Original Article

In vitro Models for Adipose Tissue Engineering with Adipose-Derived Stem Cells Using Different Scaffolds of Natural Origin

(adipose-derived stem cells / adipogenic differentiation / natural scaffolds)

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Abstract. Soft tissue regeneration with cell and tissue engineering-based approaches has numerous potential applications in plastic and reconstructive surgery. Adipose-derived stem cells (ASC) have been proved as a feasible source for adipose tissue engineering as they possess high proliferative and differentiation capacity. The purpose of our study was to evaluate adipogenic differentiation of human ASC in four different 3D scaffolds of natural origin, namely human platelet-poor plasma, alginate, fibrin gel and collagen sponge, to define their suitability for adipose tissue engineering and potential clinical applications. ASC were isolated from lipoaspirates of three adult female patients, seeded in the scaffolds, and adipogenic differentiation was induced. After two weeks of cultivation, the constructs were assessed for their mechanical and handling properties, cell viability and adipogenic differentiation. Additionally, the expression of vascular endothelial growth factor (VEGF) was analysed in different culture systems. The results indicate that the levels of specific adipogenic markers and *VEGF* expression were increased in 3D cultures, as compared to 2D culture. Among 3D scaffolds, fibrin gel showed optimal combination of mechanical characteristics and support of adipogenic differentiation; it was easy to handle, allowed high cell viability, and at the same time supported adipogenic differentiation and *VEGF* expression.

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Abbreviations: 2D – two-dimensional, 3D – three-dimensional, ADIPOQ – adiponectin, ASC – adipose-derived stem cells, LPL – lipoprotein lipase, PEGDA – polyethylene glycol diacrylate, PET – polyethylene terephthalate, PGA – polyglycolic acid, PLGA – poly(lactic-co-glycolic acid), PPARγ – peroxisome proliferator-activated receptor γ, PPP – platelet-poor plasma, PRP – platelet-rich plasma, PTFE – polytetrafluoroethylene, VEGF – vascular endothelial growth factor.

Introduction

Subcutaneous adipose tissue defects are associated with numerous conditions including traumatic injury, congenital birth defect and oncologic resection. Therefore, soft tissue reconstructions present an ongoing challenge in plastic and reconstructive surgery. Current clinical strategies for soft tissue reconstructions involve local flaps, microvascular adipocutaneous flap transfer, free fat grafting and implantation of alloplastic materials (Katz et al., 1999; Sterodimas et al., 2009). These approaches have disadvantages including donor-site morbidity, unpredictability of graft survival due to the lack of vasculogenesis, graft migration or resorption, failure to integrate into the host tissue and immune rejection (Katz et al., 1999; Baran et al., 2002; Sterodimas et al., 2009).

The strategy that promotes adipose tissue regeneration instead of damaged tissue substitution is usually based on the use of tissue engineering-techniques. The tissue-engineering approach combines an appropriate cell source with a biocompatible scaffold material (Hemmrich and von Heimburg, 2006).

An appropriate 3D scaffold should facilitate normal cellular organization and behaviour, define and maintain the desired tissue volume, and at the same time promote host integration and implant vascularization (Katz et al., 1999). Ultimately, the scaffold should undergo non-toxic degradation as it is replaced by the healthy host tissue. A number of different scaffolds, synthetic as well as naturally derived, have been investigated for adipose tissue-engineering purposes. Among synthetically derived scaffolds, polytetrafluoroethylene (PTFE) (Kral and Crandall, 1999), poly(lactic-co-glycolic acid) (PLGA) (Patrick et al., 2002), polyglycolic acid (PGA) (Fischbach

et al., 2004), polyethylene glycol diacrylate (PEGDA) (Alhadlaq et al., 2005) and polyethylene terephthalate (PET) (Kang et al., 2005) were examined. Naturally derived materials such as matrigel (Kawaguchi et al., 1998), fibrin (Schoeller et al., 2001; Cho et al., 2005), derivates of hyaluronic acid (Halbleib et al., 2003; Hemmrich et al., 2005), collagen (Gentleman et al., 2006), alginate (Marler et al., 2000) and decellularized matrices (Flynn et al., 2007) have also been studied. However, the optimal scaffold for adipose tissue engineering has not yet been defined and its use in clinical application also remains elusive.

The cellular component for most tissue-engineering strategies involves adipogenic precursor cells, mainly adipose-derived stem cells (ASC). ASC can be isolated from the stromal vascular fraction of excised or lipoaspirated adipose tissue (Zuk et al., 2001; Fraser et al., 2006). In comparison to mature adipocytes, ASC display higher resistance to mechanical damage and ischaemic conditions (Katz et al., 1999; von Heinburg et al., 2005). Furthermore, while terminally differentiated adipocytes have lost their capacity to divide, ASC possess high proliferative and differentiation capacity, thus increasing their regenerative potential (Ailhaud et al., 1992; Heimmrich et al., 2005).

Most frequently used *in vitro* models for studying adipogenesis involve two-dimensional culture systems, although planar 2D monolayers do not represent the complex three-dimensional (3D) architecture of adipose tissue *in vivo*. Additionally, it is well accepted that cell behaviour in 2D and 3D environments tends to be very different (Cukierman et al., 2002). Recent studies in tissue engineering research demonstrated the feasibility of using 3D scaffolds to promote adipogenesis of adipocyte precursor cells (Flynn and Woodhouse, 2008; Stacey et al., 2009).

In the present study we have explored the effect of four naturally derived scaffolds, namely, human platelet-poor plasma, alginate, fibrin gel, and collagen sponge, on ASC viability and differentiation capacity. Adipocyte-specific gene expression was obtained in 3D scaffolds and 2D monolayer culture and compared to mature adipocyte samples. Additionally, as the angiogenic potential of the engineered adipose tissue construct is of great importance for the tissue regeneration potential, gene expression of vascular endothelial growth factor (VEGF), secreted by differentiating ASC, was analysed.

Material and Methods

1. ASC isolation and expansion

Adipose tissue was obtained by elective lipoaspiration of subcutaneous fat of the abdominal region from three female donors (age range 47–54 years). They all consented with the procedure and agreed with further research of aspirated fat in accordance with the ethical guidelines of the National Medical Ethics Committee (code 21/09/07). ASC were isolated as described previously (Zuk et al., 2001) with slight modifications. Briefly, the lipoaspirate samples were washed extensively with phosphate-buffered saline (PBS, Gibco, Invitrogen, Camarillo, CA) and digested with 0.075 % (w/v) collagenase type I (Gibco) at 37 °C for 45 min while shaking. The digestion process was stopped by adding basal medium: DMEM/F-12 (Gibco), supplemented with 10 % human serum (Blood Transfusion Centre, Ljubljana, Slovenia) and 50 μg/ml gentamycin (Gibco). After the cell suspension was centrifuged at 1200 *g* for 5 min, the pellet was resuspended in the basal medium and filtered through 100 μm nylon mesh (BD Biosciences, San Jose, CA). The isolated stromal vascular fraction cells were plated at 100,000 cells/cm^2 in 6-well tissue culture dishes (TPP, Trasadingen, Switzerland). After 24 h non-adherent cells were gently removed by washing with PBS. To obtain primary culture cells (P0), ASC were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were detached by 0.05% trypsin-EDTA solution (Sigma, St. Louis, MS) when 90% confluence was obtained.

First-passage (P1) ASC were plated in tissue culture flasks at 3000 cells/cm^2 and cultured in the basal medium with addition of 1 ng/ml bFGF (Gibco). When the cells were 80% confluent, they were re-plated to obtain second-passage cells (P2). Medium was changed twice per week. Cell viability was assessed by the Trypan Blue exclusion method.

2. Culture systems for adipogenic differentiation

Adipogenic differentiation was induced and maintained in all culture systems with adipogenic medium: HG-DMEM/F-12 (Gibco), 3% human serum (Blood Transfusion Centre), 2 mM glutaMAX (Gibco), 10 μg/ml recombinant human insulin (Sigma), 1 μM dexamethasone (Sigma), 100 μM indomethacin (Fluka, Buchs, Switzerland), 500 μM IBMX (Sigma) and 50 μg/ml gentamycin (Gibco).

2.1 Monolayer culture

P2 ASC suspended in the basal medium were placed in 12-well plates (TPP) at a density of 20,000 cells/cm^2 and cultured overnight. The next day differentiation was induced with adipogenic medium. The medium was changed twice per week and after 7 or 14 days of cultivation in differentiation medium cells were harvested for analysis.

2.2 3D culture systems

P2 ASC were seeded into four different scaffolds: human platelet-poor plasma (PPP), alginate hydrogel, fibrin gel and collagen sponge. The volume of all scaffolds was 300 μl and cells were seeded at a final concentration of 5×10^6 cells/ml.

Platelet-poor plasma

Human PPP from four healthy donors was collected and pooled (Blood Transfusion Centre). Pelleted cells were resuspended in PPP and transferred to 24-well plates. PPP was polymerized by adding 10 μl of 500 mM $CaCl_2$ (Sigma). After 5 min the construct was covered with DMEM/F-12.

Alginate

For alginate hydrogel constructs, cells were pelleted and resuspended in 1% alginate solution (MediAlg MG17, Medipol, Lausanne, Switzerland). The suspension was transferred into 24-well plates and polymerized by adding 500 µl of 125 mM $CaCl_2$ (Sigma) on the top of the construct.

Fibrin gel

Fibrin gel was prepared from a commercially available fibrin gel kit (Beriplast®, ZLB Behering GmbH, Marburg, Germany). Pelleted cells were resuspended in 200 µl of adipogenic medium, transferred into 24-well plates, and polymerized by addition of 50 µl thrombin (500 IU/ml) and 50 µl fibrinogen (90 mg/ml).

After 2 h, all three cell-seeded scaffolds were placed into 12-well plates and covered with adipogenic medium.

Collagen sponge

Prior to cell seeding, the collagen sponge (TachoSil®, Nycomed GmbH, Linz, Austria) was soaked thoroughly with DMEM/F-12 to fill all the cavities. Pelleted cells were resuspended in adipogenic medium, seeded drop by drop on collagen sponge and allowed to adhere overnight. The next day constructs were transferred to 12-well plates and covered with differentiation medium.

The constructs were incubated at 37 °C and 5% CO_2 for 7 and 14 days. Medium was changed every second day. Macroscopic scaffold architecture, mechanical and handling properties of the constructs were evaluated on days 7 and 14.

3. Cell viability assay

Cell viability in the scaffolds was assessed on days 7 and 14 with calcein-AM and ethidium homodimer-1, according to manufacturer's instructions (LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells; Molecular Probes, Eugene, OR). A thin layer of the inner part of the construct was cut, stained and imaged by fluorescent microscopy in NIKON Eclipse 80i (Nikon Instruments Inc., Melville, NY). Viable cells were semi-quantitatively determined by counting viable cells and total cells in three areas per scaffold cross-section (three sections/sample, N = 3).

4. Oil Red O staining

Oil Red O staining was performed to qualitatively examine accumulation of cytoplasmic lipid vesicles during adipogenesis. For 3D scaffolds, staining was performed on a thin cut slice of the construct in the same manner as for 2D cultures.

Briefly, 0.5 % (w/v) of stock solution of Oil Red O in isopropanol was diluted 3 : 2 with distilled water and filtrated to prepare Oil Red O working solution. Samples were rinsed with PBS, fixed with 3.7% formaldehyde and stained for 10 min with Oil Red O working solution. Residual dye was washed with distilled water. Counterstaining with haematoxylin was omitted since all the constructs showed strong background staining. Representative pictures of Oil Red O-stained cells were taken using an inverted microscope (NIKON Eclipse TE 300).

5. RNA isolation and cDNA synthesis

Total RNA was isolated from ASC cultures on days 0 (undifferentiated ASC), 7 and 14 using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). Additionally, RNA was isolated from mature adipocytes, and samples were pooled to obtain the reference value. The amount and purity of RNA were determined spectrophotometrically by measurement of A_{260} and $OD_{260/280}$ ratio, respectively.

Two hundred ng of total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. cDNA was stored at -20 °C before use.

6. Quantitative PCR

Gene expression levels were analysed with the ABI Prism® 7900HT Sequence Detection System and 2.3 SDS software (Applied Biosystems) using TaqMan® Gene Expression Assays for *ADIPOQ* (Hs00605917_m1), *LPL* (Hs00173425_m1), *PPARG* (Hs01115513_m1) and *VEGFA* (Hs00173626_m1) according to manufacturer's instructions. Samples were analysed in duplicates. As the expression of human *GAPD* (*GAPDH*) Endogenous Control (VIC/TAMRA Probe, Primer Limited, Applied Biosystems) was stable between experimental conditions, this gene was chosen for data normalization. Amplification efficiencies for all the genes, determined using standard curves run, were in the range of 88–98 % (data not shown). The Ct value of endogenous control was substracted to the Ct value of the sample, to derive ΔCt. Relative expression values indicate whether the gene of interest is expressed to a lower or higher extent than the endogenous control gene. The normalized level of expression of each gene was then calculated as $(1+Eff)^{abs(\Delta Ct)}$, reported in arbitrary units, and thus can only be used for comparative analysis among samples.

7. Statistical analysis

To assess differences in the differentiation capacity of ASC in various scaffolds and 2D monolayer culture, non-parametric Mann-Whitney test was used. All data are presented as median ± minimal and maximal value (N = 3).

Results

1. Macroscopic architecture of cell-scaffold constructs

The macroscopic architecture of each construct is shown in Fig. 1. The scaffolds were approximately 1 cm in diameter and had an equal form immediately after

fabrication. After one day, the polymerized human PPP had shrunk to about 30 % of its volume. However, after the initial shrinkage its volume remained the same until the end of the experiment. The rest of the scaffolds maintained the original form throughout the experiment. PPP, fibrin gel and collagen sponge were strong, compact and easy to handle. On the other hand, alginate constructs were difficult to handle and tore easily.

2. Cell morphology and viability inside the scaffolds

The cellular morphology and viability of the ASC after 7 and 14 days are shown in representative images of the scaffolds (Fig. 2). Cells were evenly distributed throughout all the scaffolds. In the alginate scaffold, the cells maintained rounded shape. On the other hand, cells

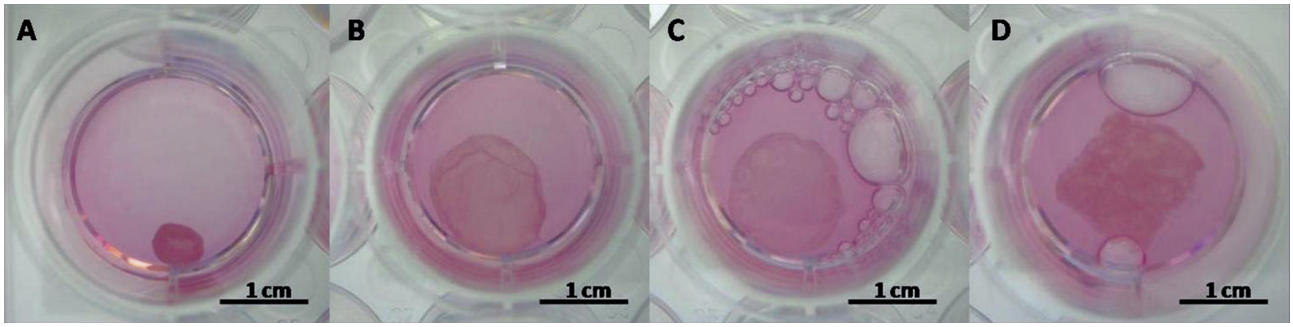

Fig. 1. The macroscopic architecture of different 3D scaffolds seeded with ASC after 14 days of culture: PPP (A), alginate (B), fibrin glue (C) and collagen sponge (D). Bars represent 1 cm.

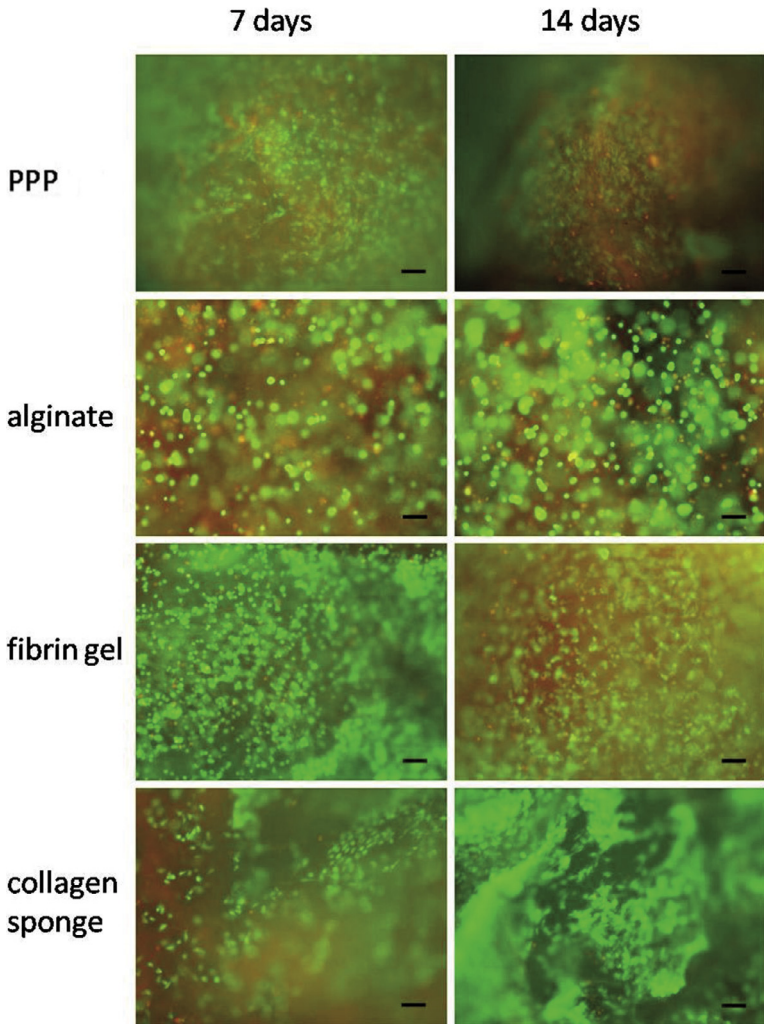

Fig. 2. Cell viability in 3D cultures at 7 and 14 days of culturing. Visualization was done using fluorescent microscopy ((green = live cells (calcein-AM), red = dead cells (ethidium homodimer-1)). Bars represent 100 µm.

seeded in the collagen sponge revealed fibroblastic shape. Morphologically in-between were the cells seeded in fibrin gel and PPP, where most of the cells were rounded while some of them were fibroblastic.

Live/dead staining revealed that the majority of the cells were viable both at 7 and 14 days after seeding. Qualitatively, the highest cell viability was observed in collagen sponge and fibrin gel, followed by PPP, while the lowest was observed in the alginate hydrogel.

3. Accumulation of lipid vesicles

Representative images of the Oil Red O-stained cells in different scaffolds and in conventional 2D cultivation conditions are shown in Fig. 3. The lipid accumulation could be visualized in all of the scaffolds already after 7 days of differentiation. At 14 day time-point, the cells had a more differentiated phenotype, with larger lipid droplets present within the cells. The extent of lipid accumulating cells was qualitatively similar in PPP and fibrin gel, both having higher extent than collagen sponge and alginate hydrogel. The collagen sponge showed lipid accumulation and intracellular distribution similar to conventional 2D cultivation, where cells containing small lipid droplets could be observed. In the alginate hydrogel, lipid accumulation could be visualized in only a small number of individual cells. However, in differentiated cells, the entire volume of the cell was filled with lipid vesicles.

4. Gene expression analysis

To quantify the extent of adipogenic differentiation, gene expression of three important adipogenic markers, namely, adiponectin (ADIPOQ), lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor γ (PPARγ), was analysed. All 3D culture systems supported higher adipogenic gene expression in compari-

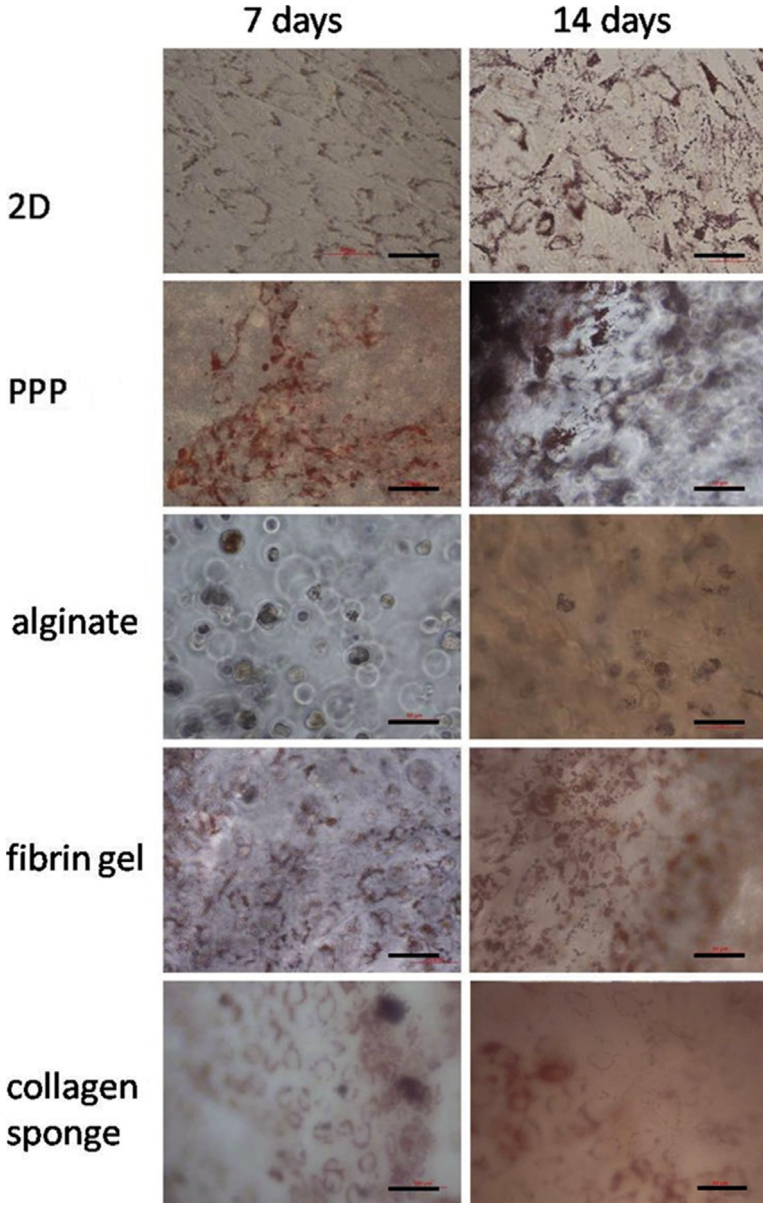

Fig. 3. Oil Red O staining of ASC-seeded scaffolds at 7 and 14 days of cultivation. Bars represent 50 μm.

son to 2D (monolayer) culture. Increased expression of adipogenic marker genes was observed with longer time in differentiation medium in all 3D culture systems, whereas in monolayer cultures it remained on the same level during the experiment. The highest expression of all three markers was found in PPP and alginate, while in fibrin gel and collagen sponge the expression was lower. The only statistically significant differences were observed when comparing the expression of *ADIPOQ* in the plasma, alginate and fibrin gel with the expression in 2D culture (Fig. 4). None of the tested scaffolds reached the level of expression of mature adipocytes.

To study the effect of adipogenic differentiation on the angiogenic capacity of ASC, expression of *VEGF* was measured in 2D monolayer culture and the four tested constructs. After two weeks, the expression of *VEGF* followed the expression of the three adipogenic markers. Highest levels were obtained in PPP and alginate. The expression level of *VEGF* was high in undifferentiated ASC and dropped after the first week of differentiation. After 14 days, the *VEGF* expression in PPP, alginate and fibrin gel increased again, while in 2D culture and collagen sponge the expression remained on the same level (Fig. 5).

5. Summary of results

The results are summarized in Table 1 for better transparency and easier evaluation of the scaffolds. Among all tested 3D scaffolds, PPP exhibited the poorest volume retention. In alginate, the highest scaffold fragility and lowest cell viability were observed. On the other hand, in alginate all the cells showed rounded morphology. The highest adipogenic differentiation and *VEGF* expression was observed in PPP, alginate and fibrin gel constructs. Collagen sponge had good mechanical properties and cell viability, but poor adipogenic differentiation.

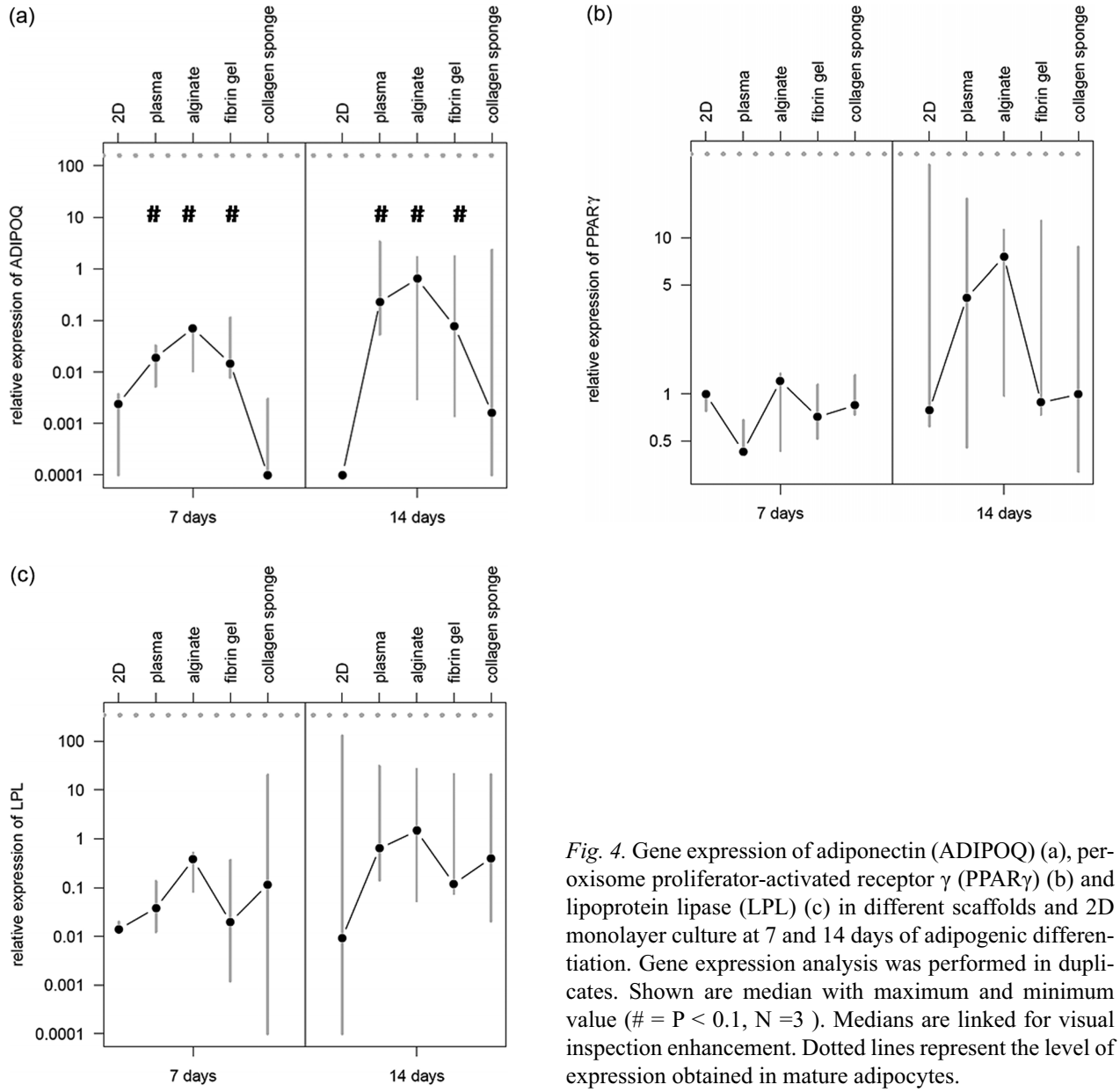

Fig. 4. Gene expression of adiponectin (ADIPOQ) (a), peroxisome proliferator-activated receptor γ (PPARγ) (b) and lipoprotein lipase (LPL) (c) in different scaffolds and 2D monolayer culture at 7 and 14 days of adipogenic differentiation. Gene expression analysis was performed in duplicates. Shown are median with maximum and minimum value (# = $P < 0.1$, N =3). Medians are linked for visual inspection enhancement. Dotted lines represent the level of expression obtained in mature adipocytes.

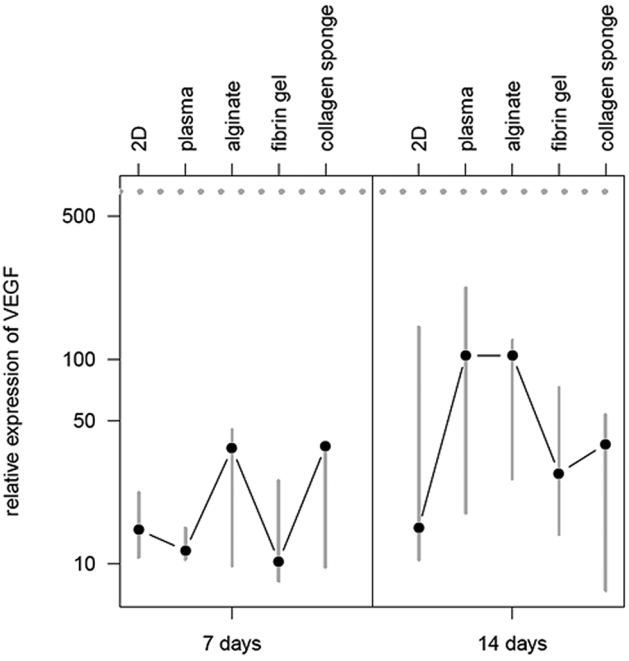

Fig. 5. Gene expression of VEGF in different culture systems at 7 and 14 days of adipogenic differentiation. Gene expression analysis was performed in duplicates. Medians with maximum and minimum value are presented. Medians are linked for visual inspection enhancement (N = 3). Dotted line represents the level of expression obtained in undifferentiated ASC.

Discussion

The needs in adipose tissue engineering have promoted testing of new scaffolds, in order to find the material that would promote cell attachment, maintain cell function and would as well be handled easily by the surgeons. Besides focusing on new innovative scaffolds, there should be more concentration on re-evaluation of the materials that have been used so far and have demonstrated promising results in *in vivo* testing as well as safe use in existing clinical applications (Hemmrich and von Heinmburg, 2006).

In our study, we have investigated four naturally derived biomaterials, already used by the surgeons for different clinical applications. Scaffolds were seeded with ASC and studied for their support and promotion of differentiation of cells into mature adipocytes. Scaffolds exhibiting different mechanical and morphological properties were selected. Hydrogel scaffolds as well as sponge-like scaffold were used. Among the selected products were off-the-shelf products as well as individually prepared products. For its easy derivation, we investigated human PPP as an autologous, easily prepared scaffold, which is cheap and plastic enough to allow the optimal diffusion of nutrients (Trombi et al., 2008). Instead of other plasma preparations (e.g. PRP – platelet-rich plasma), PPP was chosen to minimize the known inhibiting effect of PDGF on the adipogenic differentiation of ASC (Hauner et al., 1995; Koellensperger et al., 2006). Alginate is being used in a variety of medical applications because it gels under gentle conditions, has low toxicity and is readily available (Drury and Mooney, 2003). Fibrin gel is used routinely during operations since it promotes haemostasis, wound healing, tissue connection and prevents infections, and is therefore well used and accepted by clinicians (Srinivasan et al., 2009). Moreover, it has been previously investigated as a delivery vehicle for preadipocyte cultures (Schoeller et al., 2001; Wechselberger et al., 2002). Another widely used material is a collagen sponge, used for stopping internal bleedings and promoting tissue regeneration by supporting cellular ingrowth and new matrix synthesis (Silver and Pins, 1992).

All the scaffolds were investigated for the ease of handling, their support of ASC attachment, viability, and differentiation towards adipogenic lineage. Additionally, the effect of scaffolds on ASC secretion of angiogenic factor VEGF was studied.

The ease of handling and robustness of the scaffolds are very important to clinicians working with the endpoint product. The scaffolds we studied were robust, stable and easy to handle with the exception of alginate. The alginate scaffold was fragile, fragmented easily and needed to be handled with care. Another important scaffold property is the ability to maintain the original volume and shape. All the scaffolds, except for PPP, maintained their volume and shape. In our manufacturing conditions, PPP shrunk in the first 24 h to about 30 % of

Table 1. Summary of macroscopic construct characteristics, cell viability, morphology and differentiation potential of ASC cultured for 14 days in different culture systems

	2D	PPP	Alginate	Fibrin gel	Collagen sponge
Volume retention	n.a.	-	+++	+++	+++
Scaffold compactness	n.a.	+++	-	+++	+++
Cell viability	+++	++	+	+++	+++
Rounded cell morphology	-	++	+++	++	+
Lipid vacuole formation	+	+++	++	+++	+
Adipogenic gene expression	+	+++	+++	++	++
VEGF expression	+	+++	+++	++	++

+++ strong positive effect; ++ moderate positive effect; + weak positive effect; - negative effect; n.a. not applicable

its original volume, which is unsuitable for any kind of reconstructive surgery.

As cell-matrix interactions regulate a variety of cellular responses including survival and proliferation (Gumbiner, 1996), cell morphology and viability were investigated. The highest cell viability was found in the scaffolds where at least a part of the cell population retained fibroblastic morphology. These results are in accordance with the study of Flynn et al. (2007), where lower cell viability was observed when ASC were encapsulated in the scaffold in comparison to cells adhered to the scaffold matrix.

Cell-matrix interactions also affect the differentiation capacity of the cells (Spiegelman and Ginty, 1983; McBeath et al., 2004). Therefore, Oil Red O staining was performed to qualitatively assess the extent of differentiation in different scaffolds. In all cultivation conditions there was an obvious increase in accumulation of lipid droplets with time of differentiation. The morphology and extent of lipid accumulation differed heavily between 2D culture and 3D scaffolds. Poorer differentiation was obtained in 2D culture in comparison to 3D scaffolds, as also observed by Stacey et al. (2009). ASC in the collagen sponge had similar morphology and extent of differentiation as it was seen in 2D culture. This observation could be explained by the fact that PPP, alginate and fibrin glue were true 3D scaffolds, where the cells were in contact with the scaffold throughout their surface. On the other hand, collagen sponge was a porous sponge, where cells attached to the scaffold in a comparable manner as they attach to the culture dish in 2D culture. A similar observation was reported by Flynn et al. (2007), where non-adhesive scaffold promoted adipogenic differentiation in comparison to cell-adhesive conditions. Among the three 3D scaffolds, PPP and fibrin gel had higher numbers of differentiated cells than alginate, probably due to low cell viability observed in the alginate scaffold. However, the extent of lipid accumulation in the remaining cell population in the alginate was high since all the intracellular space was occupied with large red-stained lipid vacuoles, indicating a more differentiated phenotype.

Quantification of adipogenic differentiation was assessed by quantitative PCR assay. We determined the expression of three genes involved in different stages of adipogenic differentiation. The peroxisome proliferator-activated receptor γ (PPARγ) induces adipocyte differentiation by playing a key role in regulating several genes critical to adipogenesis, lipid uptake and lipid metabolism (Schoonjans et al., 1996; Rosen et al., 1999) and is regarded as a mid differentiation marker (Flynn and Woodhouse, 2008). Lipoprotein lipase (LPL) is expressed in the early stages of adipocyte development, while adiponectin (ADIPOQ) represents one of the terminal differentiation markers for adipogenesis (Flynn and Woodhouse, 2008). As expected, in comparison to 2D culture conditions, expression of all three genes was higher in the scaffold cultures. The difference was statistically significant ($P < 0.1$) only for *ADIPOQ* expression. Differences between 7 and 14 days of cultivation were more pronounced for *ADIPOQ* and *PPARG* gene expression, revealing more mature differentiation stages, especially in the case of PPP and alginate culture. A small increase of *LPL* expression from day 7 to day 14 was also noted for all the scaffolds, but not for 2D culture conditions. Due to high interindividual variability of the primary culture cell samples the differences in gene expression among different scaffolds were not statistically significant. Nevertheless, comparison of median values on day 14 for *ADIPOQ* expression showed that most adipocyte-like cells were obtained in alginate, PPP and fibrin gel scaffolds. This finding is in accordance with the results obtained with Oil Red O staining. The full adipocyte phenotype was not reached in any of the tested scaffolds. This lower extent of differentiation might be a consequence of different *in vivo* and *in vitro* adipocyte development, as discussed by Soukas et al. (2001) and the fact that *in vitro* differentiation conditions are not adequately mimicking *in vivo* conditions.

The absence of adequate blood supply is thought to be responsible for progressive resorption of adipose tissue construct when implanted *in vivo* (Patrick, 2000; Sterodimas et al., 2009). Thus, the vascularization of engineered adipose tissue is a crucial step for graft survival after the implantation. Several groups have demonstrated that ASC secrete numerous factors involved in new blood vessel formation (Rehman et al., 2004; Nakagami et al., 2006; Murohara, 2009). Recently, a significant increase of VEGF secretion was demonstrated during short-term adipogenic differentiation of ASC in 2D culture conditions (Verseijden et al., 2009). In our culture system, however, we observed decreased *VEGF* expression after adipogenic differentiation of ASC in 2D culture conditions. We hypothesized that 3D environment, as it promotes adipogenesis of ASC, would also promote secretion of angiogenic factors such as VEGF. Therefore, we investigated which of the tested scaffolds best supported the angiogenic activity of differentiating ASC by *VEGF* expression. In agreement with the results obtained for adipogenic marker expression, 3D environment positively stimulated the expression of *VEGF*. Expression of *VEGF* increased from 7 to 14 days of adipogenic differentiation, reaching the highest level in PPP and alginate hydrogel. Again, due to high interindividual variability among primary cell samples, no statistically significant differences could be confirmed. In any case, the observed stimulating environment of 3D scaffold during short-term adipogenic differentiation of ASC suggests potentially better angiogenic response of engineered tissue after *in vivo* implantation.

In conclusion, according to all summarized results, we would encourage the use of fibrin gel in adipose tissue engineering, as it has good mechanical properties, preserves high cell viability and stimulates adipogenic and angiogenic factor secretion. Certainly, for long-term stable adipose tissue formation, additional *in vivo* experiments should be performed.

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