Chapter 4
Adipose-Derived Stem Cells

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Abstract Human adipose tissue has been shown to contain a population of cells that possesses extensive proliferative capacity and the ability to differentiate into multiple cell lineages. These cells are referred to as adipose tissue-derived stem cells (ADSCs) and are generally similar, though not identical, to mesenchymal stem cells (also referred to as marrow stromal cells). ADSCs for research are most conveniently extracted from tissue removed during an elective cosmetic liposuction procedure but may also be obtained from resected adipose tissue. This chapter describes surgical procedures associated with improved ADSC recovery and the processes by which aspirated adipose tissue is washed and digested with collagenase to yield a heterogeneous population from which ADSCs can be expanded. The large volume of tissue obtained from a liposuction procedure (average ~2L), combined with the relatively high frequency of ADSC within the digestate, yields substantially more stem cells than can be realized from marrow without extensive expansion in culture.

Keywords Adipose tissue; mesenchymal stem cell; adipose tissue-derived stem cell; liposuction; CFU-F.

1 Introduction

The term mesenchymal stem cell refers to the plastic adherent adult stem/progenitor cells from bone marrow originally referred to as fibroblastoid colony forming units, then in the hematological literature as marrow stromal, subsequently as mesenchymal stem cells, and most recently as multipotent mesenchymal stromal cells (MSCs). However, although MSC are the best-described population of cells that exhibit extensive proliferative capacity and the ability to generate progeny of the connective tissue lineages (bone, cartilage, tendon, fat, etc), other cells have been shown to exhibit a similar phenotype. In 2001 Zuk et al. described multilineage differentiation from a population of cells derived by enzymatic digestion of human adipose tissue (1). This work was followed by studies using clonally derived populations
demonstrating that multilineage differentiation was the property of single cells within the population (2); an observation that has now been confirmed in both human (3) and murine (4) adipose tissue-derived cells. These adipose tissue-derived stem cells (ADSC; also referred to as adipose-derived adult stem cells (5), adipose-derived stromal cells (6), and human multipotent adipose-derived stem cells (7,8)) are characterized by extensive proliferative capacity and multilineage differentiation. In our hands and those of others, ADSC express CD105/endoglin, CD44, CD90/Thy1, and SH2 but are negative for expression of CD45 and CD31 (2,6,9). However, differences in the expression of certain surface molecules including VCAM-1 and VLA-4 have been reported (9) as have differences in the ability to differentiate towards the osteogenic and chondrogenic lineages (10,11) and in basal gene expression (12). Further, we have reported that ADSC are less stringent than MSC in their requirement for prescreened lots of sera for their growth (1,2). This may reflect the relative frequency of the stem cells within the starting population; MSC, as measured by clonogenic cell assays are generally reported at a frequency of between 1 in 50,000 and 1 in 1 million in the marrow of skeletally mature adults (13–17) whereas digestion of adult human adipose tissue releases buoyant adipocytes yielding an ADSC frequency in the nonbuoyant fraction of between 1 in 1,000 and 1 in 30 (see the following) (18,19).

The relative frequency of clonogenic cells is one reason for interest in adipose tissue as a stem cell source. Another is the relative ease and low morbidity with which adipose tissue can be harvested. The American Society for Aesthetic Plastic Surgery reported that 478,251 people underwent cosmetic liposuction in the USA during 2004 (20). This represents an enormous volume of immediately available tissue that can be obtained following a very simple informed consent process, without risk to the donor provided that due attention is paid to protecting privacy. The purpose of the present chapter is to describe the methods by which this material can be processed to yield ADSC for research purposes.

2 Materials

1. Sterile saline for tissue washing.
2. Dulbecco’s phosphate buffered saline supplemented with 25 mM HEPES, and 2% human serum albumin or fetal calf serum.
3. Type IA Collagenase (Sigma, Catalog #C-2674). Other commercially available collagenase preparations such as Roche’s Blendzyme family also provide satisfactory results.
4. Sterile separatory funnels with stop cock.
5. Shaking water bath or incubator.
6. DMEM/F-12 50/50, 1× with L-glutamine (Mediatech Catalog # 10-090-CV).
7. Antibiotic/Anitmycotic 100X solution (ABAM), sterile, contains 10,000 units Penicillin-G/mL; 10,000 mcg Streptomycin/mL; 25 mcg Amphotericin B/mL (Omega Scientific Catalog # AA-40).
8. Complete culture medium: DMEM/F-12 based commercially prepared cell culture media in which 10% fetal bovine sera (FBS) and 1% v/v antibiotic/antimycotic have been previously added.
9. Falcon 3046 Multiwell 6-well tissue culture plate, Becton Dickinson.
10. 10% formalin.
11. Hematoxylin (Gill III formula, Surgipath Medical Industries, Cat# 01542).

3 Methods

3.1 Tissue Harvest

Liposuction is generally performed in a process in which tumescent solution, a mixture of saline, epinephrine (added as a vasoconstrictor to reduce blood loss), and lidocaine (for local anesthesia), is injected into the subcutaneous space (21,22). Most commonly a 2–5-mm diameter steel cannula is then inserted through a small (0.5 cm) incision in the skin and rapidly and repeatedly moved within the space to disrupt the tissue (a process referred to as tunneling). Tissue is then aspirated through holes in the cannula into a collection trap. Variations on this general theme include; use of manual aspiration via syringe for small volume tissue removal (usually <100 mL), use of powered cannula in which a reciprocating motion increases tissue disruption, and the application of ultrasound energy through the cannula, which further disrupts tissue and adipocytes. Further, in many procedures the surgeon will change the cannula multiple times to obtain greater control over the new body contour. Clearly, for ethical reasons the researcher can have no impact on the approach chosen by surgeon and patient, yet these variables can have substantial impact on the quality of the ADSC-containing population. Generally speaking, the less energy applied to the tissue, the greater the viability of the final product. Thus, manual or simple suction-assisted lipoplasty using a large diameter (>3 mm) cannula will yield tissue that has been subject to less shear force within the system than high-powered, machine-assisted suction through a small diameter cannula. By the same token, we have made anecdotal observations that ultrasound-assisted lipoplasty is associated with a substantial (>70%) decrease in the yield of ADSC. Thus, in preparing for research using human liposuction-derived material it is essential to evaluate the approach most frequently applied by the surgeon potentially providing access to tissue and to select those surgeons whose practice population and clinical preferences provide the most suitable aspirate. Because of subtleties in individual practice (for example, dose and use of epinephrine, cannula diameter, applied suction force, etc.), we suggest that investigators initially work with a number of different surgeons to build a sense of which surgeons provide the most reliable material.

Tissue may also be obtained following informed consent during an unrelated procedure such as hip surgery or resection of excess skin, or by small volume, syringe-mediated suction of research volunteers under local anesthesia.
3.2 **Tissue Processing**

Aspirated tissue is generally collected into a sealed nonsterile container that is disposed of as biohazardous medical waste. The material collected is a mixture of tumescent solution, blood, free lipid released from lysed adipocytes, and aspirated tissue fragments the precise proportions of which are largely determined by physician practice. Following informed consent this material should be transported to the laboratory for processing as quickly as possible; we have noted that the yield of ADSC, as measured by the fibroblast colony-forming unit (CFU-F) assay, falls by approx 50% for 24 h of storage before initiation of processing. Because the common collection containers are not designed for transport, precautions must be taken to avoid contamination and spills during transfer from the surgical facility to the processing laboratory. We suggest bagging in a sealed, spill-proof pouch, placing this bag within a rupture-proof secondary container such as a Tyvek® bag, and finally use of a crush-resistant outer container bearing labeling consistent with local and federal regulations.

Human lipoaspirate is frequently obtained without the researchers being aware of the infectious disease status of the donor; that is, without available results of serologic testing for biohazardous agents such as human immunodeficiency virus (HIV) and hepatitis B. For this reason all procedures involving manipulation of tissue should be performed in a protective environment such as a biological safety cabinet and operators should wear appropriate protective clothing and equipment at all times during tissue processing.

3.2.1 **Tissue Washing**

The buoyancy of adipose tissue is such that processing can be performed using approaches similar to those used in organic chemistry. Specifically, we apply sterile (autoclaved) separator funnels in tissue processing to separate buoyant tissue fragments from tumescent solution and blood. Washing may be repeated until the buoyant fraction is a vivid orange color and the infranatant is clear. Use of large volume funnels allows maximization of the ratio of saline:aspirate and more efficient washing. Alternatively, washing may be performed in beakers and the infranatant removed by aspiration.

1. Place stopcock to the closed position and decant lipoaspirate into the sterile separatory funnel.
2. Add sterile saline, prewarmed to 37 °C, and invert the funnel 4–5 times with the cap in place. Return to the upright position and allow 3–5 min for phase separation.
3. Remove the cap, open the stopcock and let blood-saline mixture flow into a liquid pathological waste container. Close the stopcock before the fat-blood/saline interface.
4. Repeat steps 2 and 3 until the infranatant is clear or residual opacity no longer declines substantially with additional wash cycles.
3.2.2 Tissue Digestion

A number of different enzymes and enzyme combinations have been described for digestion of human adipose tissue (2,23,24). We have developed a proprietary mixture of enzymes (Celase®) that optimizes processing. However, off-the-shelf enzymes such as the collagenase preparations listed above will yield satisfactory results.

1. Estimate the volume of washed fat (volume of fat after the last wash).
2. Prepare an equal volume of warm, sterile buffered saline containing 500 CDU/mL (equivalent to 0.5 Wünsch units/mL) collagenase.
3. Pour washed fat from the separatory funnel into a 600 mL, 1,000 mL, or 2,000 mL sterile bottle, depending on the estimated volume of washed fat (container volume should be at least 4 times that of the aspirate).
4. Add the buffered saline/collagenase mixture, seal the container and place on a thermal shaker, prewarmed to 35–38 °C for 20 ± 5 min. Initiate shaking.
   a. The frequency and amplitude of shaking should be set such that it is just sufficient to prevent separation of the buoyant tissue from the collagenase solution. Excessive amplitude or frequency can cause loss of cell recovery.
5. Inspect the digestion frequently after the first 15 min to ensure that overdigestion does not occur. Digestion time will vary with different tissue donors and physicians. For example, a larger cannula may generate larger fragments of tissue that may take longer to digest.
   a. The digestion may be halted when the quantity of residual fragments of adipose tissue is approx 5% of the initial amount.
6. On completion of digestion transfer the digestate to a fresh sterile glass separatory funnel. Allow the solution to sit for 5–10 min for phase separation to occur. Undigested and partially-digested adipose tissue, free adipocytes, and free lipid will float.
   a. The speed of phase separation may be increased by adding additional warm, sterile buffered saline to the funnel.
7. Open the stopcock and transfer the nonbuoyant fraction through a sterile 265 μm filter and into a sterile beaker.
8. Add warm, buffered sterile saline to the separatory funnel and invert the funnel 4–5 times with the cap in place. Return to the upright position and allow 3–5 min for phase separation.
9. Open the stopcock and transfer the nonbuoyant fraction through a sterile 265 μm filter into the material collected in step 7.
10. Aliquot the nonbuoyant solution collected in the beaker into multiple 50-mL centrifuge tubes.
11. Centrifuge at 400 g for 5 min at room temperature with a low-medium brake speed.
12. Gently pour off or aspirate the supernatant (top layer) into a liquid pathological waste container without disturbing the cell pellet.
13. Resuspend the pellets in buffered saline and combine the pelleted cells.
14. Repeat wash/centrifugation twice more to remove residual collagenase.
15. Pass the cell suspension through a 100 μm cell strainer and collect into a new, sterile 50-mL centrifuge tube.
16. Perform cell counting using fluorescent live/dead dyes such as 7 amino-actinomycin D or propidium iodide in combination with a nuclear counterstain such as Acridine Orange (25,26) or systems that use esterase substrates (27). Simple vital dye exclusion systems (for example Trypan Blue) that do not detect cell activity or the presence of a nucleus can be confounded by residual small lipid droplets.

In general this procedure yields a heterogeneous mixture of vascular cells, preadipocytes, lymphoid cells, blood cells, and ADSC. The process typically yields $2 \times 10^4$ nucleated cells per milliliter of human adipose tissue processed.

### 3.3 Assay of CFU-F

The CFU-F assay measures the presence of cells within the population capable of clonal expansion over 2 wk. Immunohistochemical staining has shown that these colonies are composed of cells that express CD105.

1. Centrifuge cells as above and resuspend in complete medium.
2. Plate cells in triplicate in 6 well plates at both 100 cells/cm² and 1,000 cells/cm².
3. Culture at 37 °C in humidified atmosphere of air plus 5% CO₂ for approx 2 wk.
   a. Perform scheduled media changes every 3–4 d.
4. After approx 2 wk of incubation, remove plates from the incubator and aspirate all medium from all wells.
5. Rinse each well of the plate 2–3 times with saline and then fix cells by incubating with ~1 mL of 10% neutral buffered formalin for 20 min.
6. Aspirate the formalin from each well and rinse the wells with saline.
7. Stain colonies by incubating wells with ~1 mL of hematoxylin Gill III formula for 5–10 min at room temperature.
8. Aspirate the stain and rinse gently with tap water.
9. Remove excess water by inverting and patting the plate onto a paper towel to dry the plate(s) and count the number of purple-stained colonies in each well consisting of more than 50 cells within a week of staining using an inverted microscope.

In our experience, application of the method described herein with freshly harvested human adipose tissue generates a population in which CFU-F represent approx 0.1–5% of nucleated cells.
4 Notes

1. **Effect of body mass index on cell processing.** In general we have found that overweight persons (persons whose body mass index (BMI) is between 27 and 30 kg/m\(^2\)) yield fewer nonbuoyant nucleated cells per unit volume of tissue than persons of lower BMI. This is likely owing to adipocyte hypertrophy (increased adipocyte size) in overweight persons such that there is more lipid and fewer cells per unit volume of tissue. Yield from tissue of obese persons is generally lower still. Data from one cohort of 23 donors (19 females, 4 males; median age 45; range 24–72 yr) is shown in Table 4.1.

   Body Mass index also affects the yield of CFU-F such that overweight persons yield significantly fewer clonogenic cells than persons of normal BMI (normal BMI 5.3 ± 0.8 × 10\(^3\) colonies/mL; overweight BMI 1.4 ± 1.1 × 10\(^3\) colonies/mL; \(p = 0.012\)). However, clonogenic cell yield from obese persons is highly variable perhaps as a result of the relative contribution of adipocyte hypertrophy and preadipocyte and stem cell hyperplasia in response to the increased demand for lipid storage in obese persons.

2. **Rodent adipose tissue.** Harvest of rodent adipose tissue is performed by lipectomy usually by dissection of the inguinal fat pad followed by mincing with scissors or scalpels. However, this tissue invariably contains lymph nodes which, if care is not taken to dissect them during mincing, will lead to considerable contamination of the nonbuoyant cell fraction with CD45-positive lymphoid cells and dilution thereby of the ADSC population. In one study digestion of murine inguinal adipose tissue without removing lymph nodes yielded a population in which CD45-positive cells comprised 94% of all nucleated cells and CFU-F frequency was 0.1%. By contrast, tissue in which major lymph nodes were dissected out before digestion yielded a population in which CD45-positive cells comprises 31% of all nucleated cells and CFU-F frequency was 4.7%.

3. **Porcine adipose tissue.** Contrary to the popular conception, pigs, especially juvenile farm swine bred for research purposes, are not particularly fat. Further, the tissue of these animals tends to have greater connective tissue than human fat rendering it less amenable to liposuction and to digestion. Hence, working with porcine adipose tissue requires more extensive digestion (0.4 U enzyme/mL of tissue, a prolonged digestion time (30–40 min), and care to ensure adequate mixing of tissue during digestion. In our hands porcine adipose tissue yields approx 5–10 × 10\(^6\) nucleated cells/mL of tissue and a CFU-F frequency of 0.05–0.10%).

4. **Sterility.** Though liposuction is performed in a sterile surgical field, the containers into which lipoaspirate is collected during cosmetic liposuction are not usually sterile. As a result, testing of the crude lipoaspirate and the digestate sometimes shows the presence of bacterial contaminants. In our experience the washing and digestion processes, if performed properly, tend to reduce the content of bacteria as evidenced by the rate of positive bacterial cultures. Further, the presence of standard antibiotics in complete tissue culture medium is usually sufficient to avoid bacterial outgrowth and loss of cultures.

### Table 4.1: Effect of body mass index on cell yield

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<thead>
<tr>
<th>Body mass index</th>
<th>Cell yield (million nucleated cells/mL tissue)</th>
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<tr>
<td>Normal (&lt;27)</td>
<td>2.9 ± 0.4</td>
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<tr>
<td>Overweight (27–30)</td>
<td>2.2 ± 0.7</td>
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<tr>
<td>Obese (&gt;30)</td>
<td>1.8 ± 0.7</td>
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References


