

A Novel Technique to Propagate Primary Human Preadipocytes without Loss of Differentiation Capacity

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Abstract

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Objective: The study of human preadipocytes is hampered by the limited availability of adipose tissue and low yield of cell preparation. Proliferation of preadipocytes using common protocols, including fetal bovine serum (FBS), results in a markedly reduced differentiation capacity. Therefore, we were interested in developing an improved culture system that allows the proliferation of human preadipocytes without loss of differentiation capacity.

Research Methods and Procedures: Adipose tissue samples were taken from subjects undergoing elective abdominal surgery. Cells were seeded at various densities and cultured using different formulations of proliferation media including factors such as fibroblast growth factor-2 (basic fibroblast growth factor), epidermal growth factor, insulin, and FBS either alone or in combination. Cells were counted and induced to differentiate after confluence. After complete differentiation, cells were harvested, and glycerol-3-phosphate dehydrogenase activity was measured. Cells were subcultured for up to five passages.

Results: The proliferation medium with 4 growth factors (PM4), consisting of 2.5% FBS, 10 ng/mL epidermal growth factor, 1 ng/mL basic fibroblast growth factor, and 8.7 μ M insulin, resulted in lower doubling times at all seeding densities tested (0.05×10^4 to 1.5×10^4) compared

with medium supplemented with 10% FBS. In contrast to cells in FBS medium, cells grown with PM4 medium retained full differentiation rate (glycerol-3-phosphate dehydrogenase activity, 493 ± 215 vs. 41 ± 17 mU/mg, $p < 0.01$). Differentiation capacity was fully retained at least for up to three passages in PM4 medium.

Discussion: The use of PM4 medium results in substantial proliferation of human preadipocytes with preserved differentiation capacity. This novel technique represents a valuable tool for the study of human adipose tissue development and function starting from small samples.

Key words: preadipocyte, adipocytes, growth factors, human adipose tissue, cell culture

Introduction

The trend to an increase in body weight is a worldwide phenomenon. Thus, obesity has become a growing threat to the healthcare system in many countries (1). Understanding the mechanisms of adipose differentiation could be of help in developing better strategies for the prevention of obesity and its associated disorders. Human fat cell models for in vitro studies have become a valuable tool for this purpose. One established model is the isolation of the stromal-vascular fraction of adipose tissue, culture, and in vitro differentiation for long-term incubation experiments (2). This model is based on the observation that adipose precursor cells are able to achieve complete terminal differentiation under defined conditions. This process can be followed by the expression of fat cell-specific markers e.g., glycerol-3-phosphate dehydrogenase (GPDH)¹ activity, peroxisome proliferator-activated receptor γ , leptin, adiponectin, and many others (3). Thus, in vitro-differentiated adipocytes can be used as a valid model for mature human fat cells.

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¹ Nonstandard abbreviations: GPDH, glycerol-3-phosphate dehydrogenase; FBS, fetal bovine serum; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; PM4, proliferation medium with four growth factors; PM3, PM4 without 2.5% FBS; aFGF, acidic fibroblast growth factor; SMCM, skeletal muscle cell medium.

The limiting factor for many potential applications is the restricted access to donor tissue material or small cell numbers from biopsy material. Previous attempts to promote the multiplication of stromal cells from adipose tissue, mostly by the use of fetal bovine serum (FBS) as the mitogenic principle, have not been successful because cells usually lose the ability to undergo adipose differentiation. It has been shown in several studies that FBS contains an undefined variety of anti-adipogenic factors (4). Addition of single mitogenic factors such as epidermal growth factor (EGF) or endothelin 1 to either rat or human preadipocyte cultures has resulted in a markedly diminished capacity of the cells to undergo adipose differentiation (5–7). The aim of the present study was to develop an improved culture model that allows controlled proliferation of adipose precursor cells without losing the ability for differentiation.

Research Methods and Procedures

Materials

1-Methyl-3-isobutylxanthine was purchased from Serva (Heidelberg, Germany). Rosiglitazone was obtained from Axxora (San Diego, CA). Collagenase CLS type I and FBS were obtained from Biochrom (Berlin, Germany). Recombinant human EGF and basic fibroblast growth factor (bFGF) 2 were purchased from Immunotools (Heidelberg, Germany). All other chemicals were obtained from VWR (Darmstadt, Germany). Sterile plastic ware for tissue culture was purchased from Corning (Corning, NY).

Subjects

Adipose tissue samples were obtained from 14 subjects (10 women and 4 men; age, 41.0 ± 12.7 years; BMI, 25.2 ± 3.6 kg/m²) undergoing elective plastic surgery in the abdominal region. All subjects were free of metabolic or endocrine diseases as assessed by routine clinical examination and laboratory tests.

Cell Culture and Assessment of Proliferation

Stromal cells from human adipose tissue were prepared as described previously (8). Briefly, after removal of all fibrous material and visible blood vessels, adipose tissue samples were cut into small pieces (~10 to 20 mg) and digested in 10 mM phosphate-buffered saline containing 200 U/mL crude collagenase and 20 mg/mL bovine serum albumin (pH 7.4) for 90 minutes in a shaking water bath. After short centrifugation at 200g, the floating fat cells and the incubation solution were aspirated and discarded. The sedimented cells were resuspended in an erythrocyte lysing buffer consisting of 154 mM NH₄Cl, 5.7 mM K₂HPO₄, and 0.1 mM EDTA for 10 minutes to remove contaminating red blood cells. The dispersed material was filtered through a nylon mesh with a pore size of 150 μm. After short centrifugation, the sedimented cells were resuspended in Dul-

becco's modified Eagle's medium (DMEM)/Ham's F-12 medium (w/w, 1:1) supplemented with 10% fetal calf serum and inoculated into culture dishes at the density indicated. After a 16-hour incubation period for cell attachment, cells were washed twice with phosphate-buffered saline and incubated for proliferation with FBS, bFGF, EGF, and insulin, either alone or in combination, or immediately used for differentiation experiments. After confluence, medium was changed to DMEM/F12 medium containing 10 μg/mL transferrin, 66 nM insulin, 100 nM cortisol, and 1 nM triiodothyronine. To induce differentiation, confluent cells were additionally exposed to 0.5 mM 1-methyl-3-isobutylxanthine and 2 μM rosiglitazone for 72 hours. The medium was changed every 2 to 3 days. Cell number was assessed by counting the same three representative squares from two wells every day using a counting grid with a square of 1.1 mm³. For some proliferation experiments, cells were trypsinized and counted after trypan blue staining.

Proliferation Media

Preliminary experiments were performed using the commercially available skeletal muscle growth medium (Promo-Cell, Heidelberg, Germany), supplemented with 5% FBS, 10 ng/mL EGF, 1 ng/mL bFGF, 0.5 mg/mL fetuin, and 17.5 μM insulin. The new formulation, referred to as PM4 (proliferation medium with four growth factors) was composed of DMEM/Ham's F12 (1:1, w/w) supplemented with 10 ng/mL EGF, 1 ng/mL bFGF, 8.7 μM mg/mL insulin, and 2.5% FBS. These proliferation media were compared with DMEM/Ham's F12 supplemented with 10% FBS (shortly referred to as 10% FBS). All media contained 50 μg/mL gentamicin as an antibiotic.

Subculture and Differentiation of Adipose Precursor Cells

For subculture experiments, freshly isolated cells from small adipose tissue samples were initially seeded at a density of 0.5×10^4 cells/cm². At 70% to 80% confluency, cells were trypsinized (0.05% trypsin/0.02% EDTA) (PAA Laboratories, Cölbe, Germany) and seeded again at a density of 0.5×10^4 cells/cm² in 25-cm² flasks. In parallel, to assess the differentiation capacity of cells from each subculture, cells were either seeded at a high density of 4.0×10^4 cells/cm² and induced to start differentiation the next day or seeded at a density of 0.5×10^4 cells/cm² in 12-well culture plates, proliferated with PM4 medium, and induced to differentiate after having reached confluence. After the 16-day differentiation period, cells were harvested in GPDH buffer [0.05 M Tris/HCl, 1 mM EDTA, 1 mM Me-OH (pH 7.4)] to assess enzyme activity as a marker of adipose differentiation.

Measurement of GPDH Activity

GPDH was determined according to an established protocol as described previously (9). For the measurement of

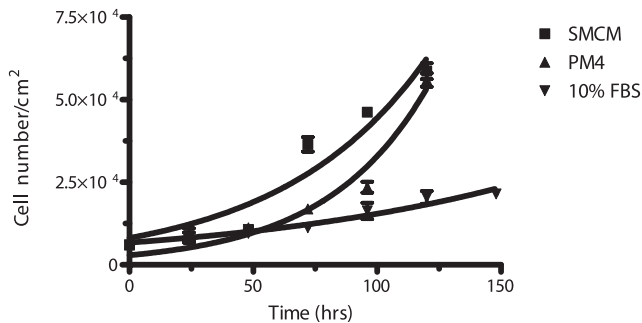


Figure 1: Effect of different media on the proliferation of cultured human adipocyte precursor cells. Cells were seeded at a density of $0.5 \times 10^4/\text{cm}^2$, and cell number was assessed by daily counting of eight defined areas from two wells (each area representing 1.1 mm^3) under the microscope until confluence. Data are given as mean \pm SE of three independent experiments in triplicate.

the protein content of the cultures, trichloroacetic acid precipitation was used to avoid lipid interference, according to a modification of the method of Lowry et al. (10).

Statistical Analysis

Cell growth was calculated using the logistic function $y = a/(1 + b \times e^{-cx})$, with the appropriate coefficients calculated by SAS (version 9.1.3; Heidelberg, Germany). Furthermore, differences between culture conditions were assessed by ANOVA. p Values < 0.05 were considered statistically significant.

Results

Effect of Different Proliferation Media on Doubling Times of Cultured Human Preadipocytes

In the first set of experiments, different media were compared for their ability to induce proliferation in human adipose precursor cells in primary culture. A commercially available proliferation medium for skeletal muscle cells was used and compared with the proliferative capacity of standard medium DMEM/F12 (1:1, w/w) supplemented with either 10% FBS or 2.5% FBS together with 10 ng/mL EGF, 1 ng/mL bFGF, and 8.7 μM insulin (PM4). In these first experiments, a doubling time of 43 hours (33 to 59 hours) was determined in the presence of the skeletal muscle medium, compared with 27 hours (23 to 32 hours) and 87 hours (67 to 117 hours) in the presence of the PM4 medium and of 10% FBS, respectively (Figure 1). Control cultures kept in medium without proliferation factors exhibited constant cell numbers throughout the culture time without evidence of proliferation (data not shown).

Effect of Seeding Density on Doubling Times in the Presence of PM4 or 10% FBS

To assess mean doubling times in dependence of the seeding density and the proliferation medium (either with

PM4 or 10% FBS), we inoculated cells at densities of $0.05 \times 10^4/\text{cm}^2$ to $1.5 \times 10^4/\text{cm}^2$. At higher cell densities ($1.5 \times 10^4/\text{cm}^2$ and $0.3 \times 10^4/\text{cm}^2$), the doubling times were 178 (57.4 to 161) and 139 (71.4 to >360) hours in the presence of 10% FBS compared with 72.1 (44.7 to 118) and 57 (47.1 to 103 hours) hours in the PM4-treated cells, respectively. At the low inoculation densities ($0.15 \times 10^4/\text{cm}^2$ and $0.05 \times 10^4/\text{cm}^2$), doubling times ranged from 141 (55.4 to >360) and 116 (37.5 to 144) hours in the FBS-treated cells to 57.7 (17.8 to 73.6) and 34.6 (21.1 to 106) hours in the PM4 medium, respectively (Figure 2, A and B).

Effect of Defined Mitogenic Factors on the Proliferation of Human Preadipocytes

To assess the relative contribution of the single mitogenic factors from the new PM4 formulation, we studied the effect of the single components vs. the complete PM4 formulation

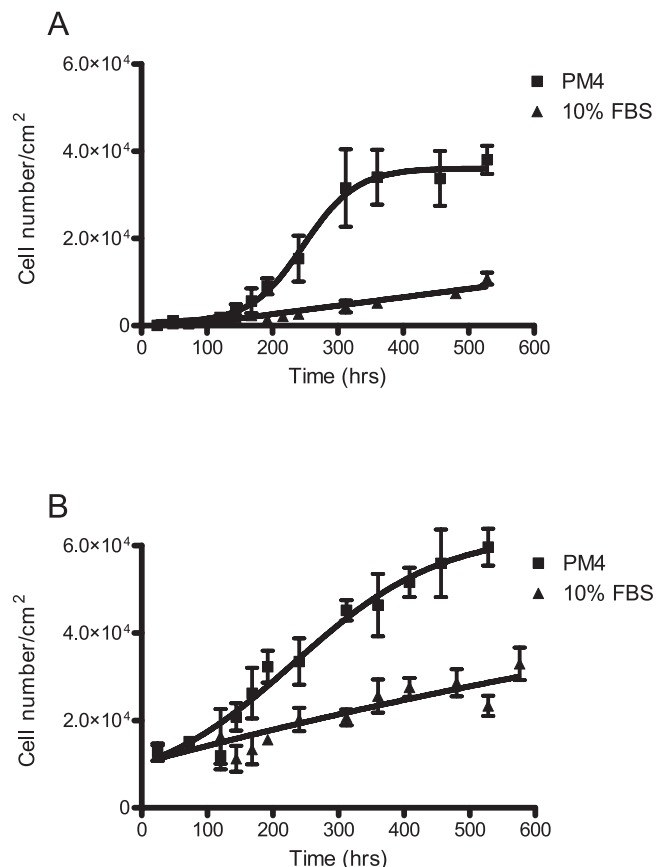


Figure 2: Effect of seeding density on the proliferation of cultured human adipocyte precursor cells. Cells were incubated with DMEM/F-12 medium supplemented with either 10% FBS or PM4 medium until confluence at a density of 0.05×10^4 cells/cm² (A) or 1.5×10^4 cells/cm² (B). Cell number was assessed by trypsinizing and counting after trypan blue staining. Data are given as mean \pm SE of five independent experiments in quadruplicate.

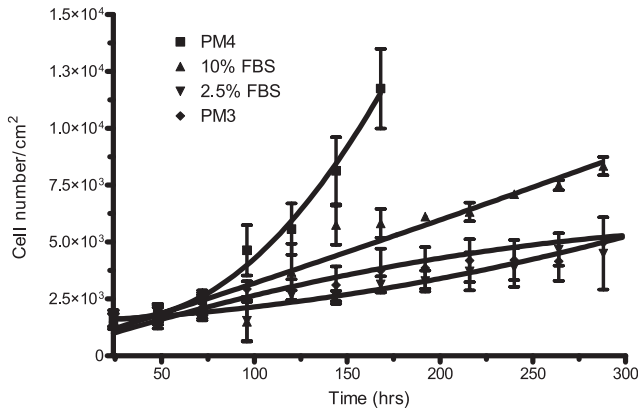


Figure 3: Summary of the effect of selected single mitogenic factors on the proliferation of cultured human adipocyte precursor cells. Cells were seeded at a density of $0.15 \times 10^4/\text{cm}^2$, and the cell number of defined areas was counted daily until preconfluence or up to 12 days. Data are given as mean \pm SE of five independent experiments in triplicate.

in cell cultures seeded at a density of 0.15×10^4 cells/cm². Additionally, a serum-free formulation, PM4 without 2.5% FBS (PM3), was tested. Control cultures exhibited rapid proliferation, with doubling times of 47 (37 to 64) hours with PM4 and 86 (72 to 106) hours with 10% FBS. Single use of DMEM/F12 medium supplemented with 2.5% FBS resulted in a doubling time of 180 (158 to 208) hours, whereas the complete serum-free formulation PM3 exhibited dramatically reduced proliferation (doubling time, >600 hours; range, 402 to 1374 hours) (Figure 3). Use of bFGF or EGF alone under serum-free conditions resulted in prolonged doubling times (data not shown). Cultures treated with a high dose of $8.7 \mu\text{M}$ insulin or acidic fibroblast growth factor (aFGF) alone did not show any significant proliferation.

Because FGFs and EGF are known to have heparin as a cofactor at a molar ratio of 1:1 (11), we also included heparin in combination with these proliferation factors or heparin alone as control. Neither heparin nor its combination with a growth factor showed any proliferation-promoting effect in cultured adipose precursor cells (data not shown). Thus, only the combination of the mitogenic factors EGF, bFGF, insulin, and 2.5% FBS proved to be effective in promoting the proliferation of human adipocyte precursor cells.

Effect of Proliferation on Differentiation Capacity of Human Preadipocytes

It is well established that proliferation is associated with a reduced capacity of differentiation in human preadipocytes, which is reflected by reduced specific GPDH activity and/or reduced accumulation of lipid droplets (11). Therefore, we proliferated adipose precursor cells seeded at a

density of 0.5×10^4 cells/cm² with 10% FBS, skeletal muscle cell medium (SMCM), or PM4 until confluence and tested the differentiation capacity after 16 days in culture under standard serum-free adipogenic conditions compared with unproliferated (control) cultures (initially seeded with 4.0×10^4 cells/cm² and induced for differentiation). On Day 16, cells were harvested for the measurement of GPDH activity to assess the degree of differentiation. Use of 10% FBS resulted in a marked decrease of GPDH activity compared with control cultures without proliferation (81 ± 19 vs. 421 ± 85 mU/mg; $p < 0.01$). Induction of differentiation after proliferation with either SMCM or PM4 resulted in a significant increase of GPDH activity compared with control cultures (986 ± 295 and 858 ± 140 mU/mg, respectively; each $p < 0.05$) (Figure 4). This increased GPDH activity was also reflected in an increased percentage of differentiating preadipocytes (Figure 5).

Effect of Subcultivation on the Proliferation of Human Preadipocytes

Subculture of human preadipocytes using standard proliferation protocols is associated with an increase in doubling time and a marked reduction in differentiation capacity (12). Therefore, we tested whether the effect of PM4 on proliferation and differentiation is preserved in subcultures. Initially, we seeded preadipocytes at a density of 0.5×10^4 cells/cm² in 25-cm² flasks and stimulated cell proliferation by using PM4 medium. The average doubling time subsequently increased in a non-significant manner from subculture to subculture from 86 (68 ± 114) hours in passage P0

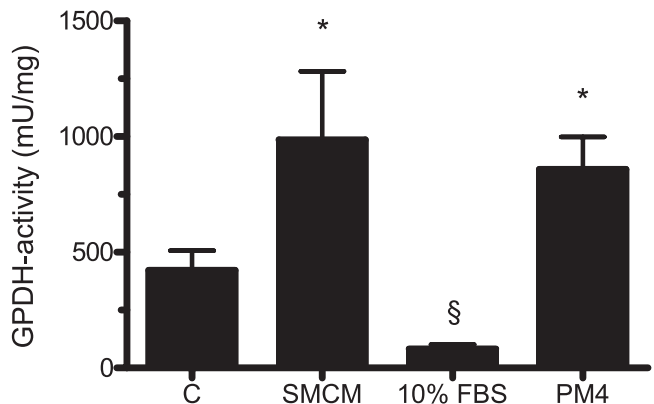


Figure 4: Effect of various culture media on the differentiation capacity of cultured human preadipocytes. Human preadipocytes were seeded at a density of $0.5 \times 10^4/\text{cm}^2$ and incubated with DMEM/F-12 proliferation medium until confluence. Thereafter, cells were induced to differentiate as described in "Research Methods and Procedures." On Day 16, cells were harvested for the measurement of GPDH activity. Data represent mean \pm SE from five independent experiments in duplicate. * $p < 0.05$ vs. controls; § $p < 0.01$ vs. controls.

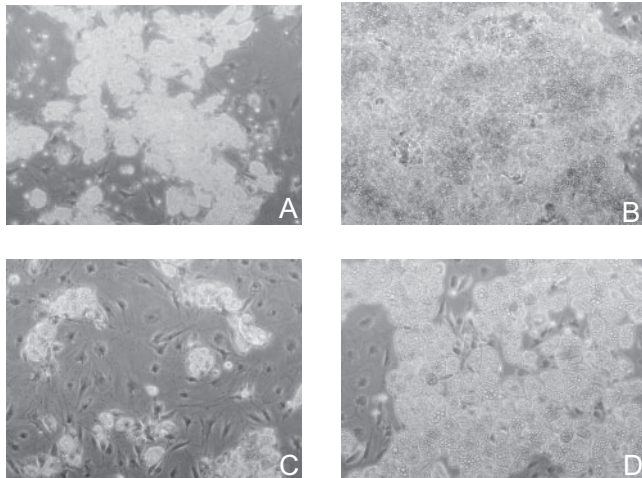


Figure 5: Representative micrographs of in vitro differentiated human adipocytes after 16 days under various adipogenic culture conditions. Control cells (A) were seeded at a density of $4.0 \times 10^4/\text{cm}^2$ and were induced for differentiation as described in “Research Methods and Procedures.” The other cultures were seeded at a density of $0.5 \times 10^4/\text{cm}^2$ and treated with DMEM/F12 medium supplemented with SMCM (B), 10% FBS (C), or PM4 (D) until confluence. After confluence, cells were also induced for differentiation as in the control cultures. Pictures were taken 16 days after induction of differentiation using a Leica DMIL microscope (Leica, Wetzlar, Germany) with a digital camera. Magnification, 100-fold.

to 97 (73 ± 146), 103 (81 ± 144), 122 (95 ± 171), 131 (98 ± 200), and 168 (133 ± 230) hours in passages P1, 2, 3, 4, and 5, respectively (Table 1).

Table 1. Effect of subcultivation on doubling times of primary human preadipocytes proliferated with PM4

Passage	Mean doubling time (hrs)	Range (hrs)
P0	85.8	68.5 to 114.9
P1	97.4	73.1 to 146.0
P2	103.4	80.8 to 143.7
P3	122.2	95.1 to 170.8
P4	131.2	97.6 to 200.0
P5	168.4	132.9 to 229.7

PM4, proliferation medium with four growth factors. Cells from every passage were seeded at a density of $0.5 \times 10^4/\text{cm}^2$ and counted every day until confluency. Data show doubling times of proliferating cells from five independent experiments in triplicate.

Effect of Subcultivation on the Differentiation of Human Adipose Precursor Cells

Use of PM4 resulted in a slightly enhanced differentiation capacity after confluency in P0 compared with standard conditions. The cells further subcultured and proliferated with PM4 retained a high capacity for adipose differentiation throughout the three passages studied (Figure 6), whereas cells cultured under standard conditions using 10% FBS exhibited a rapid loss in differentiation capacity (data not shown) (13).

Discussion

The aim of our study was to develop a method that allows for the proliferation of adipose precursor cells from a small amount of starting material without loss of differentiation capacity. For this purpose, we defined culture conditions based on DMEM/F12 medium supplemented with a combination of EGF, bFGF, insulin, and 2.5% FBS. The results of our experiments clearly demonstrate that this medium is suitable for a high proliferation of stromal cells. Under these conditions, confluent cultures and subcultures fully retained their capacity for differentiation on exposure to adipogenic factors. This improved method may represent a substantial progress for the primary culture of human adipose precursor cells because their number can be increased by >30 -fold. Thus, small amounts of human adipose tissue (0.5 to 1 grams) as primary source may be sufficient for larger series of experiments. This may also apply for biopsy material, although such material was not used in this study. It turned

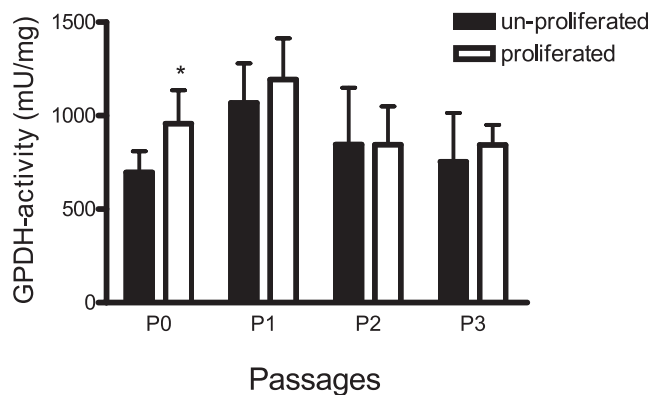


Figure 6: Effect of subcultivation and proliferation with PM4 on the differentiation capacity of cultured human adipose precursor cells. In each passage, cells were seeded at $0.5 \times 10^4/\text{cm}^2$ and proliferated with PM4 until confluence. Then, cells were allowed to differentiate by being transferred to adipogenic medium. In parallel, control cells were seeded at a density of $4.0 \times 10^4/\text{cm}^2$ and induced for differentiation. On Day 16 after starting differentiation, cells were harvested for measurement of GPDH activity. Data represent mean \pm SE of four independent experiments in triplicate. * $p < 0.05$ vs. un-proliferated cells.

out that the differentiation capacity expressed as GPDH activity was higher under these conditions as compared with conventional conditions.

Exposure of preadipocytes to FBS results not only in a marked stimulation of cell growth but also in a dramatic reduction of adipose conversion due to the presence of anti-adipogenic factors (4,12). The observation that the differentiation of human preadipocytes is reduced by exposure to FBS was also confirmed by other studies (7,14–16). Therefore, in search of a better alternative, we tested commercially available growth media, among them a growth medium for skeletal muscle cells (SMCM). In particular, the SMCM proved suitable for stimulating the proliferation of human adipocyte precursor cells but retained the capacity for differentiation. Because there was no additional effect on proliferation and differentiation, we reduced FBS and insulin concentrations (data not shown). We finally composed the PM4 medium, which consisted of standard medium for human preadipocytes supplemented with 10 ng/mL EGF, 1 ng/mL bFGF, 8.7 μ M insulin, and 2.5% FBS. Both the SMCM and the newly formulated medium PM4 were similar in their potency to stimulate proliferation without losing the capacity for differentiation.

All single factors added to the PM4 medium are known to be potent proliferation-stimulating factors for various cell types. In human stromal-vascular cells, proliferation was successfully induced by EGF and bFGF as single components, both in the presence of 10% FBS (7). Both factors signal by membrane-associated receptors and share common mitogenic pathways including phospholipase C- γ and consecutive activation of protein kinase C (17,18). However, when these factors were used during the exponential growth phase of human preadipocytes in combination with 10% FBS, a marked decrease in differentiation was observed (7). In contrast, other more recent studies showed that growth factors such as FGF-10 or aFGF (FGF-1) are able to promote lipid accumulation and adipogenesis under defined conditions (11,19). In general, FGF signaling seems to be an essential event for preadipocyte differentiation (20). FGF-1 was found to be secreted from microvascular endothelial cells in adipose tissue and is supposed to participate in the control of proliferation and differentiation of preadipocytes (11). A very recent paper reported that human multipotent adipose-derived stem cells obtained from young donors showed a progressive decrease in proliferation and differentiation potential along with a decrease in FGF-2 expression, which could be circumvented by FGF-2 treatment (21). The authors further showed that use of an FGF-2 receptor blocker dramatically reduced differentiation. In a similar study, Chiou et al. (22) recently demonstrated the mitogenic effect of FGF-2 in adipose-derived mesenchymal cells that were examined for their potential to undergo chondrogenic differentiation, indicating a role of FGF-2 in potentiating chondrogenesis. Another group also reported

the significant effect of FGF-2 on chondrogenic and adipogenic differentiation. The interesting finding in this study is that this effect was dependent on the dose of FGF-2 used (23), which is in some contrast to earlier studies (12) but may be explained by the different culture substrates used.

In a set of experiments, we studied single mitogenic factors on their potential to promote proliferation under serum-free conditions. EGF, aFGF, or bFGF alone did not stimulate proliferation to a significant extent. FGFs and EGF were reported to form heteroduplexes with heparin to exhibit closer interaction with their specific receptors (11). It was originally suggested to use a combination of a growth factor together with heparin in a molar ratio of 1:1 to enhance the growth factor effect of aFGF (24). However, also in our experiments, heparin did not show any supportive proliferation-stimulating effect either in the presence of aFGF or bFGF or in combination with EGF (data not shown). The same was true for a combination of EGF, bFGF, and insulin without the use of FBS (=PM3) (Figure 4). It clearly turned out that cells were not viable for a longer period of time in the complete absence of serum. Which serum components are essential for cell growth is unknown but may comprise a variety of factors.

In other experiments, we defined the optimal cell seeding density to obtain the highest proliferation activity of the adipose precursor cells. As expected, preadipocytes seeded at higher densities exhibited a reduced growth activity, whereas inoculation densities between 500 and 3200 cells/cm² resulted in rapidly growing cultures. Cells inoculated at very low densities (<500 cells/cm²) proliferated slowly in PM4 medium but showed substantial inconstant proliferation in medium containing 10% FBS (data not shown).

Another interesting finding was that proliferation of preadipocytes in PM4 medium caused a moderate enhancement of differentiation compared with cultures under standard conditions without subcultivation. This observation lends support to the notion that besides FGF-1 (11,25), FGF-2 also may act as a putative enhancer of adipose differentiation.

Furthermore, we were also interested in the effect of the PM4 medium in subcultures of human preadipocytes. It was recently shown that repeated subculturing dramatically reduces differentiation capacity of preadipocytes despite the presence of a combination of potent adipogenic factors, including a thiazolidinedione (13). However, the use of the PM4 medium resulted in a highly preserved capacity of the cells to undergo differentiation for at least three passages despite a high number of cell divisions in vitro. Thus, the use of the PM4 medium may open a new option for the subcultivation of human adipocyte precursor cells in primary culture for extended periods.

In conclusion, the results of this study suggest that a combination of bFGF, EGF, insulin, and a low dose of FBS

not only causes effective proliferation but also retains differentiation of human preadipocytes. The newly described PM4 medium appears to be a valuable tool for obtaining substantially higher cell numbers from small human adipose tissue samples. Thus, this new technique may further facilitate in vitro studies in human adipose tissue.

Acknowledgments

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