



Evaluation of Canine Adipose-derived Stem Cells in a Healthy Mice Subcutaneous Model

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Abstract

Canine adipose-derived stem cells (cASCs) have great interest for cell-based therapies in Veterinary Medicine. As the behaviour of these cells in non-autologous recipients is not deeply characterized, it is mandatory to study them in new animal models previously to canine specie. In this work, cASCs were injected subcutaneously in mice and these cells were detected by immunohistochemistry using vimentin, CD44 and keratin. The local response evaluated by histology did not reveal signals of significant inflammatory reaction neither in the lymph nodes or other organs. This study showed the implantation of cASCs induced a scarce inflammatory response. Hsd:CD1 (ICR) mouse can be proposed as an animal model to study the *in vivo* behavior of the cASCs and to validate new cASCs-based approaches avoiding or reducing the use of dogs in research.

Keywords: Adipose-derived stem cells; Dog; Xenotransplantation; Host response; Mice model

Introduction

The use of Mesenchymal Stem Cells (MSCs) has been proposed for a wide number of Regenerative Medicine strategies, mainly due to their ability to self-renew and potential to differentiate towards different cell lineages. These cells have been initially isolated from the bone marrow [1] but, since then, it has been reported its existence in many other adult tissues, including the adipose tissue [2,3], placenta, amniotic fluid [4], umbilical cord blood [5] and periodontal ligament or other dental tissues [6,7].

Adipose-derived stem cells (ASCs) have shown remarkable properties with great potential to be used in Tissue Engineering and other cell-based therapies [8], regarding their ability to differentiate into several cellular lineages, easiness of harvesting with low morbidity and discomfort to the patient [8].

The canine adipose-derived stem cells (cASCs) have recently been reported in several studies in Regenerative Medicine with promising results. Concerning clinical studies, cASCs have been used in combination with scaffolds based on hydroxyapatite and chitosan (30:70 wt%) for the treatment of radius and ulna nonunion fracture in dog [9], or in the treatment of dogs with lameness associated with osteoarthritis of the coxofemoral [10] and humeroradial [11,12] joints. Although without effective clinical improvement, cASCs have also been tested in the treatment of canine atopic dermatitis [13].

At a preclinical stage, cASCs have been studied in canine models for the regeneration of intervertebral disc [14] or the inhibition of its degeneration [15], and for the regeneration of cranial bone defects by autologous [16] or allogenic [17] cASCs combined with coral scaffolds. Additionally, cASCs have also been assessed as cellular delivery system of interferon- β gene for cancer therapy using a mouse melanoma model [18].

Despite the increasing interest in this cell source, there is not enough information to enables to assess cASCs in smaller animal models. Cell-based therapies in dogs are quite promising in Veterinary

Medicine, thus, prior to apply these strategies in dogs, it is mandatory to study the behavior of cells, for example cASCs, in animal models such as small laboratory mammals. The transplantation of stem cells in dogs, alone or as part of tissue-engineered constructs, could also be further translated (or be a model) to Regenerative Medicine in humans.

In this work, the cASCs were xenotransplanted in a mouse subcutaneous model without any specific induced disease. The implanted cells were immunodetected throughout the timeline of the experiment and the host response was evaluated. The obtained data contributed to the characterization of mice as model to assess cASCs before their application in veterinary clinical practice.

Materials and Methods

Preparation of the canine adipose-derived stem cells

Surgical harvesting of the canine adipose tissue: Subcutaneous abdominal adipose tissue was harvested from three adult healthy female dogs, between 1 to 3 years old, subjected to elective ovariohysterectomy at the Veterinary Hospital of University of Trás-os-Montes e Alto Douro with previous informed consent of the owners.

Dogs were sedated by an intramuscular administration of 0.2 mg/kg IM butorphanol tartrate (Torbugesic 1%; Fort Dodge, the Netherlands) and 30 μ g/kg IM acepromazine maleate (Vetranquil; CEVA Sante Animal, France). Anaesthesia was achieved by an intravenous

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administration of 0.25 mg/kg IV diazepam (Diazepam MG; Labesfal, Portugal), 4 mg/kg IV ketamine (Imalgene 1000; Merial, France) and 4 mg/kg IV propofol (Lipuro 2%; Braun, Germany), and was maintained with 1% isoflurane (IsoFlo; Abbott Animal Health, USA) administered in oxygen through an endotracheal tube.

After being anesthetized, the dogs were placed in dorsal recumbence and it was made a careful trichotomy and asepsis of the ventral abdomen from the xiphoid to the pubis. Briefly, an incision was made just caudal to the umbilicus in the cranial third of the caudal abdomen through skin. Samples of adipose tissue were collected from the abdominal subcutaneous region, before the perform the ovariohysterectomy.

Finally, linea alba was closed with simple interrupted suture using polyglyconate (Monosyn; BBraun, Portugal), the subcutaneous tissue with a simple continuous suture using the same material, and the skin with a simple interrupted pattern using silk (Silkan, BBraun, Portugal).

Isolation and expansion of the canine ASCs: The subcutaneous adipose tissue samples were processed within 12 hrs upon harvesting, and the canine adipose-derived stem cells (cASCs) were isolated by an enzymatic digestion method, as described previously [2]. Briefly, adipose tissue samples were washed with PBS containing 10% antibiotics/antimycotic and minced into small fragments. The fragments were digested with a solution of 0.1% collagenase type IA (Sigma Aldrich, Germany) in PBS at 37°C under shacking at 200 rpm for 40 min. The digested tissue was filtered with a 100 µm nylon meshe and centrifuged at 1,250 rpm for 5 min at 20°C and the supernatant removed.

The obtained cells were resuspended and expanded in basal medium composed of Dulbecco Modified Eagle Medium (DMEM) (Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA) and 1% antibiotic/antimycotic (amphotericin B/streptomycin sulfate; Sigma, USA) up to passage 2 as reported before [2] in order to obtain the necessary number of cells to use in this study.

Preparation of the canine ASCs suspension: The cASCs were resuspended in phosphate buffer solution (PBS; Sigma Aldrich, Germany), obtaining a cellular suspension of 1×10^7 cells ml^{-1} to be subsequently 100 microliter injected in the mice.

From the same culture, a cellular suspension of 2×10^6 cASCs ml^{-1} was also obtained, washed with PBS and fixed in 4% phosphate-buffered formalin for further cytology and immunostaining assays.

Mice: Thirty five immunocompetent 10 weeks old Hsd: CD1 (ICR), outbred and SPF male mice (Harlan Laboratories, Spain) were used in this protocol. As the objective of the study was to evaluate the inflammatory reaction of mice against xenogenic transplanted cells, therefore the selected animal model had to be immunocompetent.

The housing care and experimental protocol was performed according to the national guidelines, after approval by the National Ethical Committee for Laboratory Animals and conducted in accordance with Portuguese legislation (Portaria 1005/92) and international standards on animal welfare as defined by the European Directive 2010/63/EU.

The animals were housed in groups of five individuals, fed ad libitum with maintenance diet for mice (4RF21/C diet, Mucedola, Italy) and autoclaved water, and bedded in corn cob (Scobis Due, Mucedola, Italy) with suitable environment enrichment and with a day/night cycle of 14/10 hrs.

Subcutaneous injection of the canine ASCs

Mice were placed in dorsal recumbence for subcutaneous injection in the abdominal right flank with 100 µl of suspension containing 1×10^7 cells ml^{-1} by using a 1 ml syringe with a 26 G needle.

Euthanasia and explants collection

All animals were euthanized by an intravenous injection of sodium pentobarbital (Eutasil, CEVA, France). In order to the assess the effect of the injection on the skin and its histology of without the influence of the cASCs, a group of five animals injected with PBS with no cells were, considered the negative control for cASCs and euthanized at 12 hrs. Throughout the following 28 days, the remaining six experimental groups (n=5) were sacrificed at 12 hrs, and 1, 3, 7, 21 and 28 days after injection.

The abdominal skin and adjacent muscle, around the site of injection, were collected with a safety margin of 1 cm, in order to assess the local response against the xenotransplanted cASCs.

The organs, namely, mesenteric and inguinal lymph nodes, liver, kidney, lung, heart, spleen, brain, and intestine were also collected, in order to evaluate the presence of cASCs at distance and also the host response against these cells. All samples were fixed in 4% phosphate-buffered formalin (Inopat, Portugal) during 24 hrs.

Preparation of the canine ASCs' cytoblocks

A cellular culture suspension of 2×10^6 cASCs ml^{-1} was fixed in 4% phosphate-buffered formalin and centrifuged in the Shandon CytoSpin 3 centrifuge (Thermo Scientific, USA) to a cytoblock cassette.

Hematoxylin and eosin

Explants retrieved from mice and the cASCs' cytoblock were processed in an automatic tissue processor Shandon Hypercenter XP (ThermoFisher Scientific, USA) and embedded in paraffin. Paraffin blocks were cut at 3 µm to silane-coated slides and stained by routine with hematoxylin and eosin (H&E) and mounting with Entellan (Merck, Germany).

Immunohistochemistry

Mouse monoclonal antibodies anti-human Vimentin, (clone V9, Leica Biosystems, UK) and anti-human Keratin (clone AE1/AE3, Dako, USA), and rat monoclonal anti-mouse CD44 (clone IM7, Santa Cruz Biotechnology, Germany) (Table 1) were used to perform indirect avidin-biotin immunohistochemistry (IHC) technique in order to characterize the injected cASCs in the host tissue.

Antigen retrieval was performed in a water bath at 96°C for 20 min, in citrate buffer (pH=6). The slides were washed with PBS and

Antibody	Vimentin	Keratin	CD44
Type	Monoclonal	Monoclonal	Monoclonal
Clone	V9	AE1/AE3	IM7
Manufacturer	Leica Biosystems, UK	Dako, USA	Santa Cruz Biotech, Germany
Dilution	0.111111111	0.319444444	0.111111111
Antigen retriever	Sodium citrate, pH 6, 90°C, 20 min	Sodium citrate, pH 6, 90°C, 20 min	
Incubation	Room temperature, 4 hrs	Room temperature, 4 hrs	4°C, overnight

Table 1: Antibodies used in this study.

endogenous peroxidase was blocked with 3% hydrogen peroxide (Sigma, Germany) at room temperature for 30 min.

RTU Vectastain Universal Elite ABC Kit (Vector PK-7200, UK) in combination with the Mouse-on-Mouse (MOM) Basic Kit (Vector BMK-2202, UK) was used for antibody incubation, according to the instructions of the manufacturers, in order to reduce the background and antibody cross reactivity.

The mouse-on-mouse kit aimed to block the endogenous mouse's immunoglobulins was essential to avoid the detection of the studied antibodies in the host tissue, ensuring that only the canine xenotransplanted cells were identified through histological stainings of the explants. Briefly, non-specific binding of primary antibodies was blocked using a horse serum for 5 min, and the mice's immunoglobulins blocked for 1 hour. Then, after an overnight incubation with the primary antibody, at 4°C in a humidified atmosphere, tissue sections were incubated with biotinylated antibody, followed by incubation with streptavidin-peroxidase.

After washing with PBS, antibody detection was revealed using the peroxidase substrate kit DAB (Vector SK-4100, UK). Slides were washed in water for 5 min and then counterstained with Gill's hematoxylin (Sigma, Germany).

The sections of the cASCs' cytoblock were submitted to the same procedure and used as positive control of the immunohistochemistry analysis. Finally, the sections were observed under a light microscope (E600 Nikon Instruments, UK) and the images were obtained using the digital camera Nikon DXM200. The cells were interpreted as positive when revealed brown cytoplasm.

Results

The subcutaneous injections of cells promoted the formation of small papules immediately after the injection of the cellular suspension. A few minutes upon implantation, it was observed the regularization of the skin and no signs of local inflammation were detected after that. In each timepoint, all the animals revealed good body condition and also no clinical signs of inflammation were noticed.

During the samples collection for histological processing, it was observed a certain degree of local skin swelling in first experimental group corresponding to 12 hrs post-transplantation.

Histology

All organs and skin from all animals were evaluated hematoxylin and eosin-stained slides. The organs did not show any significant histology disorder. Histological analysis of the local of injection in the abdominal skin (Figures 1 and 2) revealed normal skin architecture in the negative control group (Figure 1), comprising the animals where no cells were transplanted. On other groups, at 12 hrs, one and seven days after cells inoculation, cellular agglomerates on the dermis, morphologically compatible with the injected cells were observed in 60%, 20% and 20% of the animals, respectively. In the later time points, these cells were no longer perceived.

Around the clusters it was observed a mild inflammatory infiltrate, mainly at 12 hrs, comprising mostly lymphocytes and macrophages that decreased along the experiment. At the 7th day of inoculation, a central necrosis with macrophages was observed inside the clusters.

Moreover, the histological observation of the organs did not reveal any inflammatory, degenerative or necrotic reaction which could be associated to the xenotransplanted cells. Local and regional

lymph nodes presented normal morphology with brown pigment (hemosiderin) in some cases.

Immunohistochemistry

The skin samples were submitted to IHC in order to identify if the cellular agglomerates match with cASCs according to their phenotype.

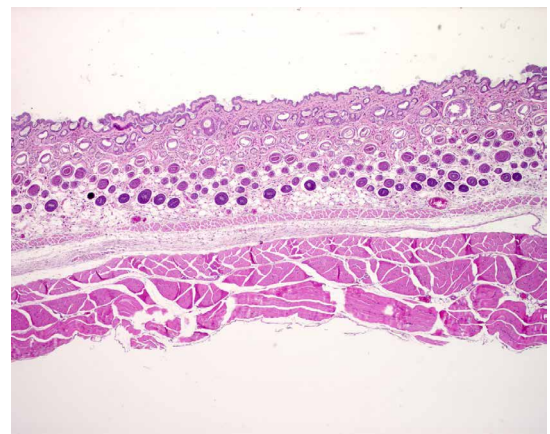


Figure 1: Histological image of the skin from control animal. Sample was obtained from the correspondent site of the inoculated PBS. Hematoxylin and eosin.

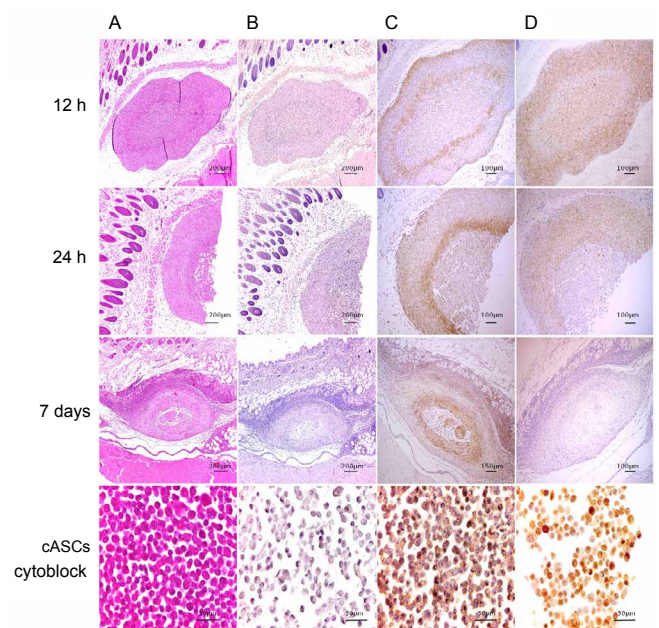


Figure 2: Histological images of the clusters present on the dermis and the cASCs' cytoblock. Observe the histology stained with hematoxylin and eosin at different times on the column (A). Note that the cASCs cluster is solid at 12 hrs and progressively necrotic in the centre of the cells. The column (B) presented the same tissues on the column (A) immunostained with antibodies against large spectrum keratin which did not labeled the cluster. (C) The same tissues on the column (A) marked with antibody against vimentin. The positive cells are diffused in the cluster with higher density inside. (D) Same tissues as in the column (A) to (C) labeled with CD44 antibody. Almost all cells present inside the cluster are positive to this marker.

The cytoblock comprises the same cells inoculated in mice and the immunohistochemistry were performed as in the skin. Observe the negative stain to keratins and strong positivity to vimentin and CD44. In the immunohistochemistry, all slides were counterstained with Gill's hematoxylin.

As shown in Figures 2A-2D, those cells were positive both to vimentin and CD44 and negative to keratins overlapping the cASCs' cytochrome positivity used as control. The intensity was strongest in the cytochrome cells then in the skin that shows different intensity for the markers used that also varied along the experimental period.

Discussion

The cASCs used in this study were isolated from dog subcutaneous tissue by a previously optimized methodology [2]. These cells display properties of MSCs namely adherence to culture flasks, positivity to the three main characteristic MSCs markers, namely, CD105, CD73 and CD90, as well as the potential to differentiate into osteogenic and chondrogenic lineages [2], fulfilling the minimal criteria for been considered MSCs [19].

Although different cell tracking methodologies could have been implemented in this study, we were interested in assessing the implanted cells in its pure form, as they are envisioned to be used in future clinical applications and, therefore without any permeabilization steps or using any type of cell surface modification such as fluorochromes, or genetic modifications such as transfection. Taking this into consideration we decided try to detect cASCs in tissues according to their phenotype, through the positive expression of vimentin and CD44, and the negative expression of keratins.

Vimentin and keratin are cytoskeleton intermediate filaments. MSCs are known to contain vimentin [20] which is involved in modulating cell-matrix interactions [21] and it has already been detected in cASCs after inducing neurogenic differentiation [22] and was highly expressed in MSCs isolated from rodent skin [23].

The antibody against large spectrum keratin used in the present work is a combination of acidic and neutral-basic keratins, thus covering broad spectra of this protein and, in that sense, minimizing the probability of false negatives for keratins' expression on the injected cells. In human ASCs committed to the epithelial lineage, cytokeratin-18 has been detected, while vimentin reduced its positive expression [24].

The CD44 is a membrane glycoprotein and functions as the major hyaluronan receptor on most cell types [25], and is one of primary stable positive ASCs' markers [26]. The expression of this marker on cASCs has already been shown by Kang (2008) who found immunomodulatory behaviour on co-cultured with allogenic leukocyte [27]. Others studies on cASCs revealed the positive expression of CD44 by flow cytometry and immunocytochemistry [22,28,29], however, in our knowledge, the detection of this marker in cASCs after xenotransplantation in an *in vivo* model was not yet been reported.

Several mouse models of disease, such as muscular dystrophy [30], colitis [31], rheumatoid arthritis [32], acute kidney injury [33], autoimmune hearing loss [34] and systemic lupus erythematosus [35] have already been used in the study of the human ASCs behavior and therapeutic application.

In this work, it was aimed to assess the response of an immunocompetent and healthy model against transplanted cells. This absence of disease and chemotactic stimuli in mice could justify that no inflammatory reaction was observed in the organs distant from the injection site.

The presence of the cASCs in the subcutaneous tissue could be related to the physical trauma, induced during the inoculation, which could have mimetized a disease model and, thus, promote the only a

mild inflammatory response and the formation of the cluster of cells [36].

Apart from the subcutaneous injection, other ways of inoculation have been reported in different disease models or clinical studies, namely, the intraarticular [10,11], the intravenous injection [13] or the direct injection into a spinal cord injury [28]. Transplantation of cASCs have been described to be carried out by combining them with different vehicles such as platelet rich plasma [12], hyaluronic acid [12] or PBS [11].

The immunomodulation of the ASCs has been examined in a variety of animal models [36]. One of the first *in vivo* studies on systemic infusion of *ex-vivo* expanded allogenic ASCs dates of 2006, when Yanez and colleagues reported that these cells were able to control an induced graft-versus-host disease in mice [37]. Human ASCs have been shown not to express the MHC-II and did not stimulate allogenic peripheral blood mononuclear cells when in co-culture [38]. The immunomodulatory potential of the studied cASCs needs to be deeply assessed, paving the way for the application of cASCs in medicine regenerative approaches not only in the donor patient but also in allogenic patients.

The present work also gave a contribution to the study of cASCs behavior in a non-autologous host. Envisioning the use of cASCs in allogenic therapies, it is important to assess their behavior also in canine recipients in the future. Taking into consideration that long-term cryopreservation of cASCs did not affect their stem-like features [39], it is feasible to create a cell bank of cASCs ready to use in autologous or allogenic approaches.

The present study demonstrated, for the first time in our knowledge, the presence of cASCs in healthy mice tissue after transplantation. Moreover, the obtained data showed the cASCs induced a local mild inflammatory reaction in the host. The scarce inflammatory response against the cASCs could also be useful to propose this mouse as an animal model to study the *in vivo* behavior of the cASCs and to validate cASCs-based therapies avoiding or reducing the use of dogs in research.

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