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Bone regeneration in a canine cranial model using allogeneic adipose derived stem cells and coral scaffold

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ABSTRACT

Adipose tissue derived stem cells (ASCs) based therapies for the repair and regeneration of various tissues have been widely investigated recently because of their multilineage potential and self-renewal capability. Our previous study demonstrated that autologous ASCs loaded onto natural coral scaffolds could repair cranial critical-sized defects (CSDs) in a canine model. The objective of this study was to determine whether the use of allogeneic ASCs could heal the same defect without the use of immunosuppressive therapy. The pedigree mismatch, mixed lymphocyte reaction assays (MLRs) and allogeneic skin graft experiments were performed to confirm unrelated ASC donors and recipients. A total of 12 adult Beagle dogs were enrolled in this study and divided into two groups. Bilateral cranial CSDs were created in each animal. The right-side defect was treated with allogeneic ASCs delivered onto a coral scaffold, and the left defect was either filled with an autologous ASC/coral composite (Group 1, $n = 5$) or with one coral scaffold alone (Group 2, $n = 5$). The systematic immune response and bone healing were evaluated postoperatively. The results showed that allogeneic ASC transplantation did not induce a systemic immune response by the hosts, and allogeneic ASCs could repair the cranial CSDs in an analogous way to that of the autologous cells. Moreover, both the green fluorescently labeled allogeneic and autologous ASCs were detected within the lacunae of newly formed bone in the defect site at 24 weeks, illustrating that the grafted ASCs contributed directly to bone regeneration *in vivo*. Thus, we concluded that allogeneic ASCs have the capacity to regenerate bone within craniofacial defects, providing an alternative source of seed cells for bone tissue engineering.

1. Introduction

Repair of large bone defects remains to be a big challenge to craniofacial surgeons. As to the proposed therapeutic approaches of bone defects, tissue engineering has gained universal agreement as a promising alternative to the traditional autologous bone grafts which are still considered as the gold standard in clinic [1]. By delivering osteogenic-induced seed cells combined with degradable biomaterials to bone defects, the efficiency of bone tissue engineering technique has been well demonstrated [2–4].

As a potential seed cell source for tissue engineering, adipose tissue derived stem cells (ASCs) have attracted attention in this field recently because of their capabilities of rapid proliferation and multilineage differentiation [5,6]. The osteogenic capacity of ASCs has been studied extensively both *in vitro* and *in vivo* [5–8]. In combination with proper biomaterials, ASCs have been shown to produce osteoid *in vivo* [9], and regenerate bone tissue in preclinical models [10–12]. Some clinical studies also reported that human ASCs can be used to heal craniofacial bone defects in an autologous setting [13–15].

However, the drive to push these advances from the bench to bedside application of ASCs in regenerative medicine requires not only autologous but also allogeneic cell source, since the procedures of tissue harvesting, cell isolation, expansion and then implantation back to patients are all time-consuming. If the allogeneic ASC-based bone regeneration is equivalent to that of autologous ASCs, the need for harvesting and expanding cells for each patient is eliminated. Recently, human ASCs have been reported to exhibit properties of immune privilege suggesting that

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they can be successfully transplanted from one individual to another [16–19]. *In vitro* studies demonstrated that these properties are maintained even after differentiation along the osteogenic lineage [20]. It is found that allogeneic ASCs can facilitate and accelerate spinal fusion as the syngeneic ASCs do in a rat model, with no significant inflammatory reaction observed [21,22]. However, whether allogeneic ASCs can be used to repair bone defects in large immunocompetent animals remains to be explored.

Our previous study documented the ability of autologous osteo-induced ASCs on a natural coral scaffold to heal the cranial critical-sized defect (CSD) in a canine model [11]. The present study was then designed to investigate the effects of allogeneic ASCs in the same animal model, seeking to address the following three specific questions. First, can allogeneic ASCs repair the canine cranial CSD as the autologous cells did? Second, does integration or elimination of allogeneic ASCs into the bone tissue occur after transplantation? Third, does the transplantation of these allogeneic cells without the use of immunosuppressive therapy provoke an immunological rejection by the host?

2. Materials and methods

2.1. Allogeneic pair determination of experimental animals

A total of twelve healthy adult Beagle canines aging from 12 to 18 months (22.6–27.2 kg) were enrolled in this study, and the experimental protocol was approved by the Animal Care and Experiment Committee of Shanghai JiaoTong University School of Medicine. To determine the possible donor-recipient pairs, a combination of three techniques was carried out. First, the pedigree mismatch was addressed by obtaining donor dogs from one colony (6 males) and choosing recipient animals from a separate colony (6 females). Then mixed lymphocyte reaction assays (MLRs) were performed with randomly-chosen donor-recipient combinations between the two unrelated pedigrees as previously described [23,24]. MLRs represent an *in vitro* model of alloreactivity [23]. When the peripheral blood mononuclear cells (PBMCs) from completely mismatched animals are co-cultured together, a proliferative lymphocyte response will be evoked, indicative of an allogeneic mismatch between the donor and recipient combination. Briefly, PBMCs were isolated from whole blood samples drawn from the recipient (responders) and donor (stimulators) dogs using the density gradient centrifugation method (Ficoll-Pague™ PLUS solution, 1.077 g/mL, Amersham Biosciences, Uppsala, Sweden). Stimulator cells, which had been treated with mitomycin C (50 μg/mL, Sigma Aldrich, St. Louis, MO) for 20 min at 37 °C to prevent cell division, were mixed in a ratio of 1:1 with responder cells in triplicate in 96 well tissue-culture plates (BD Falcon, Franklin Lakes, NJ) at a density of 1×10^5 cells per well. At day 5, the plates were pulsed with 5 Ci/mM 3H-thymidine (3H-TdR; Radiochemical Centre, Amersham, UK). Sixteen hours later, cells were harvested over glass fiber filters and thymidine uptake was quantified in a microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, CT). Results of thymidine incorporation from triplicates were presented as mean counts per minute ± standard deviation (CPM ± SD). The stimulation index (SI), a ratio of allogeneic to autologous proliferation, was calculated by using the following formula: CPM of responder (recipient) lymphocytes incubated with stimulator (donor) lymphocytes/CPM of responder lymphocytes alone. A proliferative response from the responder cells (SI > 3) is indicative of an allogeneic mismatch between the donor and recipient pair [25].

Finally one randomly-chosen donor-recipient pair (#1002 from Pedigree one vs #2002 from Pedigree two) was confirmed by the allogeneic skin graft experiment. Briefly, under general anesthesia, the donor skin was shaved, cleaned and scrubbed using standard aseptic surgical techniques. Then one full-thickness skin graft (about 6 cm^2 in size) was harvested from the donor dog, placed on the recipient flank area, and kept under moist compressive dressing for three days. An autologous skin graft was also used as a control for the recipient. Thereafter the wounds were dressed every other day up to 14 days. Skin grafts were evaluated by gross observation daily and biopsies were performed at serial time points. The eschar formation or the sloughing of epidermis was defined as the criterion for acute skin graft rejection.

2.2. In vitro immunologic characterization of undifferentiated and osteogenic differentiated ASCs

Isolation and *in vitro* expansion of canine ASCs were performed as previously reported [11], and their multilineage potential to differentiate into osteoblasts and adipocytes was confirmed in monolayer culture as previously described [6].

Canine ASCs of passage 3 were divided into two parts. One part was cultured in the basic culture medium (containing low-glucose Dulbecco's modified Eagle's medium (LG-DMEM, Invitrogen, Grand Island, NY), 10% fetal bovine serum (FBS, Invitrogen), 100 μg/mL streptomycin and 100 U/mL penicillin), and the other part was cultured in osteogenic medium containing the basic medium further supplemented with 1α,25-dihydroxy cholecalciferol (vitamin D3, 10 μM), β-glycerophosphate (10 mM) and ascorbic acid (50 μg/L) (all from Sigma Aldrich, St. Louis, MO). The immunologic characterization of ASCs after osteogenic induction for 2 weeks was evaluated by flow cytometry analysis and MLRs. For flow cytometry, osteo-induced and undifferentiated ASCs were screened on a FACScan (Coulter Epics Altra; Becton Dickson, San Jose, CA) using antibodies against canine major histocompatibility complex antigens (MHC I and MHC II) (from Sigma Aldrich). To address whether interferon-γ (IFN-γ) treatment could influence the expression of MHC molecules, osteo-induced and undifferentiated cells were also exposed to IFN-γ (100 U/mL, Sigma Aldrich) for 48 h before flow cytometry analysis.

For mixed lymphocyte culture, mitomycin C (50 μg/mL, Sigma Aldrich) pretreated osteo-differentiated and non-induced canine ASCs of passage 3 (1×10^5) were used as stimulator cells and incubated with an equal number of allogeneic lymphocytes isolated from MHC-mismatched dogs. To test whether ASCs had an effect on the proliferation of lymphocytes in response to alloantigen, responder and stimulator lymphocytes were incubated together, and osteo-induced or non-induced ASCs (administered at 1×10^5, 10^4, 10^3, 10^2 cells/well, respectively) were added on day 0 of co-culture to evaluate their effect on the proliferation of responder cells.

2.3. Preparation of ASC/coral implants

Natural coral scaffolds (Hainan island, China) were fabricated with size of 20 mm × 20 mm × 3 mm and sterilized by autoclaving before use. They have a volume porosity of 57.5 ± 6.5% with pore sizes ranging from 100 to 300 μm [11], and their main component (97–99%) is calcium carbonate [4].

Osteo-induced ASCs at passage 3 were harvested and resuspended in the osteogenic medium at a density of 2×10^7 cells/mL. The cell suspension was slowly pipetted onto the scaffold using a 1000 μL pipetter to draw the suspension bi-directionally three times. After being incubated at 37 °C for 4 h to allow cell attachment, the ASC/coral composites were cultured in 20 mL of osteogenic medium for further 7 days before implantation with the medium changed every 3 days. Coral scaffolds without loading cells but cultured under the same condition for 7 days were used as controls.

To trace the *in vivo* fate of implanted cells, one donor-recipient pair was chosen randomly (#1007 from Pedigree one vs #2004 from Pedigree two), and their defects were repaired with engineered bone using green fluorescent protein (GFP) transfected osteo-induced ASCs seeded onto porous coral scaffolds. GFP-gene transfection was performed as previously reported with minor modification [26]. Firstly, Lenti-X 293T packaging cells were transfected with the empty Lentivirus vector pLVX-shRNA2, together with pMD2G and psPAX2 vector. After 24 h fresh medium were fed. Supernatants containing lentivirus were collected at 48 h, and stored at 4 °C for use. Then subconfluent canine ASCs of passage 2 were cultured in the GFP lentivirus supernatants (10 mL/flask, containing polybrene (4 mg/L, Sigma Aldrich)) overnight. Cells were fed with fresh basic culture medium the next day, and the transduction efficiency was evaluated using fluorescence microscope.

To visualize the cell attachment and spatial distribution on the scaffold, GFP-labeled ASCs were subjected to the confocal laser scanning microscopy (CLSM, Leica Microsystem, Germany) examination and scanning electron microscopy (SEM, Philips XL-30, Amsterdam, Netherlands) examination at day 7 after cell seeding.

2.4. Surgical procedure

Our previous study suggested that a 20 mm × 20 mm full-thickness canine calvarial bone defect fulfills the criteria of CSD with advantages of permitting a pair-designed experiment and avoiding the inclusion of the sagittal suture in the osseous defect [11]. So in the present study the bilateral CSD was created at the parietal bones of each animal. Based on the results of allogeneic pair determination, animals were divided into two groups. The right defect was grafted with one allogeneic ASC/coral composite in each animal, and the opposing defect (left side) was either grafted with one autologous ASC/coral composite (Group 1) or with one coral scaffold alone (Group 2) ($n = 5$ for each group).

2.5. Detection of systemic immunological reactions

To detect the general immune rejection, 5 mL of blood samples were drawn from the jugular vein of each animal at selected time points postoperatively. The ratios of CD4 positive lymphocytes versus CD8 positive lymphocytes (CD4/CD8) were determined using flow cytometry analysis at days 0, 2, 4, 6, 8, 10, 12 and 14. Serum contents of immune cytokines including interleukin 2 (IL-2), IL-4, IL-10, IFN-γ and transforming growth factor β1 (TGF-β1) were measured using ELISA kits according to the manufacturer's protocols (Jiancheng Biotechnology Company, Nanjing, China) at weeks 0, 1, 2, 4, 12 and 24 post-surgery.

2.6. Three-dimensional CT evaluation, gross observation and micro-CT measurement

Under general anesthesia, CT scanning (GE Lightspeed Ultra 16, GE, Milwaukee, WI) was performed for each animal at 12 and 24 weeks post-surgery, and 3D images were reconstructed to evaluate the repairing process of bone defects. After CT