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Experimental

Noninvasive Monitoring of Allograft Rejection Using a Novel Epidermal Sampling Technique

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Summary: Despite promising short- and long-term results to date in vascularized composite allotransplantation (VCA), acute rejection remains the most common major complication in recipients. Currently, diagnosis of acute rejection relies on clinical inspection correlated with histopathological analysis. However, disagreement exists regarding the value of full-thickness skin and mucosal biopsies and histopathology remains semiquantitative, subject to sampling bias, and prone to intra- and inter-observer variabilities. Additionally, biopsies may cause infection, scarring, and/or potentially incite rejection through immune activation after injury. Noninvasive methods to diagnose rejection represent a critical unmet need for the emerging field of VCA. Here, we propose a novel technique utilizing skin stripping of the epidermis and subsequent molecular analysis to detect known markers of acute rejection. Using a small animal VCA model, we sought to validate our epidermal sampling technique as a noninvasive diagnostic test for acute rejection.
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INTRODUCTION

Vascularized composite allotransplantation (VCA) has become a viable reconstructive option with promising short- and long-term results achieved in hand,[1] face,[2] and penis[3–5] transplants. However, acute rejection remains the most common major complication in VCA recipients, with an incidence exceeding 80% in the first post-transplant year alone.[6] Current diagnostic methods rely on correlation of clinical examination and skin histopathology.[7] However, disagreement exists regarding the value of full-thickness skin and mucosal biopsies[8] and histopathology remains semiquantitative, subject to sampling bias, and prone to intra- and inter-observer variabilities.[9] Additionally, biopsies may cause infection, scarring, and/or incite rejection through immune activation after injury. These difficulties are magnified in facial transplantation, where allograft skin is sparse and frequent biopsies can compromise aesthetic outcomes.[10]

Noninvasive methods to diagnose rejection represent a critical unmet need for the emerging field of VCA. Based on recent studies demonstrating high diagnostic value of mRNA analysis from tape stripping in specific cutaneous disorders,[11] our proposed alternative method similarly uses stripping of the epidermis and subsequent molecular analysis to detect known markers of acute rejection. Using a VCA animal model, we sought to validate our epidermal sampling technique as a noninvasive diagnostic test for acute rejection.

MATERIALS AND METHODS

Animals and Transplant Model

Procedures were approved by the Institutional Animal Care and Use Committee at NYU School of Medicine. Adult male Brown-Norway rats and Lewis rats were used as donors and recipients, respectively. Superficial inferior epigastric composite flaps were harvested, transplanted to recipients using 10–0 nylon sutures for vessel anastomoses, and inset using 6–0 sutures as previously described.[12,13] Recipients received a 5-day course of cyclosporine (15 mg/kg) and were examined daily for clinical signs of acute rejection.[14]

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Epidermal Sampling/Skin Stripping Technique

A full description of our protocol is provided in Figure 1. Adhesive discs (D-Squame Standard Sampling Discs; CuDerm, Dallas TX) were individually applied to the allograft skin paddle, removed, and vortexed in a mixture of RLT lysis buffer (Qiagen, Valencia, CA) to lyse adhered cells. An equal volume of 100% isopropyl alcohol was added, and the sample was stored at –80°C before mRNA extraction. Total RNA was then extracted using the RNeasy Mini Kit (Qiagen), reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Qiagen), followed by quantitative polymerase chain reaction (qPCR) on a QuantStudio 7 (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems). Results were normalized to 18 seconds. A panel of 6 cytokines and chemokines (MCP1, MIP1α, MIP1β, MIP3α, CXCL9, and CXCL10) was selected based on their previously implicated role in early skin rejection.[15–18] Transplanted flaps followed a predictable timeline of rejection, and allograft epidermal sampling was performed at 3 separate time points representing different clinical stages of rejection (nonrejecting, early rejection, and advanced rejection) (**see figure, Supplemental Digital Content 1**, http://links.lww.com/PRSGO/B170, which displays clinical stages of rejection/epidermal sampling time points). Recipients were monitored daily, and epidermal sampling occurred at 3 defined clinical stages of rejection characterized based on appearance and established classifications. Nonrejecting allograft tissue (left) was sampled on postoperative day (POD) 5, whereas the animal was still receiving systemic cyclosporine and remained immunosuppressed. Advanced rejection (right) was defined as progressive erythema encompassing 100% of the allograft, as well as presence of edema or any signs

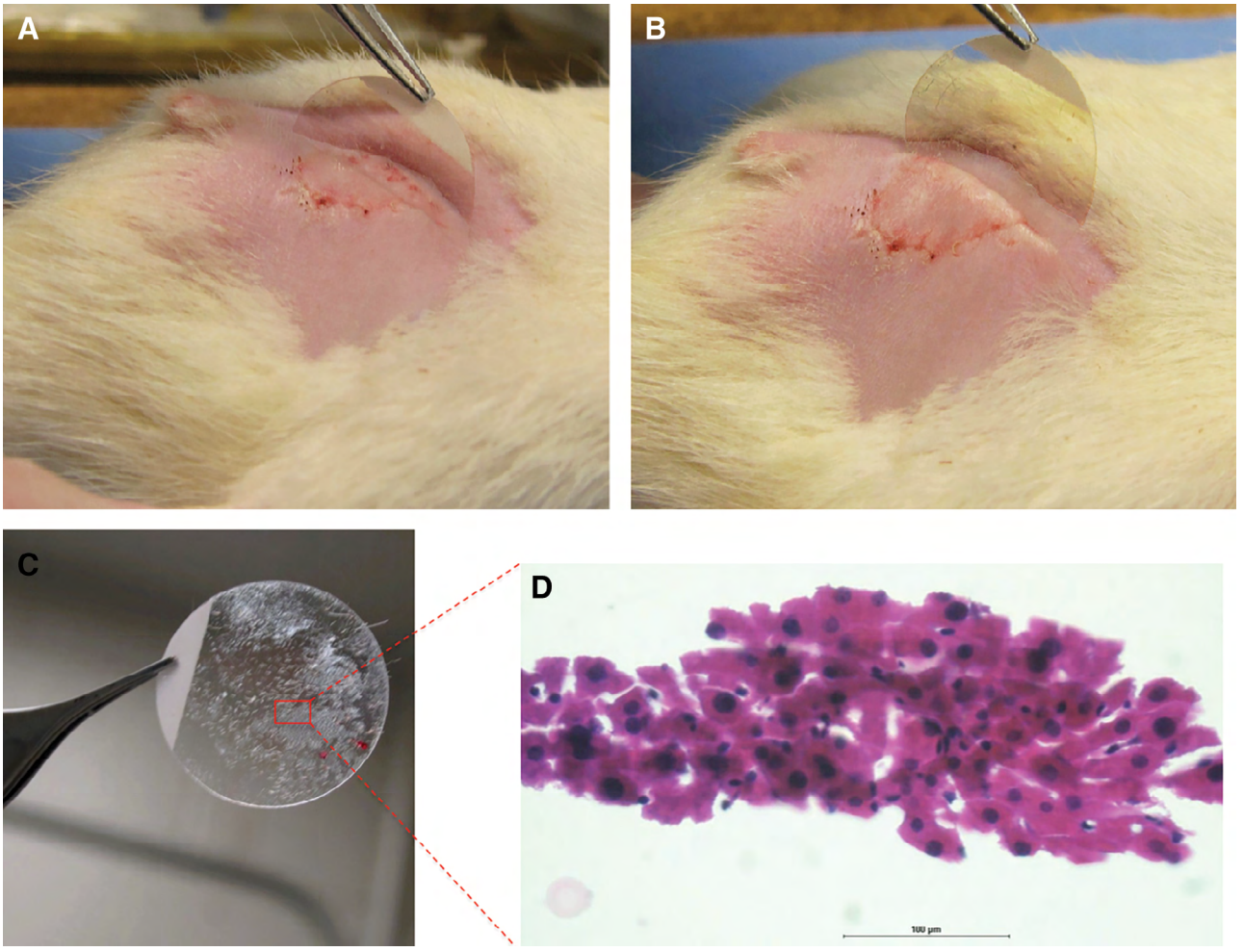

Fig. 1. Epidermal sampling/skin stripping technique. The sampling site, 2.5 cm × 2.5 cm, is first marked and cleaned with an alcohol prep pad. Five individual adhesive discs are then individually applied to the site, removed, and discarded in series to remove the outer layer of dead cells. Next, a fresh disc is applied, removed, and carefully placed into a 2.5-mL tube containing a mixture of 1 mL of RLT lysis buffer and 200 µL of β-mercaptoethanol. The tube is then vortexed for 1 min to lyse adherent cells; the disc is then carefully removed from the tube and discarded. This process is repeated for a total series of 10 discs, using the same buffer-containing tube to maximize RNA concentration, an equal volume of 100% isopropyl alcohol is added, and the sample is stored at –80°C before mRNA extraction and quantitative PCR (qPCR). A, application and B, removal of an adhesive disc. C, An adhesive disc after application to skin with adherent cells clearly visible. D, A magnified image of the cells removed on the disc stained with hematoxylin and eosin for visualization.

of epidermolysis or desquamation, and occurred predictably on POD 11–12. Early rejection (middle) was defined to represent the earliest clinical sign of rejection, specifically allograft erythema, and occurred 2–3 days before the advanced rejection time point. Both early and advanced rejection stages were confirmed histologically. Depicted above are representative images of allografts at each of the three stages of rejection during which sampling occurred.

For comparison of skin stripping cytokine profiles to tissue biopsy, select early rejection and nonrejecting tissue was collected with a 2-mm punch biopsy and mRNA was isolated using the RNeasy Mini Kit and similarly analyzed with RT-PCR.

Statistical Analysis

qRT-PCR results were analyzed and compared using the ΔΔCt method[19] and are expressed as mean fold change ± SD. All experiments were performed at least in triplicate. Statistical significance was set at $P < 0.05$.

RESULTS

Epidermal sampling at the early and advanced rejection time points revealed significant and progressive increases in the expression of MCP1 (fold change: 27.8±17.9 and 64.5±25.8, respectively), MIP1α (fold change: 11.7±5.4 and 499.5±226.8, respectively), MIP1β (fold change: 5.4±3.4 and 190.7±92.3, respectively), and CXCL10 (fold change: 8.4±6.1 and 93.2±70.0 fold, respectively) at each of the 2 clinical stages of rejection, as compared with the "nonrejecting" immunosuppressed stage ($p<0.05$) (**see figure, Supplemental Digital Content 2**, http://links.lww.com/PRSGO/B171, which displays cytokine profiles at progressive stages of rejection). A, Cytokine profiles were generated for several markers of rejection at each of the clinical stages of rejection (nonrejecting, early rejection, and advanced rejection) from mRNA obtained via epidermal sampling technique (n = 3 for each stage) (*$P < 0.05$). B, Cytokine profiles obtained via epidermal sampling were also compared with those obtained directly from biopsied tissue at nonrejecting and early rejection stages to confirm sensitivity and efficacy of the assay (n = 3 for each method at each stage of rejection). Expression of MIP3α and CXCL9 was similarly upregulated during early rejection (19.4±4.5 and 84.3±69.1 fold increases, respectively; $P < 0.05$). However, although expression remained significantly increased during advanced rejection relative to nonrejecting tissue (5.0±3.2 and 17.3±12.1 fold increases, respectively; $P < 0.05$), levels were downregulated relative to the early rejection stage. Interestingly, this suggests that certain markers or a combination of markers may predict early rejection, whereas others may be more indicative of later stages.

To verify the sensitivity and efficacy of our epidermal sampling technique, we also collected tissue biopsies from allografts at the nonrejecting and early rejection stages for comparison. For the majority of markers analyzed, results were comparable between epidermal sampling and tissue biopsy, with both methods demonstrating a significant upregulation during early rejection relative to nonrejecting samples Detection of CXCL9 and CXCL10 was significantly lower with epidermal sampling compared with biopsy.

DISCUSSION

Detection of acute VCA rejection relies on invasive tissue biopsies and histological analysis, generating interest in the development of noninvasive diagnostic tools. Several modalities show promise, including sentinel flaps,[10] serum protein biomarkers,[20] ultrasound biomicroscopy,[21] and vascular magnetic resonance imaging.[22] We present a novel method of epidermal sampling that is simple, noninvasive, and shows promise in a VCA small animal model. Preliminary results indicate that our technique is capable of generating cytokine profiles that can reliably detect and distinguish various stages of rejection and represents a potential noninvasive immune monitoring assay.

Our study's limitations include its small sample size and use of a small animal model, with limited translation to human patients. We plan to validate our findings using larger samples and human VCA recipients to determine repeatability, sensitivity, and specificity of this assay. Additionally, we are evaluating any correlation between cytokine profiles and Banff classification grade.

The high incidence of acute rejection in VCA demands the development of minimally invasive methods of surveillance to reduce the morbidity associated with skin biopsies and allow more dynamic monitoring of response to therapy. To our knowledge, this study is the first demonstrating the predictive utility of mRNA cytokine profiles in the diagnosis of acute rejection in VCA. These results demonstrate the potential of our epidermal sampling technique as a noninvasive tool to predict and diagnose allograft rejection and complement traditional clinical and histopathological data for decision making.

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