Dokkyo Journal of Medical Sciences
41(1) : 7～12, 2014

Original

Feasibility of Exploiting Celution™ System in Autologous Cell Therapy in Dokkyo Medical University Hospital : Safety and Reproducibility

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SUMMARY

In 2012, we established the Center for Regenerative Medicine in Dokkyo Medical University Hospital, and are now preparing the necessary equipment and preclinical evidence for cell therapy. Liposuction is a commonly used procedure in plastic surgery and the lipoaspirate is discarded as a medical waste. However, the lipoaspirate is known to contain abundant mesenchymal stem cells, and thus, it is currently one of the most feasible options of regenerative medicine. Several ongoing clinical trials of cell therapy (in Japan and overseas as well) are based on the Celution™ system, an automated cell-processing machine utilizing lipoaspirate. The merit of using such a machine is that collected cells are so abundant that it is not necessary to expand the cell number in a Cell Processing Center. Moreover, there are fewer risks of bacterial infection because the system is isolated from the ambient dust and operated automatically. Our group is now planning respective cell therapies for breast reconstruction after mastectomy, urinary incontinence and ischemic cardiovascular diseases, and all these protocols will be based on the Celution™ system. Here, we report the initial test run of Celution™ to confirm its safety and reproducibility.

Key Words : regenerative medicine, autologous cell therapy, Celution™ System, Mesenchymal Stem Cell

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent precursor cells that differentiate into mesenchymal lineages such as osteocytes, chondrocytes or adipocytes[1,2]. MSCs also facilitate angiogenesis and wound healing through secretion of multiple growth factors and cytokines[3,4]. For historical reasons, bone marrow has been considered to be the best cell source for MSC

Received October 9, 2013 ; accepted November 8, 2013
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therapy[1,2]. However, bone marrow aspirate does not contain enough cells and the small amount of MSCs need to be expanded in cell culture. To clinically use bone marrow-derived MSCs, a Cell Processing Center (CPC) is required to expand the cell numbers. A CPC is also critical to prevent the bacterial contamination during expansion. Thus, securing sufficient MSCs is a key issue for clinical practice.

In the plastic or cosmetic surgery field, lipoaspirate is routinely discarded as a medical waste. The lipoaspirate contains not only mature adipocytes, but also abundant stromal cells such as capillary endothelial cells and pericytes. The discovery of MSCs in fat tissue provided the impetus for scientists to direct their attention to lipoaspirate, as an alternative MSC source[5~7]. Compared to bone marrow aspirate, lipoaspirate contains sufficient number of MSCs and cell expansion by culture is not necessary.

From a clinical perspective, as much automation as possible is relevant as complicated procedures can lead to the risk of human error. In 2012, we established the Center for Regenerative Medicine in Dokkyo Medical University Hospital to promote regenerative therapy for breast reconstruction after mastectomy, urinary incontinence and ischemic cardiovascular diseases as a multi-disciplinary project. Our primary goal is to make cell therapy available in our hospital. For that purpose, we chose autologous (derived from the same individual) cell therapy using lipoaspirate as one of candidate strategies.

The Celution™ system (Cytori Therapeutics Inc., San Diego, CA, USA) is an automated cell-processing machine, which originated from a team led by a plastic surgeon in California[5,7]. The Celution™ system efficiently removes mature adipocytes from lipoaspirate and separates stromal cell fraction in the closed system. Processing is complete within two hours, in which the stromal cells are isolated for same day transplantation subsequently in patients. However, in order to use this machine in subsequent cell therapy, strict adherence to the guidelines is essential. In Japan, all the cell therapy protocols and the preclinical data must be submitted to and approved by the Ministry of Health, Labor and Welfare (Guidelines on clinical research using human stem cells, abbreviated in Japanese as HITOKAN : http://www.mhlw.go.jp/bunya/kenkou/iryousaisei.html). It is especially important to ensure safety and reproducibility by using automated cell separation system. Here we carried out an initial test run using Celution™ system and collected data for future submission to the Ministry of Health, Labor and Welfare.

METHODS

Patient and Procedure

This project was approved by an ethical committee of Dokkyo Medical University Hospital. The informed consent to use lipoaspirate for research purpose was obtained from the patient. The patient was a 65 year-old female. Previously her right breast was reconstructed because of imbalance after mastectomy for breast cancer. This time residual subcutaneous fat in bilateral chest was removed by liposuction under general anesthesia.

Test Run of Celution™ System

We isolated adipose-derived stromal cells using the Celution™ system. The process is as below. Briefly, the above lipoaspirate was washed by pre-warmed Ringer's solution and digested by Celase™ at room temperature. Celase™ is an enzyme cocktail (including collagenase) that is optimized to degrade extracellular matrix. Mature adipocytes were subsequently removed by centrifugation and stromal cell fraction was separated and washed. All of these procedures were done in closed container to be free from ambient dust.

Treatment of Adipose-Derived Stromal Cells

The residual red blood cells were hemolysed by incubating with hypotonic solution (2.06 g/l Tris base, 7.49 g/l ammonium chloride, pH 7.2) for 5 minutes at room temperature. After quenching by Hank's buffered salt solution (Life Technologies, Carsbad, CA, USA), cell debris was removed by 100 μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). Number of live cells (unstained by trypan blue) was subsequently counted with hemocytometer.

Contamination Check

Supernatant of collected cells was divided into three vials and contamination was checked. The absence of bacteria, fungi or mycoplasma was confirmed by re-

spective culture test, level of endotoxin, and DNA.

Analysis of Cell Surface Antigen by Flow Cytometry

90% of dispersed cells obtained were used for optimization and flow cytometric analysis. Antibodies used were as follows : CD45-PerCP (BD Biosciences, #340953), CD31-PE (Beckman Coulter, Brea, CA, USA, #IM2409), CD34-FITC (BD, #348053), CD90-FITC (Beckman Coulter, #IM1839U). Triple staining of PerCP, PE and FITC were done for each combination of antibodies and data were collected and analyzed by FACS Calibur and Cell Quest (BD Biosciences).

Cell Culture and Differentiation Assay

The remaining 10% of dispersed cells were cultured in standard cell culture condition (37°C, 5% CO_2, advanced DMEM-F-12, 5% fetal bovine serum, Life Technologies). Cells were divided into two groups : floating cells (e.g. leukocytes) and adherent cells. The group of adherent cells consisted of endothelial cells and mesenchymal stem cells but the endothelial cells barely proliferate and are eliminated by serial passages (subculture of a small portion in a new dish)[6]. After two passages, cells were split into three different dishes : the dish for long time culture, that for osteoblast differentiation assay, and that for adipocyte differentiation assay. In the long time culture, cells were maintained to be kept quiescent under the contact inhibition and culture medium was replaced (5% serum) twice a week. In the differentiation assays, mediums were prepared separately in each of differentiation conditions for osteoblast and adipocyte, and the medium was replaced twice a week. The constituents of each medium were advanced DMEM/F-12 (5% serum or serum free), 100 nM dexamethasone, 10 mM β-glycerophosphat and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA) for osteocyte differentiation, and advanced DMEM/F-12 (serum-free), 500 nM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, 10 μg/ml insulin, 100 μM indomethacin for adipocyte differentiation[6]. Three weeks after differentiation assay process, cells were fixed by 10% formaldehyde, and osteocyte calcification and lipid droplets were stained using alizarin red and oil red staining solution (Wako Pure Chemical, Osaka, Japan), respectively. For a positive control of osteoblast differentiation, non-transformed human fibroblasts (OUMS-36, #JCRB1006.0) purchased from JCRB cell bank (Osaka, Japan) were used.

RESULTS

Operation of Celution^{TM} System

Disposable canister and tubes were properly connected to the Celution^{TM} system machine and the lipoaspirate was successfully poured into the canister. The machine automatically washed the tissue and weighed the amount. The starting weight of the subcutaneous fat was 330 g. Then the machine enzymatically digested extracellular matrix, following the removal of mature adipocytes by centrifugation. Collected cells are known to be adipose-derived stromal cells, or coined as Adipose-Derived Regenerative Cells (ADRCs). ADRCs were a mixture of various kinds of cells such as lymphocytes, endothelial cells or mesenchymal stem cells. Clinically ADRCs are known to be ready for transplantation to the patient but this time all of the cells were used for subsequent quality check.

Bacterial Contamination

Before collecting the ADRCs, supernatant of Ringer's solution was poured into three vials. From these supernatant vials for bacterial, fungus or mycoplasma contamination check, there was no evidence of contamination of these organisms, resulting in the reconfirmation of the previous results in other facilities.

Dispersion of ADRCs and Live Cell Counting

After removal of residual red blood cells by hemolysis, the number of mononuclear live cells was around 1.5×10^7.

Analysis of Cell Surface Antigen by Flow Cytometry

We analyzed the following cell surface antigens for the collected ADRCs : CD45 ; a marker of leukocytes, CD31 ; a marker of endothelial cells, CD34 ; a marker of various progenitor cells including adipose precursor cells, and endothelial and smooth muscle precursor cells and CD90 ; a marker of MSCs. According to the literature, MSCs exist in CD45/CD31 double negative fraction[6]. Moreover, at least in adipose tissue, cell sur-

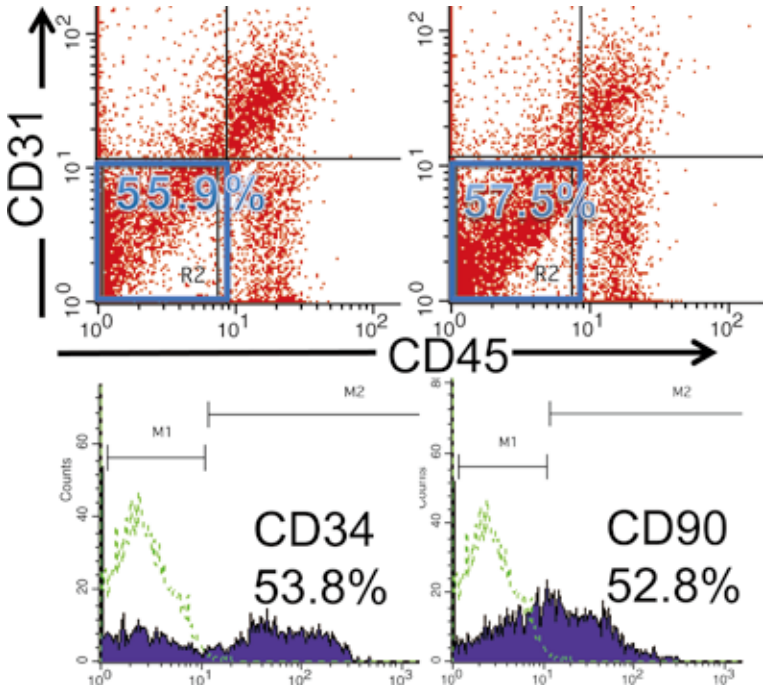

Figure 1 Flow cytomeric analysis for ADRC typing by cell surface antigens. ADRCs were triple-stained by anti-CD45-PerCP (leukocytes marker), anti-CD31-PE (endothelial cell marker) and anti-CD34-FITC (adipose progenitor marker) or anti-CD90-FITC (mesenchymal stem cell marker). CD45/CD31 double negative fraction was gated (upper, light blue rectangles, the ratio among live cells is depicted as percentage) for CD34 and CD90 analyses. The ratio of CD34 positive (lower left) and CD90 positive (lower rght) cell were 53.8% and 52.8%, respectively, in the CD45/CD31 double negative fraction. Dotted line in light green indicates isotype control signal.

face CD34 and CD90 are mostly overlapping in MSCs[8]. Therefore, we first gated cells with CD45 and CD31 and next analyzed the positivity of CD34 or CD90 in the limited fraction of CD45/CD31 double negative. As a result, 30.1% and 30.4% of total live cells were $CD45^{-}CD31^{-}CD34^{+}$ and $CD45^{-}CD31^{-}CD90^{+}$, respectively (data not shown). However, 53.8 % was CD34 positive cells and 52.8 % was CD90 positive in the limited cells that belong in CD45/CD31 double negative fraction (Figure 1).

Cell Culture and Differentiation Assay

A portion of the collected ADRCs was expanded by cell culture assay. The ADRCs actively proliferated in standard culture condition but stopped under the contact inhibition. Since the patient once had breast cancer, we investigated transformation of ADRCs into cancer cells in the long-term culture. As a result, no evidence of such a transformation was observed during 160 days cell culture. In the culture with adipocyte differentiation condition, the ADRCs actively differentiated into adipocytes that displayed massive synthesis of lipid droplets. In addition, in the appropriate culture condition, ADRCs differentiated into calcified osteocytes (Figure 2a). Osteoblast differentiation of ADRCs, however, was dependent of serum in the culture medium. Without serum, ADRCs very rarely differentiated into osteocytes and instead, lipid droplets were synthesized even in the presence of the osteoblast differentiation stimuli (data not shown). As a positive control, human embryonic fibroblast OUMS-36 intensively calcified in the culture with osteoblast differentiation, with or without the serum (Figure 2b and data not shown). OUMS-36 did not synthesize lipid droplets in any culture conditions (Figure 2d and data not shown). These data indicate that ADRCs contain substantial amount of adipose progenitors and some cells have potential to differentiate into osteocytes.

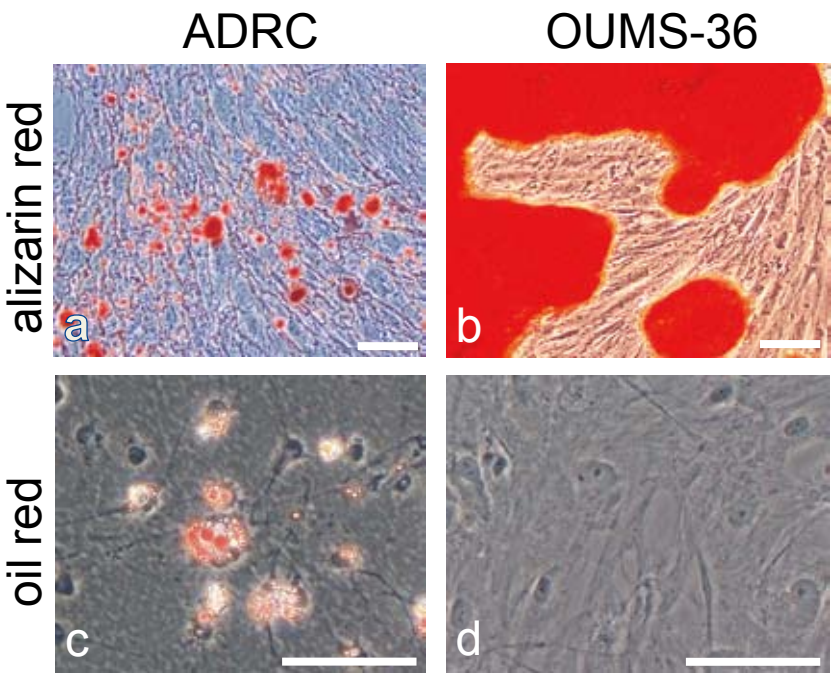

Figure 2 Osteoblast and adipocyte differentiation assay. Cultured ADRCs and human embryonic fibroblasts OUMS-36 was stimulated for osteoblast (**a**, **b**) or adipocyte (**c**, **d**) differentiation. Calcification of osteocytes and lipid droplets were visualized with alizarin red (**a**, **b**) and oil red (**c**, **d**) staining, respectively. ADRCs actively differentiated into adipocytes that displayed massive synthesis of lipid droplets (**c**). Although less intense than positive control, ADRCs differentiated into calcified osteocytes (**a**). OUMS-36 calcified in the culture with osteoblast differentiation, even in the absence of serum (**b**). The OUMS-36 could not differentiate into adipocytes (**d**). Scale bars : 200 μm.

DISCUSSION

The current study reports our bench evaluation of the Celution™ system, which prepares ADRCs in an automatic and closed system. We confirmed the absence of any bacterial contamination, consistent with the manufacturers reports (Cytori Therapeutics Inc.). When we use cultured cells, such as inducible Pluripotent Stem (iPS) cells or bone marrow derived stem cells, for regenerative medicine, a Cell Processing Center is required to prevent bacterial contamination. Although our hospital does not have such a facility, the same or a superior level of safety could be achieved when we use the Celution™ system. We also confirmed that ADRCs have substantial numbers of CD34 or CD90 positive cells. According to a report by Cytori Therapeutics, the ratio of CD34 positive cells/total live cells is around 38%[9]. In our test run, the percentage was 30.1, suggesting that the result was within the range of individual difference. In addition, the ADRCs successfully differentiated into adipocytes and osteocytes that displayed lipid droplets and calcification, respectively. Of note, one of the minimal criteria of mesenchymal 'stem' cells is multipotency to differentiate into both osteocytes and adipocyte[10,11]. In our case, however, the evidence is not enough to conclude that ADRCs contain authentic 'stem' cells. For example, heterogeneous ADRCs might contain mutually exclusive adipocyte progenitor and osteoblast. Nevertheless, from the clinical perspective, the potency of ADRCs (even though they might not be genuine 'stem' cells) to secrete multiple growth factors and cytokines seems very interesting. Since the efficacy of ADRCs depends on such secretory function, rather than differentiation potency *per se*[3,4], we are now preparing a measurement of various growth factors and cytokines secreted in the culture medium, using a multiple biomarker assay system (Luminex microbeads array system, Lu-

minex Corporation, Austin, TX, USA).

Overall, our data of initial test run of the Celution™ system indicate that using lipoaspirate and this system is a feasible option for cell therapy in terms of safety and reproducibility.

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