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Delayed skin allograft rejection following matrix membrane pretreatment[☆]

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Summary *Introduction:* No solution has been offered to induce long-term skin allograft survival in burn patients. We investigated whether transplant acceptance could be improved by a nonsystemic pretreatment of the graft and recipient wound surfaces with a bioengineered interface consisting of an acellular matrix membrane.

Methods: Group 1 ($n = 30$): Crosstransplants of untreated skin grafts between BALB/c and C57BL/6 mice.

Group 2 ($n = 30$): Crosstransplants of matrix-treated skin grafts between BALB/c and C57BL/6 mice.

Group 3 ($n = 30$): Retransplantation of skin grafts from the original donor on to the sensitised recipients. Sensitisation was accomplished by prior transplantation of an untreated skin allograft from the same donor (Group 1 mice).

Two skin grafts were transplanted: one treated and one untreated.

Results: Rejection occurred in the untreated group after a mean of 6.8 days (± 1.5 days). In contrast, treatment with the bioengineered matrix membrane was found to substantially prolong allograft survival with a mean of 28 days (± 3.8 days). Graft survival between the two groups reached statistical significance ($P < 0.05$). In the sensitised mice, the untreated skin regrafts were all rejected in an accelerated fashion with an onset of less than 4 days (mean ± 1 days). However, the matrix membrane-treated skin regrafts were maintained for a mean of 18 days (± 3 days).

Conclusion: These results show that treatment with the bioengineered matrix membrane greatly delays the onset of acute allograft rejection. The described topical application to

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the wound surfaces of both the graft and the recipient may offer a new and readily available source of wound coverage in patients with extensive burns.

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Extensive burns represent one of the most devastating injuries to the human body. Severe, deep burns cause fluid escape, loss of temperature control, disturbances of ion equilibrium, and high risk of bacterial invasion. Treatment of such injury requires skin replacement to reform the functional and protective barrier that differentiated epidermis provides. Next to using autologous skin, which is often impossible in patients with large acute wounds due to the lack of adequate donor sites, the best option is allograft skin. For deep extensive burns, temporary closure can be achieved with a cadaveric allograft, an approach that has been an integral part of burn surgery for many years. Unfortunately, the clinical use of allogenic skin is limited by the short graft survival times attributable to the inevitable immunologic rejection.^{1,2}

New technologies that provide improvements in the ability to reconstruct following a severe burn would have significant clinical impact. Ideally such innovations should have the characteristics of a grafting material with all of the relevant functions of skin. Any new technology should have the goal of providing complete coverage of the wound. Such technology should also not be dependent upon autologous tissue that results in more scarring and ideally should improve the prognosis for scarring that would result in an improvement in the patient's quality of life.

A logical approach that could potentially provide all of the features just described would be an immunomodifying therapy that leads to the long-term acceptance of cadaveric skin allografts. Long-term graft survival has been achieved in the case of solid organ transplants for many years. However, there has been no corresponding improvement in the acceptance of skin grafts.

Since the ground breaking studies of Peter Medawar in 1944 using skin grafts to demonstrate the role of the immune system in rejection, it has been understood that graft rejection is under genetic control.³ In first set skin graft rejection, infiltrating cells were usually seen by the second or third day. Medawar noted that these infiltrating cells tended to congregate at the graft/host interface and increased in numbers on subsequent days. Extensive morphologic changes were observed in rejecting skin grafts long before ischaemic death occurred, with infiltration of mononuclear cells, polymorphonuclear cells and plasma cells.⁴ An Arthus reaction then occurred with the resulting development of erythematous lesions, oedema and epidermal necrosis.^{5,6}

As a direct result of our emerging understanding of the rejection process, almost all efforts to prolong graft survival have focused on the effector arm of the immune system. With the exception of pretransplant graft treatments such as the removal of passenger leukocytes, efforts have focused on interrupting the effector cell cascade. In the present study, we investigated whether skin allograft acceptance could be improved by a targeted pretreatment

of the allograft to form a bioengineered interface consisting of an acellular matrix membrane.

The goal of this study was to investigate whether an interruption of the recipient/donor interface could result in prolonged skin graft survival. Since skin grafts are isolated from the vascular circulation when first implanted, with a corresponding delay in revascularisation, something that does not occur in the case of solid organ transplants, we hypothesised that this delay in revascularisation may provide a window of opportunity for novel methods of immunomodulation.

Materials and methods

Endomatrix membrane

The Endomatrix membrane used for immunocloaking is a proprietary technology of Breonics, Inc., Otisville, NY, USA. The matrix membrane is comprised of type IV collagen, vitrogen, fibronectin, laminin, entactin, glycosaminoglycans and proteoglycans. The components are polymerised to result in a tri-dimensional transparent membrane. The interaction between the skin and the recognition domains within the membrane is receptor specific via the laminin and fibronectin portions of the membrane.

Lysine-derived crosslinks and disulfide bonds stabilise the components in the membrane. Its barrier function is attributable, in part, to its overall anionic charge. In its present biosynthetic form the membrane appears as a fine mesh lacking the banded fibrillar structure of other collagen types. Each molecule has a globular region at one end and a disulfide region at the other end. Additionally, each molecule is interconnected with others through disulfide bonds.

Application and transplantation procedure (Fig. 1)

The process involves solubilising the synthesised membrane by acidification at 4 °C. The membrane was then neutralised with 0.1 N NaOH and 100 µl was applied to both the skin graft and wound surfaces and allowed to polymerise at physiologic temperature. A thin continuous membrane that covers the surface of the wound and the basolateral surface of the skin allograft resulted. Since the bioengineered membrane is permeable to small molecular weight compounds, free transport of nutrients and oxygen is unaffected and the tissue remains viable. Likewise, the membrane supported cellular functions similar to the role of extracellular matrices in substrata tissues.

Genetically inbred BALB/c and C57BL/6 mice were obtained from Charles River Company (Wilmington, MA, USA). The BALB/c-C57BL/6 model is considered to

Group 1 - untreated skin graft
Group 2 - treated skin graft



Group 3 - treated and untreated skin graft

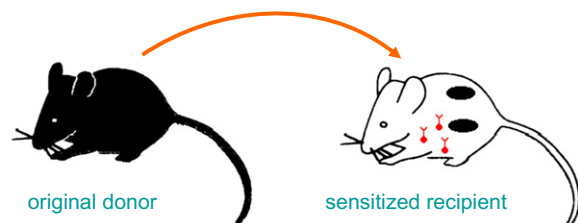


Figure 1 Treatment groups.

Group 1 ($n = 30$): Crosstransplants of untreated skin grafts between BALB/c and C57BL/6 mice.

Group 2 ($n = 30$): Crosstransplants of matrix-treated skin grafts between BALB/c and C57BL/6 mice.

Group 3 ($n = 30$): Retransplantation of skin grafts from the original donor on to the sensitised recipients (mice from Group 1). Sensitisation was accomplished by prior transplantation of an untreated skin allograft from the same donor. Two skin grafts were transplanted: one treated and one untreated.

represent an excellent model for skin allograft transplantation because of the major H-2 complex incompatibilities. Congenic BALB/c mice possess the d phenotype while congenic C57BL/6 possess the b phenotype.⁷ Mice were housed in compliance with the 'Principles of Laboratory Animal Care' with free access to food and water. Full thickness 8 mm skin grafts were crosstransplanted between the two genetically unrelated groups of mice (BALB/c and C57BL/6 mice). Control skin allografts were transplanted without treatment following 15 min incubation at 37 °C in a sterile milieu. In the case of the test skin allografts, the 8-mm skin grafts were maintained with the outer skin surface face down providing access to the exposed subdermal surface. The skin grafts were immobilised in this position using a sterile convex surface as a holder that resulted in the skin graft surface to be treated being slightly concave. The 100 μ l of neutralised and normothermic matrix membrane was carefully layered on to the subdermal surface of the skin graft using a sterile syringe. The concave holder containing the skin graft was gently rotated to ensure even distribution of the matrix membrane along the graft surface during the period of polymerisation. Treated skin grafts were then incubated at 37 °C for 15 min. During the incubation period, an additional 100 μ l of the neutralised and solubilised membrane was layered on to the wound bed on each mouse. Following polymerisation, the treated skin grafts were allotransplanted on to the surface of the also treated wound bed.

In the case of the retransplantation studies, two new skin grafts from the original donor were used to

retransplant the original recipient that had rejected its prior untreated skin graft. In the case of retransplantation, the two skin grafts were allotransplanted on to the recipient with one graft serving as an untreated control and the other paired skin graft and its wound being treated with the Endomatrix membrane as described above.

Animals were followed by daily visual inspection by two independent technologists. The daily observations from each technologist were compared and if a discrepancy was encountered, the principal investigator examined the skin graft in question in the presence of the two technologists and a consensus was rendered. The occurrence of rejection was defined as graft necrosis greater than 90% resulting in the loss of viable skin.^{8,9}

Treatment groups (Fig. 2)

Group 1 ($n = 30$): Crosstransplants of untreated skin grafts between BALB/c and C57BL/6 mice.

Group 2 ($n = 30$): Crosstransplants of matrix-treated skin grafts between BALB/c and C57BL/6 mice.

Group 3 ($n = 30$): Retransplantation of skin grafts from the original donor on to the sensitised recipients. Sensitisation was accomplished by prior transplantation of an untreated skin allograft from the same donor. Two skin grafts were transplanted: one treated and one untreated.

Immunologic screening by flow cytometric crossmatch

The development of donor-reactive antibodies was determined by flow cytometry crossmatching in order to demonstrate the immunologic basis for the graft loss. The flow cytometric crossmatch has served as a sensitive laboratory test for determining immunologic status. The flow cytometric crossmatch was performed by incubation of the recipient sera with the donor lymphocytes followed by staining with a fluorochrome-conjugated secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse IgG (H and L); Jackson Immuno Research, West Grove, PA, USA).¹⁰ Specifically, the target lymphocytes were isolated from either the spleens of naïve BALB/c and C57BL/6 mice or the crosstransplanted donors after euthanasia. The lymphocytes were used at a working concentration of 2×10^5 cells that were incubated with 50 μ l of recipient serum for 30 min, washed three times and then resuspended in 10 μ l of fluorochrome-conjugated secondary antibody. After a second 30 min incubation, the lymphocytes were again washed twice followed by fixation in paraformaldehyde. The fixed cells were then analysed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) and Cellquest software (Cellquest Inc. Largo, FL, USA). The baseline channel fluorescence was determined using negative controls consisting of both autologous sera (recipient sera tested with recipient cells) and sera from naïve mice.

The median channel fluorescence (MCF) shift used for determining positivity was calculated as the standard deviation based upon the average of the median channel fluorescence of the naïve controls as well as autologous controls. Three standard deviations were employed as

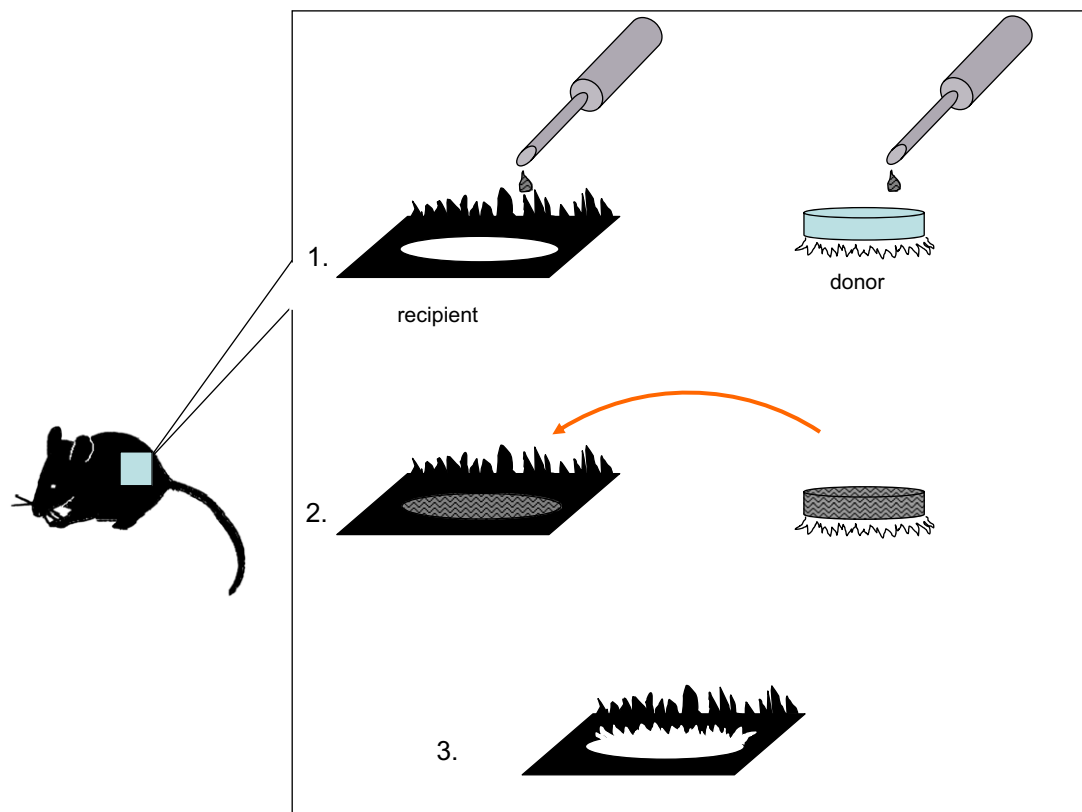


Figure 2 Matrix application.

1. The matrix is solubilised by acidification at 4 °C. Solubilised matrix is then neutralised with 0.1 N NaOH and 100 μ l applied to both the skin graft and wound surfaces.
2. Matrix is allowed to polymerise at physiologic temperature. A thin continuous membrane that covers the surface of the wound and the basolateral surface of the skin allograft results.
3. Skin allograft is then sutured into the recipient wound.

cutoff for positivity. The MCF of the negative control was subtracted from the MCF of the recipient/donor crossmatch yielding a calculated channel shift. The presence of donor-reactive antibody was assigned when the MCF shift was greater than the established cutoff for positivity.

Results

Primary skin graft survival

Rejection occurred in the Group 1 mice receiving the untreated skin allografts on days 5 through 10 posttransplantation with a mean of 6.8 days (± 1.5 days). Rejection of the untreated skin grafts resulted in open wounds that eventually formed scabs. In contrast, pretreatment of the skin allografts with the bioengineered Endomatrix membrane was found to substantially prolong allograft survival. Allograft survival in the Group 2 mice ranged from 17 to 32 days with a mean of 28 days (± 3.8 days). The observed prolongation of graft survival in Group 2 recipients reached statistical significance ($P < 0.05$). In all cases, the treated group demonstrated good hair regrowth that in some cases exceeded surrounding native hair regrowth. The eventual rejection of the matrix-treated skin allografts did not result in an open wound nor in the formation of a scab, but rather the skin graft was eventually sloughed leaving an intact pseudo-dermis.

Flow cytometry

The results of the flow cytometric analysis indicated that the Group 1 recipients of untreated skin allografts in all cases developed a humoral immune response against the donor antigens. By 14 days posttransplantation, all the control recipients in Group 1 demonstrated donor-reactive antibody levels that exceeded the cut-off of three standard deviations established for determining positivity; indicating an immunologic basis for the skin graft loss. In contrast, in Group 2 where the recipients were allotransplanted with skin that had been treated with the Endomatrix membrane prior to transplantation, a substantially reduced level of allosensitisation was observed. Only 50% of the Group 2 skin graft recipients demonstrated a MCF shift of greater than the three standard deviations determining positivity for the development of donor-reactive antibody. This observed reduction in sensitisation also reached statistical significance.

Retransplantation (Fig. 3)

Since treatment with the Endomatrix membrane led to reduced rates of allosensitisation and prolonged skin graft survival, the potential of the membrane to prolong skin graft survival was evaluated in cases of known pre-existing sensitisation where a high titre of preformed antibody was

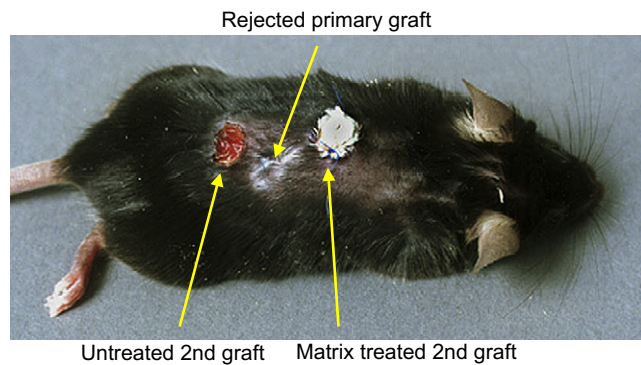


Figure 3 Retransplantation. Recipient that had previously rejected an untreated skin graft (Group 1) and was retransplanted with two skin grafts from its original donors. One graft was transplanted without treatment and in the paired skin graft Endomatrix membrane was applied. Day 12 posttransplantation.

present. The skin graft recipients that had previously rejected an untreated skin graft (Group 1) were retransplanted with two skin grafts from their original donors. One graft was transplanted without treatment and in the paired skin graft Endomatrix membrane was applied. In the presensitized mice, the untreated skin regrafts were all rejected in an accelerated fashion with an onset of less than 4 days (mean \pm 3 days). However, the Endomatrix-treated skin regrafts were maintained for >16 days (mean of 18 days \pm 3 days), in the presence of rejection of the paired skin regraft.

Discussion

The use of a systemic immunosuppressive regimen to prevent graft rejection has remained the foundation of clinical transplantation. While tolerance induction protocols possess the potential to eliminate our reliance on systemic immunosuppression, none have progressed beyond early phase clinical trials. Because immunosuppressive agents are administered systemically and are largely non-specific in function, it is currently not possible to block rejection of allografts without simultaneously suppressing other immune functions as well. The relative nonspecific nature of systemic immunosuppressive agents renders them an undesirable treatment for burned patients.

The risk of exogenous immunosuppression in the already immunocompromised burn patient has prompted interventional strategies directed at the graft itself. An attractive option that has been used is to apply allografts and then later remove the immunogenic epidermis and replace it with cultured autologous keratinocytes. Analogous options that have been used include using an artificial dermis such as Integra (Integra Life Science Corp, Plainsboro, NJ, USA) followed by the application of sheets of autologous cultured cells once circulation has been established. Alloderm (LifeCell, Woodlands, TX, USA) consists of human dermis rendered antigen free over which a thin layer of skin graft can be applied. The disadvantage of these therapies remains the vulnerability of the epidermal layer and the

lack of appendages such as hair follicles or sweat glands that breach in both the epidermal and dermal layers.

Our approach to graft immunomodification entails coating the wound surfaces of the allograft skin with a bioengineered basement membrane called Endomatrix. The goal is to interrupt the normal interface by providing a physical barrier between recipient and donor tissues. The bioengineered basement membrane is composed of a complex array of collagen type IV, glycoproteins and proteoglycans that provide an apical surface that remains nonthrombogenic and nonimmunogenic. The network of adhesive molecules constituting the basement membrane functions as a barrier to cellular migration, while simultaneously allowing the free diffusion of nutrients and oxygen.

The results of this initial feasibility study demonstrate that treatment with the bioengineered matrix membrane substantially delays the onset of acute skin allograft rejection. Interestingly, while the development of antibody to donor skin occurred in 100% of the untreated skin grafts, sensitisation in terms of a humoral response was observed in only approximately half of the treated skin grafts. Whether this can be attributable to factors associated with the methodology used to apply the membrane or alternatively relates to the immunologic status of the recipient remains to be determined in future studies. However, in the case of second set rejection where pre-existing sensitisation had already developed, treatment with the Endomatrix membrane also provided protection resulting in the significant prolongation of skin allografts. The observed prolongation of skin grafts may present the opportunity to interrupt the normal effector pathway, thereby allowing for the introduction of an immunosuppressive regimen to burn patients at a later time point that would not be an option in the early post-burn period.

These results yield tantalising preliminary evidence for the Endomatrix membrane providing protection from the immune cell allorecognition pathway leading to humoral rejection. Such protection would appear logical during the phase when graft survival is dependent upon plasmatic circulation and during the early period of communication between recipient and donor microvessels. What is not yet understood is how the membrane provided protection leading to the observed prolongation of graft survival when mature complex capillaries, arterioles and venules should have developed (>7 days post-engraftment). The goal of this study was solely to investigate whether an interruption of the recipient/donor barrier interface could result in prolonged skin allograft survival. Biopsies were not performed prior to graft rejection in this initial study out of concern that the membrane would be disrupted. Likewise, once the graft was destroyed no useful information could be obtained. Given the redundancy inherent in the immune system with most specific pathways demonstrating the ability to damage a graft, time course studies will be needed to begin to understand how a physical barrier such as the endomatrix membrane resulted in the observed prolongation of graft survival. The impact of the physical barrier on the normal immune response in terms of antigen presentation, co-stimulating signals, allograft revascularisation and leucocyte extravasation can be elucidated by histologic evaluation of the graft at sequential time points. These studies are now underway. It is anticipated that an

understanding of the underlying protective mechanisms involved will help to optimise the treatment and lead to further enhancement in graft survival.

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