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Prolonged survival of GalT-KO swine skin on baboons

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Abstract

Background—Allogeneic skin is currently the best alternative to autologous skin as a temporary treatment for severe burns, but it has several drawbacks. As a potential alternative, we have evaluated GalT-KO swine skin, which lacks expression of the Gal epitope, to investigate the effect of eliminating this epitope on survival of pig-to-baboon skin grafts.

Methods—Two adult baboons that had fully recovered from previous T cell depletion received simultaneous skin grafts from: (i) GalT-KO swine, (ii) Gal-positive swine, (iii) a third-party baboon, and (iv) self (control skin). Recipients were treated with cyclosporin for 12 days and the survival, gross appearance, and histology of the grafts were compared.

Results—In both baboons, the GalT-KO skin survived longer than either the Gal-positive swine skin or the allogeneic skin. Early rejection of the Gal-positive skin appeared to be mediated by cytotoxic preformed anti-Gal IgM antibodies, while the rejection of GalT-KO skin appeared to result from cellular mechanisms.

Conclusions—GalT-KO skin may have potential clinical benefits as an alternative to allogeneic skin as a temporary treatment for severe skin injuries.

Keywords

allografts; galT-KO; skin; xenografts; xenotransplantation

Introduction

According to the American Burn Association, there are approximately 500 000 burn injuries per year in the United States, with roughly 40 000 requiring hospitalization [1].

A treatment option that has helped to decrease mortality over the past 10 yrs has been the immediate excision of burned skin with replacement by grafted skin [2–4]. The ideal material for grafting is autologous skin, taken from a non-burned region of the patient's own skin. The supply of healthy autologous skin, however, is limited in severely burned patients, even when expansion techniques, such as “meshing,” are used [5,6]. Allogeneic skin is considered the gold standard for temporary grafts [1]. In addition, it is able to engraft temporarily before rejection occurs, and it can be frozen and stored for transportation or later use. However, disadvantages include ethical concerns, cost considerations, and possibility of disease transmission, and like all types of temporary grafts, it is more easily infected than autologous skin and not always available.

Pig skin is known to have many characteristics similar to that of humans [7–12] and glutaraldehyde-fixed pig skin has been utilized as a temporary cover for third degree burns under battlefield conditions [13]. The properties of such fixed skin are far inferior to those of living skin, and living pig skin is susceptible to rapid rejection, thought to be due, at least in part, to natural antibodies present in all humans [14,15]. The recent development in this laboratory of genetically modified swine missing the Gal epitope, the major cell surface determinant toward which these antibodies are directed, made it possible that skin from these “GalT-KO” animals might provide a new source of living skin grafts for the immediate treatment of burns. Previous studies in our laboratory have shown that the use of GalT-KO swine donor organs has greatly increased the survival of vascularized xenograft organs in baboon recipients [16,17].

In an attempt to evaluate whether the use of skin from GalT-KO swine would be of benefit in prolonging the survival of pig-to-primate skin grafts, we transplanted GalT-KO skin onto two baboon recipients and compared the survival of these grafts with that of Gal-positive and allogeneic grafts. We report here the results of this preliminary study.

Materials and methods

Animals

Two 3- to 4-yr-old baboons that were available from a previous study were used as recipients for this initial experiment. Both animals had been thymectomized and treated with an anti-T cell immunotoxin in the previous protocol and then followed for several months, during which time all immunologic parameters returned to baseline, including natural antibodies as well as numbers and phenotypes of white blood cells in both the peripheral blood and lymph nodes.

Allogeneic skin donors were unrelated baboons available in our animal facility. Xenogeneic donors were from our closed herd of MGH Miniature Swine. Animals from the standard line of SLA^{dd}, GalT^{+/+} miniature swine [18] or from our GalT^{-/-} (GalT-KO) line, derived from this standard inbred line [19], were used.

Surgery

Harvesting of donor skin was performed using a Zimmer dermatome (Medfix Solution, Inc., Tucson, AZ, USA), with depth set at 24 mm. Anesthesia consisted of induction with 2 mg/kg ketamine i.m. followed by maintenance with isoflurane administered by mask. Partial thickness sections of skin (approximately 3 × 5 inches) were taken. Grafts were stitched into place with interrupted 1-0 sutures and covered with a Duoderm dressing for 2 days, after which they were left open, protected by a loose fitting jacket. Recipients were treated with 13 mg/kg cyclosporine intramuscularly for 12 days.

Biopsies

Recipients were sedated and anesthetized to evaluate the skin grafts and draw blood at various times postoperatively. On each of these occasions, grafts were examined, graded, cleaned, and photographed, and blood was drawn for complete blood count, serum collection, and in vitro assays. At selected times, 6.0-mm full-thickness punch biopsies were taken for histologic evaluation of frozen and formalin samples.

PBMC isolation

For separation of peripheral blood leukocytes, freshly heparinized whole blood was diluted 1 : 2 with Hank's balanced salt solution (HBSS; GIBCO BRL, Gaithersburg, MD, USA) and the mononuclear cells were obtained by gradient centrifugation using lymphocyte separation

medium (Organon Teknika, Durham, NC, USA) as previously described [20] and stored in mixed leukocyte reaction (MLR) media.

Histology

Biopsy specimens were either fixed in 10% buffered formalin or immediately frozen in liquid nitrogen. Fixed samples were embedded in paraffin, and 4- μ sections were stained with hematoxylin and eosin. Immunohistochemical analysis of frozen samples was carried out using the avidin–biotin horseradish–peroxidase complex technique [21].

Complement-mediated cytotoxicity

Cytotoxic antibodies to Gal-positive and GalT-KO PBMC were detected by complement-mediated cytotoxic assays, as previously described [22]. Briefly, target cell suspensions were diluted to 5×10^6 cells/ml in Medium 199 (Cellgro, Herndon, VA, USA) supplemented with 2% fetal calf serum and serially diluted from 1 : 2 to 1 : 1024. In some cases, IgM was eliminated prior to the assay by adding dithiothreitol (DTT; Sigma–Aldrich, St. Louis, MO, USA) to the serum. In 96-well U-bottom plates (Costar, Cambridge, MA, USA), 25 μ l of the appropriate target cell suspension was incubated with 25 μ l of diluted serum or controls for 15 min at 37 °C, followed by a second incubation with 25 μ l of appropriately diluted rabbit complement. Dead cells were identified by staining for 30 min with 10 μ l of 7-AAD. Data were acquired, and the percentage of dead cells was assessed, using a Becton–Dickinson FACScan (San Jose, CA, USA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME, USA).

Results

Gross findings following skin grafts

On baboon 1, the four skin grafts (self, GalT-KO, Gal positive, and allo baboon, left to right, respectively in Fig. 1A) were placed side-by-side on a single graft bed and covered with bacitracin and a gauze dressing. On baboon 2 (Fig. 1B), the graft beds were prepared separately to avoid spreading of local infection or inflammation from one graft to another, and no dressings were applied.

Baboon 1

By day 4, the self and allo grafts on baboon 1 were warm, soft, and pink, suggesting that they had engrafted and begun to re-vascularize. The GalT-KO skin was also warm and pink, although with slight mottling. In contrast, the Gal-positive pig skin was cool and white, suggesting a “white graft,” as previously described for skin grafts that do not re-vascularize because of hyperacute rejection due to preformed antibodies [23,24]. By day 7, the self skin was still pink and healthy, but the allo skin had begun to develop a crust over the graft, as had the Gal-positive pig skin. The Gal-positive skin also appeared to be infected, producing purulent discharge, and the animal’s white blood cell count rose. In contrast, the GalT-KO skin was warm, dark pink, and mostly intact, although localized, superficial infection caused some loss of integrity. The infection in the GalT-KO skin, which damaged part of the graft as well as a portion of the self graft, appeared to have spread from the neighboring infected Gal-positive skin. The animal was treated for 3 days with 15 mg/kg per day i.v. vancomycin, after which the superficial infection cleared and the white count returned to normal. By day 9, the allo skin had been totally rejected and was covered by scab, while a portion of the GalT-KO skin still remained intact. The self skin remained normal. The GalT-KO appeared to be totally rejected by day 11.

Baboon 2

On day 4, the self and allo grafts on baboon 2 were likewise warm, soft, and pink, whereas the Gal-positive graft was bright white and cool to touch. The GalT-KO skin was warm and soft, but with some purple mottling. There was minimal bleeding when the Gal-positive biopsy was taken, suggesting poor vascularization. The grafts appeared much the same on day 7 (Fig. 1B) except that a portion of the Gal-positive graft appeared grossly necrotic and purulent. By day 11, self skin was still warm, soft, and pink, but the allo skin was fully crusted with only half of it remaining intact. The Gal-positive skin was mostly crust with two small areas that remained bright white. The GalT-KO skin showed some moderate crusting at the edges, but otherwise remained soft and warm. By day 14, a small portion still appeared viable, and the final biopsy was taken from this portion.

Histologic findings following skin grafts

As seen in Fig. 2, the histology of the self skin graft on baboon 1 remained normal except for a small amount of non-specific granulation tissue on day 7 and evidence of localized bacterial infection on day 9. The histology of the allogeneic skin graft appeared normal on day 0, showed slight vacuolization on day 4, developed a dense cellular infiltrate by day 7 and appeared to be fully rejected by day 9, with histologic evidence of a regenerating host skin bed beneath the rejected graft. Consistent with the gross observation of a white graft, the Gal-positive pig skin never showed histologic evidence of engraftment (Fig. 2). Histology on day 4 showed thrombi in small vessels, consistent with hyperacute rejection, leading to occlusion of the blood vessels. Immunohistochemistry revealed a large amount of anti-Gal but not anti-non-Gal antibody deposition by this time (Fig. 4A,B), and the graft was necrotic by day 7. In contrast, the GalT-KO skin graft appeared essentially normal on days 4 and 7, with only mild congestion. Antibody deposition was not observed by immunohistochemistry until day 9, at which point the graft showed evidence for cellular rejection similar to that seen in the allograft on day 7 (data not shown).

In baboon 2, the interpretation of early histologic samples of skin graft biopsies was less clear. However, by day 4, the Gal-positive skin showed antibody deposition, and by day 11 it appeared completely non-viable, respectively. In contrast, in the GalT-KO skin graft, antibody deposition was observed only after day 7 and the graft remained viable on day 11 (Fig. 4C,D). It still showed partial viability on day 14, although with considerable inflammation. The graft bed was visible in this sample, confirming that the viable epidermis was in fact graft-derived.

Antibody responses to skin grafts

Cytotoxicity assays with and without DTT showed that despite high levels of anti-Gal and anti-non-Gal preformed IgG in both baboons, all antibody-mediated cytotoxicity in the first week was almost entirely mediated by preformed anti-Gal IgM. After 7 days, however, both the amount and cytotoxicity of anti-Gal and anti-non-Gal IgG increased markedly (Fig. 3).

Discussion

The results reported in this preliminary study suggest that GalT-KO xenografted skin is preferable to normal, Gal-positive swine skin, and as good as or better than allogeneic skin (i.e., cadaveric human skin) for use as an acute skin transplant to cover severe burn injuries. The gross observations on the skin grafts in this study were substantiated by the histological findings in both animals.

The antigen most responsible for rapid rejection of normal pig tissues is the α -1,3-Galactose (Gal) moiety, found on the cell surfaces of all mammalian species, except Old World

primates and humans [15]. Primates have high levels of preformed antibodies to Gal and after transplantation, these antibodies bind to the Gal antigen on vascular endothelial cells. There, they fix complement, damaging the endothelium and triggering the coagulation cascade, resulting in immediate rejection by occlusion of graft vessels [25]. This process, which is dependent on both antibody levels and on the level of antigen expression on endothelial cells [26], was likely responsible for the early rejection of Gal-positive skin in both baboon recipients in the present study, leading to non-vascularized or “white grafts” by day 4 in both cases. The much lower levels of preformed anti-non-Gal antibodies were likely responsible for the much milder evidence of humoral rejection observed several days later for GalT-KO skin. Although some humans also have antibodies against non-Gal pig antigens, previous studies in our laboratory have demonstrated that these antibodies are generally of low prevalence and titer [22].

Therefore, prolonged survival of GalT-KO skin compared with Gal-positive skin confirms the importance of immune recognition of Gal. However, it is less clear why the survival of GalT-KO skin should have been prolonged over that of allogeneic skin, as in neither case would high levels of anti-donor antibody-mediated reactivity be expected. Preliminary mixed lymphocyte reaction data indicate that, in the absence of a major contribution of humoral rejection, the strength of anti-donor cellular responses may determine the kinetics of rejection (data not shown). Further experiments are in progress to determine whether or not this correlation is generalizable and will be reported in a subsequent publication.

In summary, these results suggest that xenogeneic pig skin from GalT-KO swine may provide a less expensive, more readily available and potentially long-lasting alternative to allogeneic human skin as an initial covering for extensive burn injuries.

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Patents: Alpha (1,3) Galactosyltransferase Negative (GalT-KO) Swine (00841.10)

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Fig. 1.

(A) Baboon 1 skin grafts on days 0 and 4 (from left to right): self, GalT-KO, Gal-positive, allogeneic baboon. (B) Baboon 2 skin grafts on days 0 and 7 (from left to right): self, GalT-KO, Gal-positive, and allogeneic baboon.

Fig. 2.
Histology of skin grafts on baboon 1, biopsies on days 0, 4, and 7.

Fig. 3.

Antibody-complement mediated cytotoxicity assay of sera from baboons 1 (A) and 2 (B) at 1 : 4 dilution showed increased antibody-mediated cytotoxicity after day 4, with higher baseline killing levels against Gal-positive cells than against GalT-KO cells. Addition of DTT demonstrated that early cytotoxicity was largely due to preformed anti-Gal IgM (solid black line ■ = Gal-positive cell targets without DTT, solid gray line ▲ = GalT-KO cell targets without DTT, dashed black line = Gal-positive cell targets with DTT).

Fig. 4. Immunohistochemistry of Gal-positive (A,C) and GalT-KO (B,D) skin on baboons 1 (POD 7) and 2 (POD4) showing early deposition of anti-Gal IgM but not anti-non-Gal IgM.