Flow cytometric immunophenotyping of canine adipose-derived mesenchymal stem cells (ADMSCs) and feline ADMSCs using anti-human antibodies

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Abstract: Various trials have been conducted to develop therapies for serious untreatable diseases. Among these, those using stem cells have shown great promise, and adipose-derived mesenchymal stem cells (ADMSCs) are easier to obtain than other types of stem cells. Prior to clinical trials, characterization of ADMSCs with monoclonal antibodies should be performed. However, it is difficult to use species-specific antibodies for veterinarians. This study was conducted to confirm the panel of human antibodies applicable for use in immunophenotypic characterization of canine adipose-derived stem cells and feline ADMSCs extracted from subcutaneous adipose tissue collected during ovariohysterectomy. For flow cytometric immunophenotyping, the third passages of canine ADMSC and feline ADMSC and human CD31, CD34, CD42, CD44, CD62 and CD133 antibodies were used. Of these, CD133 reacted with canine cells (3.74%) and feline cells (1.34%). CD133 is known as a marker related with more primitive stem cell phenotype than other CD series. Because this human CD133 was not a species-specific antibody, accurate percentages of immunoreactivity were not confirmed. Nevertheless, the results of this study confirmed human CD133 as a meaningful marker in canine and feline ADMSCs.

Keywords: adipose-derived mesenchymal stem cell, cats, dogs, flow cytometric immunophenotyping, human antibodies

Introduction

Mesenchymal stem cells (MSCs) are defined as multipotent cells that have ability of self-renewal, long-term viability, and multilineage potential. MSCs can regenerate into bone, cartilage, muscle, tendon, ligament, adipose, and stroma *in vitro* and *in vivo*. Because of these potencies of MSCs, they have been used for human and veterinary treatments [9].

MSCs have various origins, including the bone marrow, umbilical cord tissue, adipose tissue, tooth birth and peripheral blood [2]. When compared to other cell types, MSCs are abundant in adipose tissue, which the number of MSCs in adipose tissue have been shown to be up to 300 times than in bone marrow [9]. Adipose-derived MSCs (ADMSCs) also have the advantage of easy access and rapid expansion *in vitro* [3].

Although there have been many studies of the therapeutic uses of ADMSCs in dogs and horses [9], few studies have investigated the use of ADMSCs in cats [10, 15], despite the

high number of cats as companion animals.

Immunophenotyping is generally used to characterize ADMSCs in different animal species. However, immunophenotyping has been limited in veterinary medicine because of the lack of antibodies for the surface profile in some of the species [9]. To overcome these limitations, cross-reactive antibodies have been used; however, few studies have been conducted using cross-reactive antibodies [5]. Therefore, in this study, flow cytometric immunophenotyping of canine adipose and feline ADMSCs was conducted using anti-human antibodies.

Materials and Methods

Animals

Two beagle intact female dogs and two mixed breed intact female cats were used. Animals were determined to be healthy by physical examination. All animals underwent rou-

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tine ovariectomy, under the situation of sedation with midazolam (0.1 mg/kg) and acepromazine (0.03 mg/kg) and then anesthetization with isoflurane (0.25–3% isoflurane in 100% oxygen in an induction chamber). During ovariectomy, 2–4 g of subcutaneous fat in ventral midline abdomen of each animal was obtained [10]. This study was approved by the Kangwon National University Institutional Care and Animal Use Committee (KW-160809-1).

Isolation and expansion of ADMSCs

Subcutaneous fat (2–4 g) in ventral midline abdomen of each animal was collected in phosphate buffered saline (PBS; Gibco, USA) during ovariectomy. Each tissue sample was cut into small pieces and incubated in 0.075% of Collagenase I (Gibco) in HBSS (Sigma-Aldrich, USA) for 1 h at 37°C under 5% CO₂. After then the suspension including cells was neutralized with 10% fetal bovine serum (FBS; Gibco) and then centrifuged at $1,200 \times g$ for 10 min at room temperature. The supernatant was then eliminated and the sediment was mixed with 5 mL RBC lysis buffer (Sigma-Aldrich) for 10 min. Next, the suspension including cells got out a 100 μ m cell strainer (Falcon; Corning Life Sciences, USA) to remove excess tissue stroma and diluted with 10 mL PBS, after which it was centrifuged at $1,200 \times g$ for 10 min at room temperature. The supernatant was then eliminated and the sediment was resuspended in 5 mL Dulbecco's Modified

Eagle Medium (Gibco) with 5% FBS and plated in T-25 plastic tissue culture flasks. After the confluency of cells reached 80%, cells were detached with 0.05% trypsin (Gibco). Then the cells were resuspended in 20 mL of medium and split into 2 T-75 plastic tissue culture flasks. After this, all cell cultures were passaged along the similar lines until the third passage of cells was available for characterization [10].

Flow cytometric immunophenotyping

Flow cytometry analysis was conducted to analyze the phenotypes of ADMSCs. Briefly, ADMSCs derived from dogs or cats were collected, washed with PBS, and stained for 1 h with antibodies. The specific antibodies used were phycoerythrin-conjugated mouse anti-human CD31, fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD42b, FITC-conjugated mouse anti-human CD44, FITC-conjugated mouse anti-human CD62b, FITC-conjugated mouse anti-human CD133 (BD, USA), and FITC-conjugated mouse anti-human CD34 (Santa Cruz Biotechnology, USA). The labeled ADMSCs were analyzed using a FACScalibur (BD Bioscience, USA), after which the data were analyzed with a FACS II flow cytometer (BD Biosciences).

Results

The cells of the third passage in each dogs and cats dis-

Fig. 1. The third passages of adipose-derived mesenchymal stem cells in dogs (A) and cats (B). 50 $\times$ (A and B) and 100 $\times$ (insert).

Fig. 2. Forward scatter vs. side scatter plots of adipose-derived mesenchymal stem cells (ADMSCs) in dogs (A) and cats (B). One cellular subset was identified in each ADMSC of dogs and cats with forward scatter (FSC-H)/side scatter (SSC-H) dot plots.

Fig. 3. Reactivity of the monoclonal antibodies in dogs. X-axis indicates the fluorescence intensity (FL1-FITC; FITC, fluorescein isothiocyanate) and Y-axis indicates the fluorescence intensity (FL2-PE; PE, phycoerythrin). This results revealed that the cells had cross-reactivity 0.15%, 0.1%, 0.07%, 0.73%, 0.38% and 3.74% with CD31 (A), CD34 (B), CD42 (C), CD44 (D), CD62 (E), and CD133 (F).

played the exacted MSC phenotype. The morphology of ADMSCs in dogs was more unified than in cats (Fig. 1).

After labelling ADMSCs with monoclonal antibodies, the scatter-plot profile was determined by displaying as the forward scatter (FSC-H) vs. side scatter (SSC-H). One cellular subset was identified in each ADMSC of dogs and cats (Fig. 2). The results revealed that each set of cultured cells was of one type. To test the cross-reactivity, six monoclonal antibodies against various CD molecules were tested in each ADMSC. Flow cytometric analysis of dog ADMSCs revealed that the cells had cross-reactivity 0.73% with CD44 and 3.74% with CD133, while it was 0.15%, 0.1%, 0.07% and 0.38% for CD31, CD34, CD42, and CD62, respectively (Fig. 3). Flow cytometric analysis of cat ADMSCs revealed that the cells

had cross-reactivity or 0.62%, 0.68%, 1.34%, 0.05%, 0.07% and 0.12% with CD44, CD62 CD133, CD31, CD34, and CD42, respectively (Fig. 4).

Discussion

A great deal of attention has been focused on ADMSCs due to their potential ability as cell therapy products. In human medicine, beyond the basic study of ADMSCs, the massive production and banking of clinical applicable phase cells, issues related with legal regulations, of cell therapy in different countries currently registered are being aligned. [8, 9]. These phenomena are not relevant to human medicine, but also apply to veterinary medicine. In equine veterinary

Fig. 4. Reactivity of the monoclonal antibodies in cats. X-axis indicates the fluorescence intensity (FL1-FITC) and Y-axis indicates the fluorescence intensity (FL2-PE). This results revealed that the cells had cross-reactivity 0.05%, 0.07%, 0.12%, 0.62%, 0.68% and 1.34%, with CD31 (A), CD34 (B), CD42 (C), CD44 (D), CD62 (E), and CD133 (F).

medicine, clinical trials of ADMSCs have focused on tendon regeneration, while in small animal veterinary medicine, trials have focused on osteoarthritis and atopic dermatitis [1].

In human medicine, ADMSCs are confirmed based on cells being positive for CD13, CD73, CD90 and CD105 and negative for CD31, CD45, and CD235a upon immunophenotyping [4]. Despite the availability of ADMSCs, the use of species-specific antibodies in veterinary medicine is limited. Indeed, there have been few veterinary studies of immunophenotyping of ADMSCs. A recent study of immunophenotype showed that canine ADMSCs are positive for CD44, CD90 and MHC I, but negative for CD14, CD20 and MHC II [14]. Equine ADMSCs were shown to be positive for CD29, CD105, CD44, CD90, CD140b, and CD164 and negative for CD34, CD45, CD73 and MHC II [11]. Interestingly, other groups obtained conflicting results, with one

group finding equine ADMSCs to be negative for CD44 and CD105 and the other group finding them to be both positive and negative for CD90 and CD34 [12, 13]. In a study of feline ADMSCs, immunophenotyping showed that the cells were positive for CD44, CD90, and CD105, but negative for CD14, CD34 and CD45 [7]. As mentioned above, the opinions regarding immunophenotyping of ADMSCs in veterinary medicine are controversial. This is especially true in feline medicine because very few studies have investigated feline ADMSCs.

In this study, to test the possibility of other CDs, human CD31, CD34, CD42, CD44, CD62 and CD133 antibodies were analyzed by flow cytometric immunophenotyping. Among these, CD133 reacted with canine cells (3.74%) and feline cells (1.34%). CD133 positive cells are considered to have ability of early progenitors as self-renewing and differentiat-

ing into other cell types. Therefore, CD133 appears to be a marker of primitive stem cell phenotype [6].

The human CD133 in this study was not species-specific; therefore, percentage was not confirmed. Nevertheless, the results of this study confirmed that CD133 is a meaningful marker associated with primate stem cells in canine and feline ADMSCs.

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