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Syngeneic adipose-derived stem cells with short-term immunosuppression induce vascularized composite allotransplantation tolerance in rats

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Abstract

Background aims. A clinically applicable tolerance induction regimen that removes the requirement for lifelong immunosuppression would benefit recipients of vascularized composite allotransplantation (VCA). We characterized the immunomodulatory properties of syngeneic (derived from the recipient strain) adipocyte-derived stem cells (ADSCs) and investigated their potential to induce VCA tolerance in rats. *Methods.* ADSCs were isolated from Lewis (LEW, RT1A^{l}) rats; their immunomodulatory properties were evaluated by means of mixed lymphocyte reactions *in vitro* and VCAs *in vivo* across a full major histocompatibility complex mismatch with the use of Brown-Norway (BN, RT1A^{n}) donor rats. Two control and four experimental groups were designed to evaluate treatment effects of ADSCs and transient immunosuppressants (anti-lymphocyte globulin, cyclosporine) with or without low-dose (200 cGy) total body irradiation. Flow cytometry was performed to quantify levels of circulating $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs). *Results.* Cultured syngeneic ADSCs exhibited $CD90.1^{+}CD29^{+}CD73^{+}CD45^{-}CD79a^{-}CD11b/c^{-}$ phenotype and the plasticity to differentiate to adipocytes and osteocytes. ADSCs dramatically suppressed proliferation of LEW splenocytes against BN antigen and mitogen, respectively, in a dose-dependent fashion, culminating in abrogation of allo- and mitogen-stimulated proliferation at the highest concentration tested. Accordingly, one infusion of syngeneic ADSCs markedly prolonged VCA survival in LEW recipients treated with transient immunosuppression; of these, 66% developed tolerance. Total body irradiation provided no additional VCA survival benefit. An important role for Tregs in tolerance induction/maintenance was suggested *in vivo* and *in vitro*. *Conclusions.* Treatment comprising syngeneic ADSCs and transient immunosuppression (i) increased levels of circulating Tregs and (ii) induced tolerance in 66% of recipients of major histocompatibility complex–mismatched VCAs.

Key Words: *adipose-derived stem cell, allotransplantation, regulatory T cell, tolerance, vascularized composite*

Introduction

Vascularized composite allotransplantation (VCA) describes the *en bloc* reconstruction of a recipient's anatomical unit, such as hand/forearm, abdominal wall or face, by replacing it with a transplantation of the corresponding part from a deceased donor (1–3). The term "allotransplantation" is applied because the donor and recipient are genetically

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non-identical but belong to the same species. Unlike solid-organ transplants, VCAs characteristically contain multiple tissue types, such as skin, muscle, nerve and often bone/marrow, which exert different degrees of immunogenicity (4). The technique has the potential to revolutionize reconstructive surgery but remains hindered by the requirement for lifelong non-specific immunosuppressants and their attendant toxicities, some of which may be fatal (5,6). Conceivably, these problems could be solved by induction of donor-specific transplantation tolerance that allows complete withdrawal of immunosuppressants without harming VCA survival (7).

Mesenchymal stromal cells (MSCs) have potent immunomodulatory properties. They can suppress T- and B-lymphocyte activation and proliferation (8,9), dendritic cell differentiation and maturation (10,11) and natural killer cell activity (12). Donor bone marrow–derived MSCs (BM-MSCs) induced tolerance to semi-allogeneic cardiac (13,14), allogeneic renal (15), xenogeneic islet cell (16) and allogeneic skin transplants (17). Kuo *et al.* (18) successfully induced VCA tolerance in outbred swine with the use of multiple rounds of donor BM-MSCs combined with non-myeloablative irradiation, donor bone marrow transplantation and transient immunosuppression. Similarly, tolerance was successfully induced in a swine hemi-face VCA model by use of multiple rounds of donor BM-MSCs and transient immunosuppression but without irradiation or bone marrow transplantation (BMT) (19). However, an alternative, more accessible and plentiful source of MSCs than marrow is adipose tissue (20). Accordingly, Kuo *et al.* (21) investigated donor-derived adipocyte-derived stem cells (ADSCs) for hind limb VCA in rats, reporting successful tolerance induction across a full major histocompatibility complex (MHC) mismatch with multiple doses of ADSCs alongside transient immunosuppression. ADSCs of recipient origin, however, have not been investigated for VCA. Given that human VCA donors are deceased, recipient-origin ADSCs have a major advantage over any form of donor-derived MSCs in that they can be harvested from the recipient and stored in advance of VCA in optimized form.

Syngeneic (from the same animal strain with the same genetic background as the recipient) and autologous (from the recipient animal itself) ADSCs are equivalent in inbred animals with identical genotype. We demonstrate for the first time that syngeneic ADSCs are profoundly tolerogenic for VCA recipients, in that only one round of syngeneic ADSCs is sufficient for tolerance induction across a full MHC mismatch when given in conjunction with transient cyclosporine A (CsA) and anti-lymphocyte globulin (ALG). Additionally, it is demonstrated that VCA tolerance was accompanied by the persistent elevation of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs).

Methods

Animals

Male 8- to 12-week-old donor Brown-Norway (BN, $RT1A^n$) and recipient Lewis rats (LEW, $RT1A^l$), representing a full MHC mismatch, were purchased from the National Laboratory Animal Center, Taiwan. Rats were housed in pyrogen-free conditions under controlled temperature and lighting cycles with water and commercial rat chow freely available at the Chang Gung Memorial Hospital Animal Center. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (Bethesda, MD, USA) and following the Institutional Animal Care and Use Committee protocol authorized by Chang Gung Memorial Hospital, Taiwan.

ADSC preparation

The inguinal fat pad from LEW rats was harvested in a sterile fashion, washed with phosphate-buffered saline, minced and digested with type IV collagenase (Life Technologies, Grand Island, NY, USA) and hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) for 1 h at 37°C. After centrifugation, the supernatant was discarded and the pelleted stromal vascular fraction containing ADSCs was resuspended and plated in stromal medium (low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin) and incubated at 37°C in 5% CO_2 atmosphere. The medium was replaced every 2–3 days, and cells with 80–90% confluence were detached with the use of trypsin/ethylenediaminetetraacetic acid (EDTA) (0.5%) and passed. ADSCs at the fourth passage with 80–90% confluence were detached by light trypsinization and used for surface marker characterization, differentiation assays and *in vitro* immune function assessments. If these cells met the criteria of being MSCs, they were infused into VCA recipients.

ADSC differentiation

For adipogenic differentiation, ADSCs at 80–90% confluence were cultured with low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.5 mmol/L isobutylmethylxanthine, 100 μmol/L indomethacin, 1 μmol/L dexamethasone and 10 μg/mL insulin with medium change every 3 days. After 21 days, cells were fixed with 10% formalin for 30 min, dried