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Cultures of Human Adipose Precursor Cells

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1. Introduction

Recently, there has been rapidly growing interest in cell culture models that allow the study of the adipose differentiation process in vitro, as well as the long-term regulation of fat cell metabolism in human adipose tissue (AT) material. Although valuable clonal preadipocyte cell lines of rodent origin have been available for more than 20 yr, it became more and more obvious from subsequent studies that substantial differences exist in the developmental stage and regulation of differentiation, as well as in specific adipocyte functions, between rodent and human AT *(1–3)*. This knowledge makes it necessary to use human cell culture models, if specific questions concerning human AT metabolism and the mechanisms that may lead to either hypertrophic or hyperplastic growth in humans are investigated. Another aspect that is currently attracting considerable attention is the secretory function of adipocyte precursors and fat cells. Numerous factors are released from these cells, and maintain an intense crosstalk with distant organs, or act at the local level *(4)*.

Therefore, techniques that allow stable cultivation of human adipocyte precursor cells may provide a valuable tool for extending understanding of the mechanisms that lead to obesity and the known alterations in fat cell metabolism under defined conditions. The possibility to culture human adipocyte precursor cells and in vitro differentiated adipocytes may also open new perspectives for developing targeted therapeutic interventions to prevent further AT growth *(5)*.

Important progress in the culture of human adipocyte precursor cells was achieved with replacing the former serum-containing media by chemically defined serum-free media and by better characterization of the hormonal requirements. It was revealed in these studies *(1,6)* that the presence of serum almost completely prevents adipose differentiation in the human system, in

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contrast to the experience in cell lines or in cultured rodent adipose precursor cells *(6)*. This chapter describes in detail an improved technique that allows the study of human adipocyte precursor cells in primary culture.

2. Materials

Enzymes, media, tissue culture plasticware, and supplements may be purchased from any company supplying such products. Only a reagent-grade for tissue culture should be used. All material, including solutions that are used for cell culture must be handled under sterile conditions. Prior to use, all solutions are filtrated through filters with a pore size of 0.2-µm, to exclude bacterial contamination (e.g., by using Millipore material). The following solutions are required for the isolation, culture, and differentiation of the stromal cell fraction from human AT.

1. Basal medium: Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (50:50, v:v), supplemented with 15 m*M* HEPES, 15 m*M* $NaHCO_3$, 33 µ*M* biotin, 17 µ*M* D-pantothenate, at pH 7.4, and is used for tissue transportation from the surgery room to the laboratory.
2. Collagenase solution: Crude collagenase (e.g., Worthington CLS type 1, specific activity 172 U/mg) at a final concentration of approx 200 U/mL, pH 7.4, is dissolved in phosphate-buffered saline (PBS): 10 m*M* KH_2PO_4, 10 m*M* Na_2HPO_4, 2.7 m*M* KCl, 0.137 *M* NaCl, pH 7.4, supplemented with 2% bovine serum albumin (fraction V, according to Cohn). The solution should be freshly prepared for every preparation. However, the collagenase solution can be also stored at –20 C for a few days, with only a minor loss of enzyme activity. For use, the frozen solution is thawed at room temperature and is slowly prewarmed to 37°C. However, repeated thaw–freeze cycles must be avoided.
3. Erythrocyte lysing buffer: 155 m*M* NH_4Cl, 5.7 m*M* K_2HPO_4, 0.1 m*M* EDTA at pH 7.3. This buffer serves to remove red blood cells, which represent the major contaminating cellular component, and may reduce or prevent adhesion of the stromal cells.
4. Inoculation medium: Basal medium supplemented with gentamicin (50 µg/mL) and 10% fetal calf serum (FCS). The FCS is used to improve cell attachment, and to promote spreading.
5. Adipogenic medium: Basal medium supplemented with 10 µg/mL human transferrin (stock solution 1 mg/mL H_2O), 50 µg/mL gentamicin (stock solution 10 mg/mL), 1 n*M* triiodo-$_L$-thyronine (1 m*M* solution is alcalized with 1 *M* NaOH, and diluted to 2 µ*M* stock solution in EtOH), 66 n*M* human insulin (stock solution 22 µ*M* in 10 m*M* HCl), 100 n*M* hydrocortisone (stock solution 0.1 m*M* in 50% EtOH).
6. Adipogenic factors: To promote adipose differentiation, the following reagents may be added to the adipogenic medium: 0.2–0.5 m*M* isobutyl-methylxanthine (IBMX), a nonselective phosphodiesterase inhibitor (stock solution 20 m*M*, alcalized with Na_2CO_3) for the initial 3 d or 1 µg/mL troglitazone, a thiazolidinedione (stock solution 1 mg/mL) for the initial 3 d. Thiazolidinediones are activators of the nuclear receptor peroxisome proliferator activated receptor γ (PPARγ), which is a well-characterized master regulator of adipose differentiation *(7)*. Members of this class include troglitazone, pioglitazone, ciglitazone, and rosiglitazone among others. The authors frequently use troglitazone, which is dissolved in dimethyl sulfoxide (DMSO). The stock solution must be stored protected from light.
7. Oil red O staining: Oil red O is a specific neutral lipid marker. 0.5 g of this dye is dissolved in 100 mL 99% isopropanol giving a 0.5% solution. Six mL of this stock solution is mixed with 4 mL H_2O, resulting in a 0.3% solution ready for use.

3. Methods

3.1. Tissue Collection

1. AT specimens are usually obtained from elective surgery. Strictly sterile conditions are required, and it is recommended to collect the tissue in the surgery room. Reconstructive procedures, such as mastectomy or surgical removal of abdominal fat, are particularly suitable for tissue sampling. In studies of visceral AT, tissue collection from the intra-abdominal fat depot during abdominal surgery is needed. Because the intra-abdominal AT depots are more densely vascularized, careful electrocauterization, to avoid bleeding, is recommended. It is also possible to take tissue samples during laparoscopic interventions. However, in this case, the amount of material is usually limited. The differentiation rate depends on the age of the donor, so it is recommended to use tissue samples from donors at ages between 20 and 50 yr. The differentiation capacity is significantly higher in cultures from younger subjects, compared to older people, but the highest rates are observed in samples from children. To consider possible effects of obesity and confounding by concomitant diseases, careful documentation of basal patient characteristics is advisable.

   Another potential source of AT for primary cultures of human adipocyte precursor cells is the use of biopsy material. Between 1 and 2 g AT cylinders can be obtained by repeated aspiration using a 16-gauge steel needle *(5)*. The needle is fixed on a 20-mL sterile syringe filled with 5–10 mL 0.154 *M* NaCl and 1 mL 0.2% EDTA. However, this technique has its limitations, i.e., the amount of tissue is small, there is a high contamination by blood cells, which also reduces the yield of adipose precursor cells, and only the subcutaneous depots are accessible (*see* **Note 1**).

   For ethical reasons, informed consent must be obtained from the patient prior to tissue sampling.
2. The samples are crudely prepared in the surgical room, with surgical scissors, to remove skin and other non-AT material, such as gland tissue. The AT pieces are immediately transported to the laboratory in basal medium. Because the time of tissue collection is difficult to schedule, and sometimes may be late in the day, the samples may be stored overnight in a refrigerator at +4°C. Comparative studies (unpublished observation) have shown that there is no significant difference in adipose differentiation capacity of stromal cells isolated either immediately or after overnight storage in a refrigerator.

3.2. Cell Isolation

1. After transportation to the laboratory, the crudely prepared fat pads are carefully liberated from remaining connective tissue and blood vessels. This step is followed by collagenase digestion. Different types of collagenases are offered by several companies, and batches may differ substantially in their quality and activity. This problem renders it difficult, if not impossible, to standardize the conditions for collagenase digestion. However, variation in collagenase activity can be compensated by the adaption of concentration and duration of incubation. For the standard digestion procedure, a crude collagenase preparation at a concentration of 200 U/mL and 3 mL/g tissue is used. The incubation time is approx 60–90 min in a shaking water bath at 37°C.
2. To collect the disaggregated cells, samples will be centrifuged at $200g_{max}$ for 10 min. The supernatant with the adipocytes is discarded, or can be used for other purposes.
3. The pellet is resuspended in erythrocyte-lysing buffer, and incubated for not more than 10 min. This suspension is filtered through a 150 µm nylon mesh, and centrifuged once again. The resulting pellet is resuspended in an appropriate volume of basal medium. The cells are optionally filtered through a nylon mesh with a pore size of 70 µm (*see* **Note 2**).

3.3. Determination of Total Cell Number

1. For determination of the total cell number, a 50-µL aliquot of the cell fraction, obtained after resuspension in basal medium, is taken, and diluted with 100 µL medium and 50 µL trypan blue (0.4% in H_2O). 10 µL of this solution is transferred to a Neubauer chamber and counted under the microscope. This procedure is repeated and the mean value used to calculate total stromal cell number. The yield of isolated stromal cells/g wet AT is in the range of 100,000–350,000. In samples from severely obese subjects, the yield of stromal cells is usually lower than in samples from lean persons.
2. After cell counting, the still-concentrated stromal cell solution is diluted with inoculation medium to a final concentration of 150,000 cells/mL for subsequent seeding of the cells in a 4.5-cm^2 well. Thus, 1 mL corresponds to a seeding density of approx 33,000 cells/cm^2.

3.4. Cell Inoculation and Attachment

1. Usually, cells are resuspended in the inoculation medium containing 10% FCS, because serum contains many components that not only promote cell adhesion, but also support cell spreading and proliferation. This is the most frequently used and least expensive procedure. The advisable inoculation density is 30,000–50,000 cells/cm^2 to achieve optimal differentiation. For cell culture, 6- or 12-well plates, representing an area of 10 and 4.5 cm^2/well, respectively, are used. Although not all cells become attached to the surface of the sterile plastic dishes, the remaining cell number is sufficient to obtain a confluent cell monolayer within a few days, when cells have spread out. For optimal cell attachment, it is recommended to keep the cells for 16–24 h in the serum-containing medium. Longer exposure of the cells to serum is associated with an increased mitogenic activity, detectable by an increase in total cell number *(3)*. This phenomenon goes along with a progressive loss of differentiation capacity. When cells are incubated in serum-containing medium for the usual 16-d period in the presence of adipogenic factors, the differentiation rate is reduced by more than 90%, and, at the same time, total cell number increases by at least three- to fourfold (*see* **Note 3**).
2. It is also possible to inoculate the stromal cells in unsupplemented (without FCS) adipogenic medium. However, under this condition, cell adhesion is low and does not exceed 30–40%, on average. A small advantage is that the differentiation is higher (**Table 1**). Finally, cells can be inoculated in serum-free basal medium in culture dishes precoated with extracellular matrix proteins, such as fibronectin or laminin (commercially available). Frozen fibronectin prepared from human plasma, is thawed at 37°C and diluted to a concentration of 0.02 mg/mL, using aqua bidest. The surface of the culture dishes is precoated with a thin layer of this solution over night in an incubator at 37°C. Before being used, dishes are rinsed with basal medium. Precoating with human fibronectin significantly improves the attachment rate, but cannot fully replace FCS. Precoating of the dishes with fibronectin does not affect adipose differentiation in human tissue (**Table 1**).

3.5. Stimulation of Proliferation by FGF

The low yield of stromal cells from AT prompted attempts to stimulate stromal cell proliferation without losing the capacity for adipose differentiation. Such studies showed that fibroblast growth factor (FGF) is able to promote cell division without inhibiting adipose conversion. Cells are inoculated at a density of 10,000/cm^2 in serum-containing inoculation medium. After 16 h, cells are repeatedly washed with PBS, then fed with basal medium supplemented with 10 µg/mL transferrin, 66 n*M* insulin, 10 n*M* cortisol (reduced concentration), and a final concentration of 1 n*M* recombinant hu-bFGF (stock solution of 1 µ*M* FGF in PBS). The medium is changed every other day until confluency is reached, usually within 5–6 d; during this time, cells divide 2–3×. The FGF stock solution is stored in aliquots at –20°C for no longer than 6 mo. Repeated thaw–freeze cycles must be avoided.

3.6. Adipose Differentiation in Primary Culture (see Notes 4–6)

1. After cell adhesion, cultures are washed 2–3× with PBS to remove nonattached cells, which include most contaminating blood cells, cell detritus, and serum.
2. Cells are then incubated in a serum-free, hormone-supplemented medium, to induce adipose conversion. This adipogenic medium includes 66 n*M* insulin and 1 n*M* triiodothyronine, 100 n*M* hydrocortisone, and, for the first 3 d, 0.5 m*M* IBMX to induce the rearrangement of the gene expression pattern, which will result in the expression of genes required for adipose differentiation. To facilitate lipogenesis from glucose, 33 µ*M* biotin and 17 µ*M* pantothenate are continuously present in the culture medium. Under these conditions, up to 90% of the attached cells undergo differentiation, which can be easily followed by inverse light microscopy. The average differentiation rate is 40–50%.

Table 1
Effect of Inoculation Conditions on Cell Attachment and Differentiation Capacity[a]

Inoculation condition	Attachment rate (%)	G3PDH (rel %)
10% FCS for 24 h	60–70	100
10% FCS for 16 d	60–70	7
Fibronectin (precoating)	50–60	114
Completely serum-free	30–40	142

[a]Differentiation was assessed by determination of glycerol-3-phosphate dehydrogenase (G3PDH) activity on d 16 of culture, 100% is equivalent to a mean specific activity of 692 ± 137 mU/mg protein.

3. The adipose differentiation process can be also induced by addition of thiazolidinediones, instead of IBMX. This new class of antidiabetic drugs activates the nuclear receptor, PPARγ, and, thereby, triggers the expression of genes characteristic for fat cell development and function. Troglitazone is added, at a final concentration of 1 µg/mL, for the initial 3 d after changing to the serum-free culture medium. Continuous presence of troglitazone is not required. The average differentiation rate is in the range of 60%.

   The adipogenic medium is renewed 3×/wk. Visible lipid accumulation starts within 6–8 d under these conditions, initially around the nucleus. Within 16 d, the differentiating cells are completely filled with lipid droplets, and have changed their morphology to a spherical shape. Measurement of lipogenic enzyme activities indicates characteristics of differentiated fat cells, although cells still have an multilocular appearance (**Fig. 1**).

3.7. Oil Red O Staining

Cells cultured in a well or dish are fixed with a 10% formaldehyde solution for 2 h, then cells are washed with PBS, and subsequently incubated with 0.3% oil red O solution (400 µL/4.5-cm^2 well). After 1 h, the staining solution is removed by aspiration, then cells are exposed to 60% isopropanol (500 µL/4.5-cm^2 well) for a few minutes. Finally, cells are washed twice with PBS. Stained cultures can be stored in a refrigerator for weeks. Cells containing Oil red O dye can be easily detected, using an inverse light microscope at an appropriate magnification.

4. Notes

The establishment and in vitro differentiation of human adipose precursor cells can be complicated by a number of problems, some of which are addressed under the following points:

1. To keep contamination with other cell types low, and to achieve a good yield of precursor cells, it is essential to remove any contaminating tissue, particularly

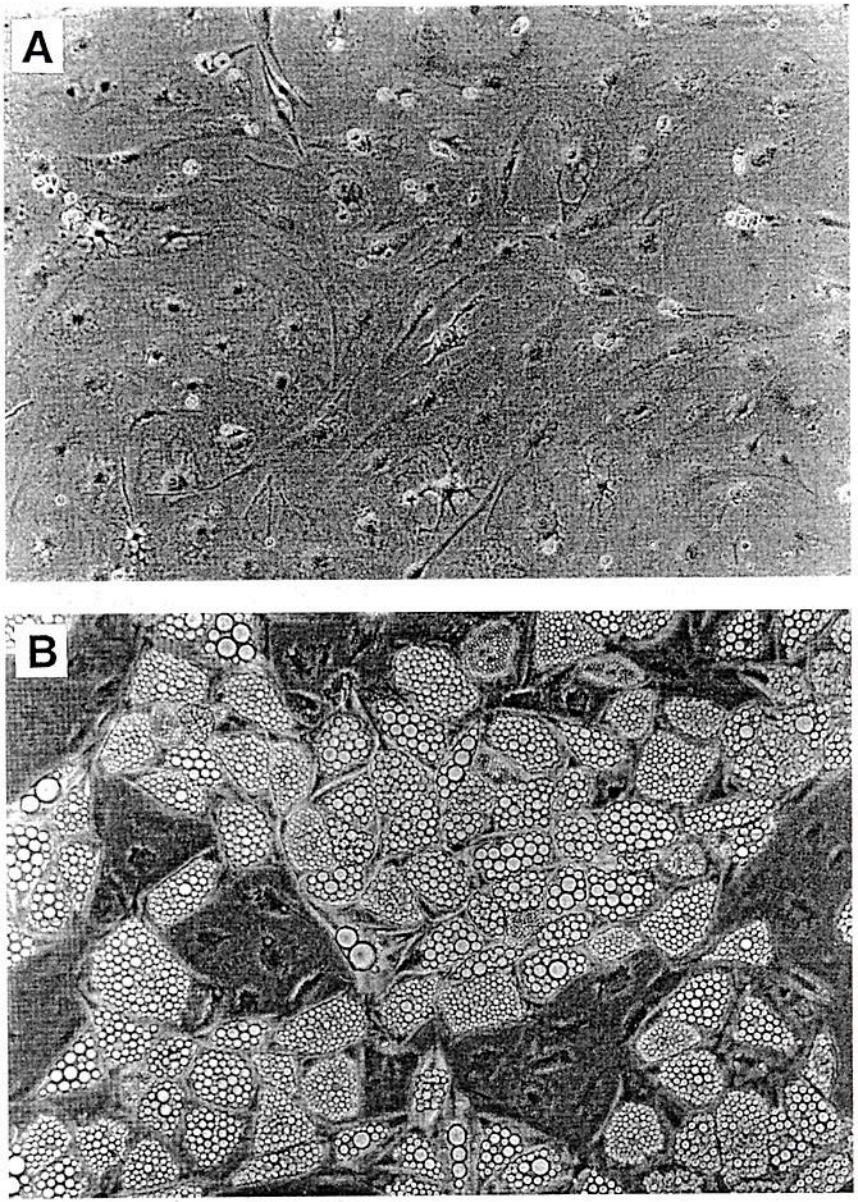

Fig. 1. Micrographs of cultured preadipose and adipose cells. (**A**) Human preadipose cells after 3 d incubation. Cells were seeded in a density of 30,000/cm^2 in inoculation medium for 1 d, and adipogenic medium supplemented with 1 µg/mL troglitazone. (**B**) Human preadipose cells from subcutaneous tissue in primary culture on d 16. Cells were cultured with adipogenic medium and, for the first 3 d, 1 µg/mL troglitazone. Note that human adipocytes are still multilocular at this stage.

blood vessels and fibrous material, prior to collagenase digestion. Moreover, the tissue samples should be thoroughly washed with PBS to get rid of blood that may reduce cell attachment and differentiation capacity. The AT should be finely minced to obtain an optimal and uniform enzymatic disaggregation of the tissue. More than 90% of the initial tissue should be digested.

2. AT samples from the intra-abdominal depots are densely vascularized. For this reason, there is usually a variable but significant contamination of the stromal cell fraction by endothelial cells (EC). In contrast to the stromal cell fraction from subcutaneous AT, which is essentially free of ECs, this cell type may cause several problems. The proportion of ECs should be reduced to less than 5% of total cell number. Therefore, after a first filtration through a 150-µm mesh in erythrocyte-lysing buffer, a second filtration is recommended, using a nylon mesh with a pore size of 25-µm to reduce this contamination *(2)*. ECs reaggregate rapidly, and are, for this reason, quantitatively retained by this filter size, but some contamination by ECs cannot be fully prevented. If precursor cells from various depots are compared, all samples must undergo the same filtration procedure.
3. Poor attachment and spreading of cells can be frequently encountered, if samples are extensively exposed to collagenase. Duration of digestion is critical, and can damage cells seriously. Therefore, too long enzymatic disaggregation of the samples should be avoided.
4. The rate of differentiation can vary considerably. Several aspects may be considered, but it is not always possible to prevent a low differentiation rate. Most important is that samples from young donors are used, that the tissue is transported in a suitable isotonic solution, and that collagenase digestion is adequate in terms of concentration and duration. A low differentiation rate, however, may be also an inherent characteristic of the donor. Moreover, a low inoculation density can result in suboptimal differentiation. Adipogenic factors should be freshly prepared, and stored appropriately.
5. Some compounds are dissolved in EtOH or DMSO. A combination of such additives can lead to subtoxic or toxic concentrations of the respective solvent. Detached and/or shrivelled cells can result from such effects. It is important that a final concentration of 0.1% DMSO and 0.1% EtOH is not exceeded.
6. With increasing lipid accumulation and rounding up, the developing adipocytes get more easily detached from the plastic surface. It is recommended to aspirate and replace the medium very gently, and to limit the culture period. We usually define d 16 as date of terminal differentiation, and use glycerol-3-phosphate dehydrogenase (GPDH)-activity as differentiation marker. By this time, cells have a multilocular morphology, but are completely filled with lipid droplets, and express almost maximal levels of characteristic genes. Then, cells can be cultured for another 10–14 d at maximum, to study AT metabolism. During this extended period, the adipocytes exhibit only one, or a few, large lipid droplets, and are very vulnerable to vibration or other mechanical damage *(8)*.

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