future studies are likely to involve modification of the treatment to increase long-term chimerism and likelihood of genuine tolerance, with the aim to produce a treatment suitable for clinical application.

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