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ORIGINAL ARTICLE

Stem Cells, Mature Adipocytes, and Extracellular Scaffold: What Does Each Contribute to Fat Graft Survival?

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Abstract
Background Soft tissue engineering offers new perspectives for improving fat graft survival, for which the appropriate association of cells and scaffold seems essential. This study aimed to analyze the survival of free-cell grafts compared with adipose-derived stem cells (ASCs) seeded on collagen scaffolds.
Methods Adipose tissue from a single volunteer was used for the following preparations: purified adipose tissue, isolated mature adipocytes (free-cell graft), cultured ASCs without scaffold (free-cell graft), collagen scaffold only, cultured ASCs in collagen scaffold without and with bioactive factors, and freshly-isolated ASCs in collagen scaffold. These were grafted on 18 nude mice for 2 months, after which specimens were evaluated grossly and histologically using hematoxylin-phloxine-safran (HPS), Oil-Red-O, and antivimentin labeling. Specimens and animals were weighed before implantation and after explantation, and weight values were statistically analyzed.
Results Free-cell grafts (mature adipocytes and free ASCs) showed complete resorption in 50 and 60% of the animals (remaining weight fraction was 22.5 and 5.3%, respectively). The survival of purified adipose tissue was 81.8% (statistically greater compared with free-cell grafts; $p < 0.05$). In the ASCs–scaffold association, the remaining weight fractions (87.3–70.4%) were statistically greater than in free-cell grafts (5.3–22.5%; $p < 0.05$), but the difference between ASC–scaffolds and fat grafts was not statistically significant. These results were confirmed by clinical and histologic observations.
Conclusion Three-dimensional collagen scaffolds seem to improve survival of ASCs compared with free-cell grafts (adipocytes and free ASCs).

Keywords Adipose-derived stem cells · Collagen scaffold · Fat graft · Survival

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Restoring soft tissue volume and defects remains a challenge in plastic surgery [12]. Both synthetic and natural materials are used as fillers, but satisfactory outcomes remain elusive [8, 25] due to resorption, complications, or both associated with foreign body reactions [1, 30].

Autologous fat transfer seems to be regaining popularity, but resorption rates remain unpredictable [18]. To increase fat graft survival, autologous transplantation and adipose tissue engineering are investigated [32].

Concerning autologous transplantation, Yoshimura et al. [37] proposed cell-assisted lipotransfer to decrease resorption rates by adding adipose-derived stem cells (ASCs) to the fat graft. The ASCs would differentiate into mature adipocytes, stimulate in vivo regeneration by

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triggering migration of host stem cells to the recipient site, and promote vascularization, which is essential for fat graft take [11, 20].

In the field of tissue engineering, adipose tissue constructs offer good perspectives for improving survival rates [19]. Engineered adipose substitutes capable of predictably imparting soft tissue volume and shape would be of significant value in both reconstructive and cosmetic applications [15, 23]. This is especially true for lean patients with a thin subcutaneous tissue layer, in whom fat grafting is difficult to perform due to the limited amount of fat tissue.

Adipose-derived stem cells are particularly promising as a cell source for a range of regenerative strategies, including adipose tissue engineering [10]. Seeded on various scaffolds, ASCs can differentiate into mature adipocytes in vivo [30]. The interest in ASCs becomes even greater because fat tissue is the most abundant and accessible source of multipotent cells [39].

Creating and maintaining the shape and dimensions of the engineered soft tissue are perhaps best accomplished by scaffold biomaterials [30], and ASCs have been associated with various scaffolds used to achieve the corresponding tissue substitute. Decellularized placenta, cartilage, and bone have been investigated in combination with mesenchymal stem cells (including ASCs) for adipogenic, chondrogenic, and osteogenic applications, respectively [7, 36, 38].

Supronowicz et al. [31] studied ASCs cultured on demineralized bone matrices that developed new bone matrix in vitro and in vivo. Gastaldi et al. [9] proposed that ASCs seeded on titanium scaffolds are able to produce a calcified extracellular matrix with proteins (fibronectin, osteocalcin, osteonectin, osteopontin, and type 1 collagen), suggesting that this scaffold–cell construct is effective for regenerating damaged bone tissue.

In soft tissue reconstruction, obvious strategies include injecting hydrogels containing preadipocytes and adipogenic/angiogenic factors or implanting a thin, flexible fabric or felt composed of a biodegradable polymer or polymer blend that can be pre-seeded with preadipocytes. The preadipocytes can be isolated from fat tissue harvested from a patient by liposuction or fat biopsy [23].

The creation of an adequate fat tissue construct capable of restoring both volume and function depends on the capacity of preadipocytes to adhere to appropriate support structures, or scaffolds. A support structure is required for cells to migrate and proliferate and to provide a tissue equivalent for final overall tissue shape. Although various implanted or injected scaffolds have been used, the optimum scaffold for adipose tissue engineering remains elusive [23]. Furthermore, the interactions between cell–cell and cell–matrix are not clearly elucidated.

Flynn [6] showed that fat tissue can be used both as scaffold and as a source of ASCs, opening promising perspectives in both autologous fat grafting and tissue engineering. In this sense, it would be interesting to investigate the survival rates of adipocytes and ASCs implanted separately and in association with a supporting scaffold. This study was designed to investigate and analyze the behavior of adipocytes and ASCs grafted alone or on three-dimensional (3D) scaffolding (natural in fat grafts or collagen) using in vivo models.

Materials and Methods

Nude mice were used as recipients after institutional board approval. The following preparations were grafted into the mice:

1. Purified adipose tissue (i.e., fat grafts)
2. Isolated mature adipocytes without a collagen scaffold
3. Cultured ASCs (passage 2) without a collagen scaffold
4. Collagen scaffold only (i.e., negative control)
5. Cultured ASCs (passage 2) without growth factors in a collagen scaffold
6. Cultured ASCs (passage 2) with growth factors in a collagen scaffold
7. Freshly isolated ASCs in a collagen scaffold.

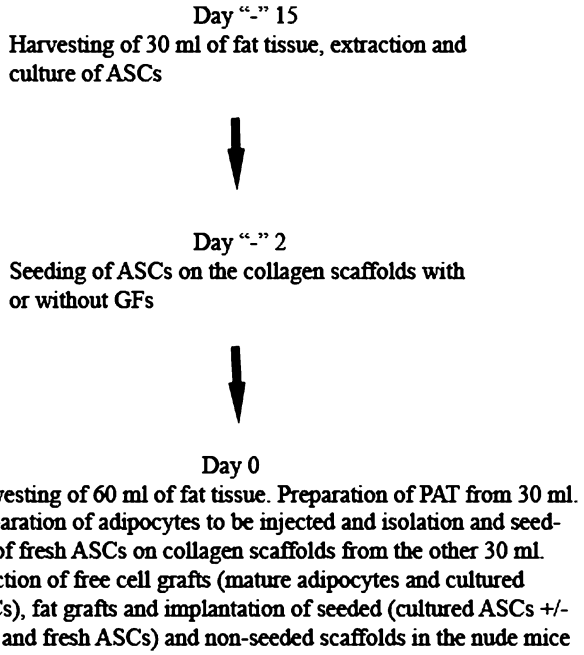

Fig. 1 Timing for the preparation of the various types of grafts to be injected or implanted in the nude mice. *ASCs* Adipose-derived stem cells, *GFs* bioactive factors, *PAT* purified adipose tissue (fat graft)

These were prepared from two portions of fat harvested from the same donor at different times, as described in the next section (Fig. 1).

Sample Preparation

Fat tissue was harvested (manually or via syringe suction) from the abdominal region of a single donor: a 35-year-old, healthy, adult, female volunteer. She had a height of 172 cm and a weight of 72 kg (body mass index [BMI] = 24.91 kg/m^2). Two harvesting procedures were performed: 30 ml of fat tissue was harvested 15 days before grafting, and then 60 ml was harvested on the day of grafting (these are quantities after centrifugation in the operating room).

After centrifugation (3,000 rpm for 3 min), the upper oily and lower debris layers were removed [3], and the purified fat was sent to the laboratory. The first portion of adipose tissue (30 ml harvested 15 days before grafting) was used for ASC cultures. The cultured ASC would be seeded on collagen scaffolds (preparations 3, 5, and 6).

The second portion of fat tissue (60 ml harvested on the day of grafting) was processed as follows. The first 30 ml was preserved at 37°C after centrifugation at 3,000 rpm for 3 min [3]. This would be injected as a fat graft (preparation 1). The second 30 ml was sent to the laboratory and used for preparation of free mature adipocytes (preparation 2) and fresh ASCs (to be seeded on collagen scaffolds, preparation 7). Mature adipocytes (preparation 2) were obtained from the supernatant after collagenase digestion. They were placed in 1-ml syringes for grafting.

Isolation of ASCs

The adipose tissue from the operating room was placed in a 37°C shaking water bath for 30 min in collagenase containing medium (0.225 U/mg of collagenase; Roche Diagnostics, Mannheim, Germany). Centrifugation was used to separate the mixture into layers. The supernatant containing mature adipocytes and the middle layer containing the media and products of digestion were suctioned off, leaving a cell pellet. The remaining red blood cells were removed by suspending the cell pellet in a lysis buffer (NH_4CL-based) for 10 min at 37°C. This was followed by centrifugation, resulting in a pellet referred to as the stromal vascular fraction (SVF), which contained the ASCs.

ASC Cultures

Only the ASCs harvested 15 days before grafting were cultured. The SVF was cultured in Dulbecco modified Eagle's medium (DMEM) with glutamax (Gibco Laboratories, Gaithersburg, MD, USA)/F12-based proliferation medium supplemented with 10% fetal bovine serum (Foetalclone II; Hyclone, Logan, UT, USA), 10 ng/ml of basic fibroblast growth factor (bFGF), antibiotics (100 IU of penicillin; Sarbach, Chatillon sur Chalarone, France), and 20 μg/ml of gentamycin (Panpharma, Fougères, France).

Cells from the SVF were seeded at a density of 7×10^3 cells/cm^2 in proliferation medium. Once the cultures reached confluence, the cells were trypsinized for further processing. A portion of the cultured ASCs was seeded on collagen scaffolds 2 days before grafting. Another portion was used as free ASCs (implanted without scaffolds, preparation 3). These were prepared from passage 2 cultured ASCs (at a concentration of 10^6) and resuspended in 0.2 ml of medium the day of grafting. This suspension would be injected in the mice as a free-cell graft.

Seeded Collagen Scaffolds

Adipose-derived stem cells were seeded on synthetic collagen circular scaffolds (4 cm^2 × 4 mm high) (LPI, Lyon, France). These scaffolds represent sponges composed of types 1 and 3 bovine collagen (72%), glycosaminoglycan (8%), and cross-linked chitosan (20%) [4]. They are fabricated as 12-well plates containing the collagen sponges (4 cm^2 × 4 mm high) or as single sponges with the same dimensions. These collagen sponges can be produced with other dimensions if needed.

In this experiment, collagen scaffolds were seeded in the 12-well plates, and each seeded sponge was implanted in the nude mice separately. Pure collagen scaffolds were implanted as controls (preparation 4). Next, collagen scaffolds were seeded with cultured ASCs (preparations 5 and 6). Finally, collagen scaffolds were seeded with freshly isolated ASCs (preparation 7).

At the confluence in passage 1, cultured ASCs were trypsinized. Trypsin containing 0.01% ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and 10^6 ASCs were seeded into each scaffold in passage 2. Ten scaffolds were seeded with cultured ASCs and bioactive factors (dexamethasone 25 nM, insulin 0.12 UI/ml, and triiodo-thyronine T3 2 nM). Ten other scaffolds were seeded with cultured ASCs that had no bioactive factors. On the day of grafting, the freshly isolated ASCs (see Sample Preparation section) were seeded on collagen scaffolds at a density of 10^6 cells per scaffold.

Graft Implantation

Eighteen 42-day-old male nude mice (NU/NU Nude Mouse Crl:NU-*Foxn1*^{nu}, Charles River, Wilmington, MA, USA) were grafted under anesthesia induced by 30 mg/kg of intraperitoneal pentobarbital. Each mouse received 4 specimens, and two mice received 3 specimens, for a total of 70 specimens. The specimens were implanted in the

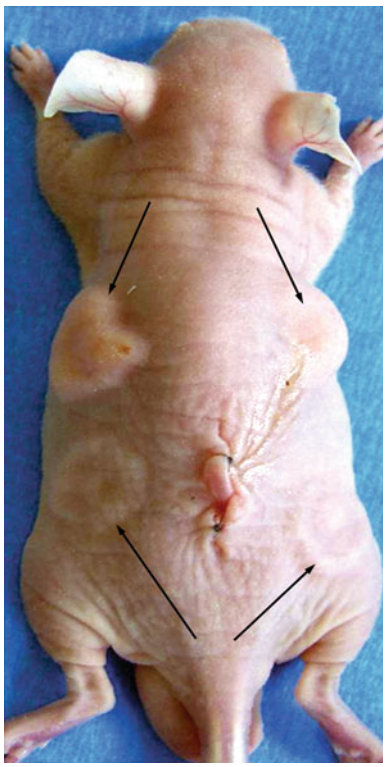

Fig. 2 Implantation and injection of the studied preparations. The scaffolds were inserted through a single dorsal incision (the sutured incision is visible in the middle of the mouse back). Fat tissue and free-cell grafts were injected. Note the four tumefactions on the right and left sides (*black arrows*). The *upper left* tumefaction represents injected fat tissue

subcutaneous plane. The distribution of the grafts in the mice was randomly accomplished.

Scaffolds were introduced through a dorsal incision under sterile conditions. Fat tissue, mature adipocytes, and cultured ASCs were injected using 18-gauge needles (Fig. 2). Both the grafts and the mice were weighed before and after implantation to record the differences in graft and animal weight. The graft weight among the various preparations was constant before implantation.

Evaluation Criteria

The mice were killed via intraperitoneal pentobarbital overdose injection 8 weeks after grafting. The methods of evaluation were

- Clinical criteria including macroscopic evaluation of specimens and animals, weighing, and photography
- Histologic evaluation.

Clinical Criteria

Macroscopic Evaluation After explantation, the specimens were grossly evaluated for newly formed fat tissue and vessels (angiogenesis). Observations were recorded and photographed.

Mouse and Implant Weight The mice were weighed before and after implantation as well as before and after explantation. The individual and average weights for each group were recorded. The implants and scaffolds were weighed before implantation and after explantation. All the specimens had constant weight before implantation (and consequently, constant individual and mean weights, see the Results section). After explantation, all the specimens (if not completely resorbed) were weighed, and the individual and mean weights were recorded. The weight loss of the specimens was computed for each mouse (value before implantation minus value after explantation). The remaining fraction of weight was calculated as the ratio of values after explantation and before implantation. The animals and implants were weighed with a precision balance.

Photography Photographs of the scaffolds were taken before implantation and after explantation. Photographs of the mice were taken before and after implantation.

Histology and Immunohistochemistry

On the average, 20 microtome sections per graft from the periphery and the center of the samples were included.

Hematoxylin-Phloxine-Safran (HPS) Staining Samples were fixed in 10% formalin solution (Sigma Chemical Co., St. Louis, MO, USA). After dehydration, they were included in paraffin blocks. Slices of 5 µm were stained with HPS. Hematoxylin stained the cellular nucleus blue, whereas phloxine stained the cytoplasm pink, and saffron stained the connective tissue orange.

Human Antivimentin Antibody Paraffin slices were labeled with monoclonal antibody specific for human vimentin (clone V9; Dako, Heverlee, Belgium) at a dilution of 1–50. Detection of the primary antibody was achieved with a biotinylated secondary antibody, horseradish peroxidase-conjugated streptavidin, and diaminobenzidine (DAB) substrate.

Finally, tissue sections were counterstained using Harris hematoxylin (Sigma) and mounted using Faramount aqueous mounting medium (Dakocytomation). Vimentin, known as intermediate filament polypeptide, typically is present in cells of mesenchymal origin. This fact was used to evaluate the presence and evolution of the human ASCs because these are cells of mesenchymal origin. Labeled with antihuman antivimentin antibodies, only the mesenchymal cells of human origin were stained. In this way, proliferation of mesenchymal cells of murine origin could be excluded.

Oil-Red-O Staining All the samples were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA, USA). Then 7-μm slices were fixed in isopropanol and stained with Oil-Red-O solution (10 mg/ml; Sigma). Oil-Red-O is adipocyte specific and stains lipid-filled vacuoles red [19].

Phenotype Analysis

Phenotype analysis was performed by flow cytometry as previously described [17]. The following mouse monoclonal antibodies were used: phycoerythrin (PE)-conjugated anti-CD14 (Abcam, Paris, France), fluorescein isothiocyanate (FITC)-conjugated anti-CD45, FITC-conjugated anti-CD73, PE-conjugated anti-CD90 (BD Biosciences, Le point de Claix, France), PE-conjugated anti-CD105 (Caltag Laboratories, Burlingame, USA), FITC-conjugated anti-HLA DR, and PE-conjugated anti-HLA ABC (Beckman Coulter, Roissy CDG, France).

Aliquots were analyzed with fluorescence-activated cell storing (FACS) using FACS Aria cell sorter and FACS Diva software (BD Bioscience, San Jose, CA, USA). Data were registered as the relative mean fluorescence intensity of the indicated markers compared with untreated cells and tested culture medium.

Statistical Analysis

Descriptive statistics were used to record and calculate the individual and mean weights of the specimens before implantation and after explantation. The remaining fraction of weight (ratio between weights after explantation and before implantation) for the different groups was computed with corresponding 95% confidence intervals and expressed in box plots of the remaining fractions of weight. Nonparametric statistics were used to evaluate the significance of the weight loss in the various groups. The nonparametric Wilcoxon signed rank test was used to compare the median values of the remaining fractions of weight. Statistical analyses were performed using SPSS (SPSS Inc., 12.0.0, Chicago, IL, USA) with two-sided p values (threshold for significance, 0.05).

Results

All the mice had survived at 8 weeks. The average mouse weight increased from 26.5 to 33.7 g (±7.2 g).

Purified Adipose Tissue

Ten mice received 0.2 ml of purified fat tissue. Each portion (of 0.2 ml) weighed 0.250 g before injection. The median weight of the explanted adipose tissue was 0.205 g (Table 1), and the remaining weight fraction was 81.8% (Table 2).

The grossly explanted fat was homogeneous and yellow with well-visible, abundant, newly formed vessels (Fig. 3). Histologically, this tissue presented the 3D structure of normal adipose tissue. Both Oil- Red-O and HPS stains confirmed the presence of large adipocytes (Fig. 4). Abundant extracellular matrix (colored orange) surrounded the adipocytes. The antihuman vimentin labeling was negative, suggesting the host origin of this matrix.

Mature Adipocytes

Ten mice received 0.2 ml of mature adipocytes with a pregrafting weight of 0.176 g. Five specimens (50%) were

Table 1 Weight repartition of various specimens between mice after explantation (in grams)

N	PAT	MA	Cultured ASCs	Scaff	Cultured ASCs + Scaf	Cultured ASCs + Scaf + GF	Fresh ASCs + Scaf
1	0.456	0.082	0.000	0.084	0.072	0.082	0.068
2	0.206	0.026	0.016	0.093	0.168	0.004	0.078
3	0.125	0.209	0.024	0.069	0.100	0.185	0.139
4	0.086	0.056	0.055	0.112	0.085	0.123	0.095
5	0.146	0.024	0.013	0.099	0.102	0.081	0.065
6	0.255	0.000	0.000	0.077	0.125	0.097	0.070
7	0.169	0.000	0.000	0.103	0.103	0.105	0.153
8	0.190	0.000	0.000	0.084	0.097	0.112	0.089
9	0.216	0.000	0.000	0.077	0.123	0.087	0.099
10	0.197	0.000	0.000	0.092	0.102	0.098	0.010
Mean[a]	0.205 ± 0.101	0.040 ± 0.066	0.011 ± 0.018	0.089 ± 0.088	0.108 ± 0.026	0.097 ± 0.045	0.087 ± 0.040

N Mice identification number, *PAT* purified adipose tissue, *MA* mature adipocytes, *ASCs* adipose-derived stem cells, *Scaf* collagen scaffold (negative control), *GF* growth factors

[a] Mean ± standard deviation

Table 2 Weight evolution of adipose tissue specimens before grafting and after explantation (in grams)

Adipose tissue specimens	PAT	MA	Cultured ASCs	Scaff	Cultured ASCs + Scaf	Cultured ASCs + Scaf + GF	Fresh ASCs + Scaf
Weight before implantation	0.250	0.176	0.200	0.123	0.123	0.123	0.123
Weight after explantation	0.205	0.040	0.011	0.089	0.108	0.097	0.087
Weight loss[a]	0.045	0.136	0.189	0.034	0.016	0.026	0.036
Remaining fraction of weight[b]: % (95% CI)	81.8 (52.9–110.6)	22.5 (0–49.3)	5.3 (0–11.6)	72.2 (64.4–80.0)	87.3 (72.0–102.7)	79.1 (53.2–105.0)	70.4 (47.1–93.7)

PAT Purified adipose tissue, *MA* mature adipocytes, *ASCs* adipose-derived stem cells, *Scaf* collagen scaffold (negative control), *GF* growth factors, *CI* confidence interval

[a] Weight loss = value before implantation − value after explantation

[b] Remaining fraction of weight = value after explantation/value before implantation

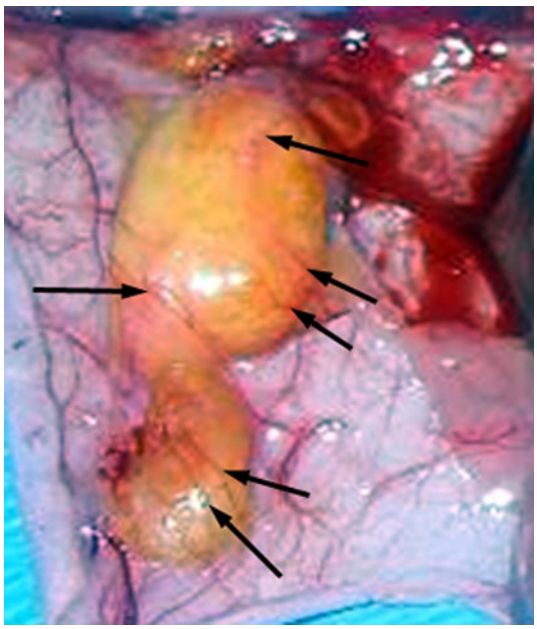

Fig. 3 Purified adipose tissue at explantation (macroscopic view). Abundance of newly formed vessels around the fat tissue can be observed (*black arrows*)

completely resorbed. The median weight of the specimens after explantation was 0.040 g, and the remaining weight fraction was 22.5% (Table 2). In the five cases of nonresorbed adipocytes, the explanted tissue was scarce and fibrous. Oil-Red-O staining showed a small number of lipid vacuoles, and HPS staining showed no newly formed adipose tissue. Newly formed vessels were scarce.

Cultured ASCs Without a Scaffold

The weight of each graft before implantation was 0.2 g. The median weight of the specimens after explantation was 0.011 g, and the remaining weight fraction was 5.3% (Table 2). Complete resorption of the graft was found in six cases (60%). When tissue was present, it was fibrous with a small number of adipocytes (Oil-Red-O) and newly formed vessels.

Negative Control Scaffold

Before implantation, the scaffolds weighed 0.123 g. The median weight after explantation was 0.089 g, and the remaining weight fraction was 72.2% (Table 2). The control scaffolds appeared white and nonvascularized, without any macroscopic evidence of newly formed adipose tissue (Fig. 5). Histologic examination (Oil-Red-O and HPS staining) showed very few cells (Fig. 6), and the antivimentin labeling was negative, suggesting that the few cells present were colonizers from the host.

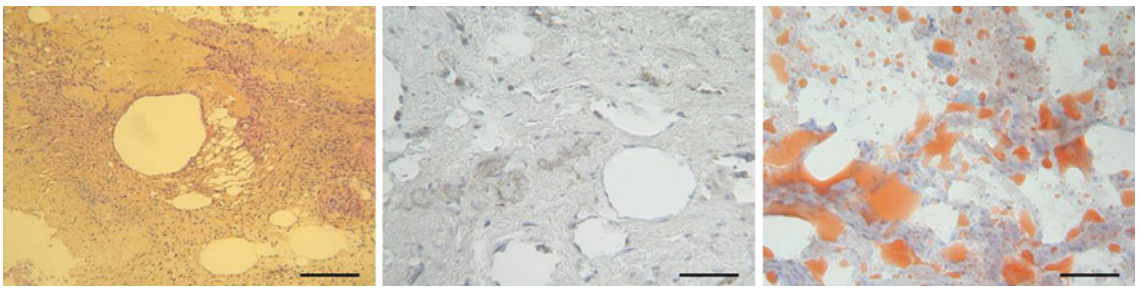

Fig. 4 Purified adipose tissue at explantation (microscopic view; scale, 200 μm). Both hematoxylin-phloxine-safran (HPS) (*left*) and Oil-Red-O (*right*) staining confirmed the presence of large adipocytes (*middle*). The negative antivimentin staining suggests that precursor cells were transformed into mature adipocytes

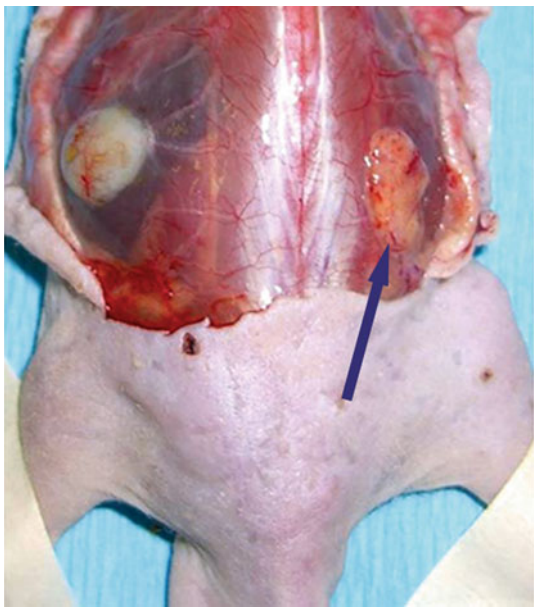

Fig. 5 A nonseeded scaffold (*left*) and a scaffold seeded with adipose-derived stem cells (ASCs) that have no bioactive factors (*arrow*) at explantation (macroscopic view). On the *left side* of the mouse, the nonseeded collagen scaffold remains white without any fat tissue growth in it. The ASC-seeded scaffold is completely colonized by abundant yellow fat tissue (*arrow*)

Scaffold Seeded with Cultured ASCs Without and with Bioactive Factors

The scaffold weight before implantation was 0.123 g in both groups. The median weights after explantation were 0.108 and 0.097 g, respectively, and the remaining weight fractions were respectively 87.3 and 79.1% (Table 2). The explanted specimens showed a macroscopic view of normal adipose tissue (Fig. 5), with both central and peripheral newly formed vessels (angiogenesis).

The newly formed tissue resembled grossly mature human fat tissue. By HPS staining, complete settlement of the scaffold was shown. Abundant newly formed extracellular matrix filled the scaffold spaces, stained in orange

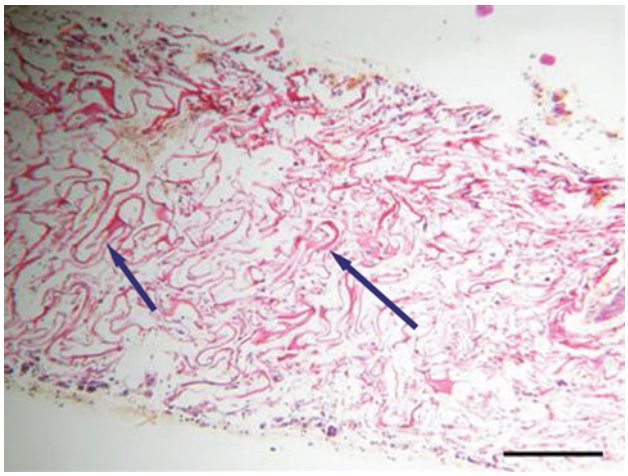

Fig. 6 Unseeded collagen scaffold after explantation (microscopic view, hematoxylin-phloxine-safran [HPS] staining; scale, 200 μm). Note the collagen matrix (*arrow*, stained in *red*) with empty spaces and few cells

on the HPS (Fig. 7). Oil-Red-O staining confirmed the presence of abundant mature adipocytes. Antivimentin labeling showed cells of human origin (Fig. 7).

Scaffold Seeded with Freshly Isolated ASCs (Stromal Vascular Fraction)

The initial weight of the specimens was 0.123 g, and their median weight after explantation was 0.087 g (Table 1). The remaining weight fraction was 70.4% (Table 2). Although the cells settled all areas of the scaffolds (HPS staining), these cells were markedly fewer compared with the other ASC-seeded scaffolds. Extracellular matrix also was less abundant (spaces were less occupied by orange-stained connective tissue, HPS). Oil-Red-O stain showed mature adipocytes in smaller numbers than in the other ASC/scaffold groups. Human antivimentin labeling showed cells of human origin (Fig. 7). The overall data for the clinical and histologic evaluation are summarized in Table 3.

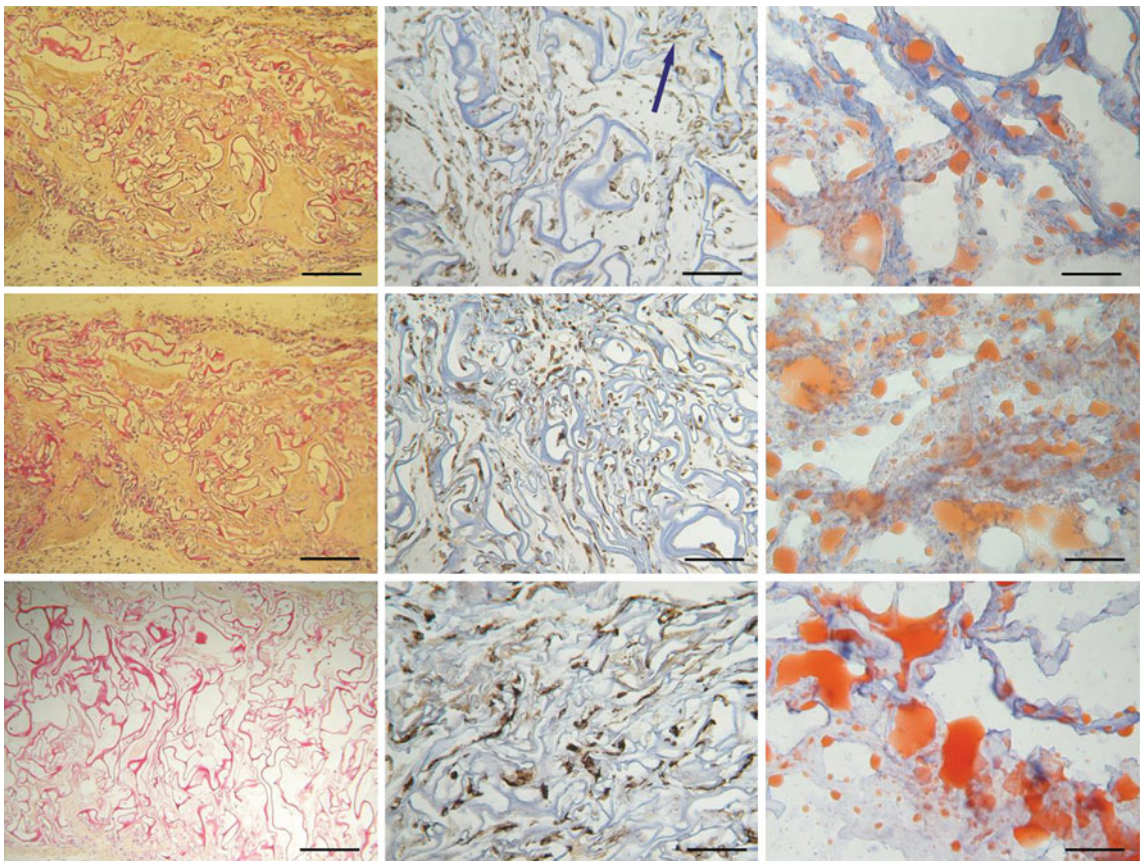

Fig. 7 *Upper row*: Scaffold seeded with adipose-derived stem cells (ASCs) that have no bioactive factors after explantation (microscopic view; scale, 200 μm). The hematoxylin-phloxine-safran (HPS) staining (*left*) shows that spaces in the collagen scaffold (stained in *red*) are filled with connective tissue (stained in *orange*) that has numerous cells (stained in *blue*). The antivimentin (*middle*) shows abundant cells of human origin (*middle*, *blue arrow*, cells are *brown* stained). Oil-Red-O staining confirms the presence of mature adipocytes via the *red*-stained lipid vacuoles (*right*). *Middle row*: Scaffold seeded with ASCs that have bioactive factors after explantation (microscopic view; scale, 200 μm). HPS staining (*left*) shows cells throughout the scaffolds (stained in *blue*). The spaces in the collagen scaffold (stained in *red*) are filled with abundant connective tissue (stained in *orange*). Human antivimentin labeling (*middle*) shows cells of human origin (cells stained in *brown*), and Oil-Red-O stain (*right*) shows the presence of mature adipocytes (*red* staining). *Lower row*: Scaffold seeded with fresh ASCs after explantation (microscopic view; scale, 200 μm). HPS staining (*left*) shows cells throughout the scaffolds. Human antivimentin labeling (*middle*) shows cells of human origin (cells in *brown*), and Oil-Red-O stain (*right*) shows the presence of mature adipocytes (*red* staining)

Phenotype Analysis with Flow Cytometry

The results of the flow cytometry showed positive expression of CD70, CD90, CD105, and HLA ABC, which are specific markers for ASC [17]. In addition, the ASC specimens were negative for CD14, CD45, and anti-HLA DR, which are hematopoetic and endothelial cell markers.

Statistical Analysis

The weight evaluation of each type of specimen is presented in Table 1, and the mean weight for each type is presented in Table 2. The mean weight fraction (range, 5.3–87.3%) (Table 2) of free-cell grafts (mature adipocytes and cultured ASCs) was significantly smaller compared with the other groups ($p < 0.05$, Sign test). There was a tendency toward higher weight loss in cultured ASCs than in mature adipocytes ($p = 0.06$, Sign test) (Fig. 8). No significant difference was found between the other groups.

Discussion

Soft tissue engineering offers new perspectives for improved fat graft survival. Appropriate association of cells and supporting material (scaffold) are essential. In this sense, coordination between cell source, scaffold material, cellular environment, and means of delivery must be successful [5, 28, 32]. Our study aimed to compare two of these components: the cell source and the scaffold material.

Table 3 Summary of the postexplantation data in the various groups

Adipose tissue specimens	PAT	MA	Cultured ASCs	Scaf	Cultured ASCs + Scaf	Cultured ASCs +Scaf + GF	Fresh ASCs + Scaf
Complete resorption (%)	0	50	60	0	0	0	0
Gross examination	Well-vascularized fat	Scarce and fibrous tissue	Scarce and fibrous tissue	White, non-vascularised tissue	Adipose tissue grown in the scaffold		
Histologic examination	Normal fat tissue with good vascularization	Scarce lipid vacuoles	Scarce no. of adipocytes and newly formed vessels	Very few cells–colonizers from the host (negative antivimentine)	Complete settlement of the scaffold with cells of human origin (positive antivimentine). Presence of mature adipocytes. Abundant newly formed ECM and vessels		Markedly fewer cells of human origin than in the groups of ASC-seeded scaffolds. Less abundant mature adipocytes and ECM
Remaining weight fraction[a], % (95% CI)	81.8 (52.9–110.6)	22.5 (0–49.3)	5.3 (0–11.6)	72.2 (64.4–80.0)	87.3 (72.0–102.7)	79.1 (53.2–105.0)	70.4 (47.1–93.7)

PAT Purified adipose tissue, *MA* mature adipocytes, *ASCs* adipose-derived stem cells, *Scaf* collagen scaffold (negative control), *GF* growth factors, *ECM* extracellular matrix, *CI* confidence interval

[a] Remaining fraction of weight = value after explantation/value before implantation

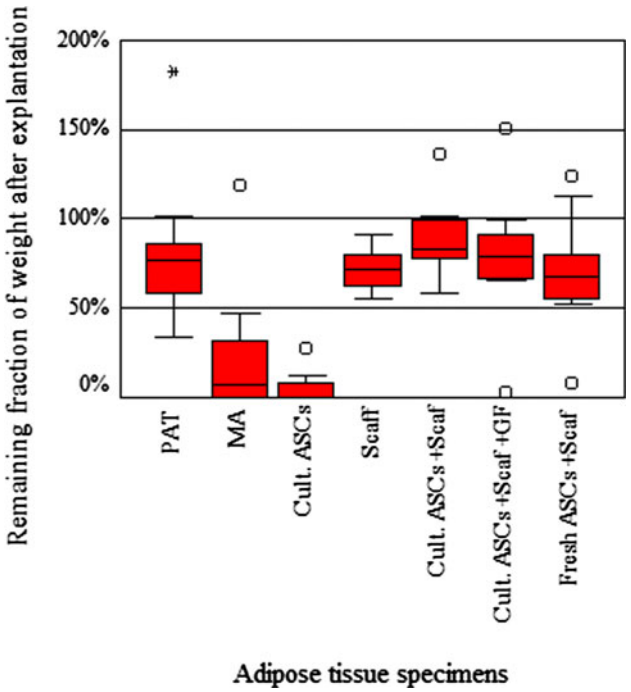

Fig. 8 Box plot of the remaining fraction of weight after explantation for each type of adipose tissue specimen. The *top* of the box represents the upper quartile, and the *bottom* of the box represents the lower quartile. The *line* in the middle represents the median. The *whiskers* represent the highest and lowest values that were not outliers or extreme values. *PAT* Purified adipose tissue, *MA* mature adipocytes, *Cult* cultured, *ASCs* adipose-derived stem cells, *Scaf* collagen scaffold (negative control), *GF* bioactive factors

We used the same technique and donor for fat harvesting to minimize any potential influence on procurement of ASCs.

Concerning the cell source, mature adipocytes were shown to be susceptible to damage during aspiration procedures [24] and to have limited capacity for proliferation due to their differentiated state [2]. Thus, the pluripotent ASCs were introduced as an alternative source of adipose cells with high proliferation capacity and adipogenic potential. They were used in both autologous fat grafting [34] and adipose tissue engineering [7] to improve fat graft survival.

The results of this study compared the in vivo viability of grafted free cells of adipose origin (adipocytes and ASCs) with that of ASC-seeded scaffolds. To provide objective evidence that the cells used were mesenchymal ASCs, a phenotype analysis was used. The positive expression of CD70, CD90, CD105, and HLA ABC as well as the negative expression of CD14, CD45 (hematopoetic markers), and anti-HLA DR (an endothelial marker) confirmed that these were indeed mesenchymal ASCs and not hematopoetic or endothelial cells.

Free-cell grafts (mature adipocytes and ASCs) showed a significant rate of resorption (50 and 60%, respectively)

and significant weight loss (Tables 1, 2). This was confirmed by the hystologic examinations (Table 3). When tissue was present, it was fibrous, with a scarce number of adipocytes (Oil-Red-O stain) and newly formed vessels.

Purified fat grafts produced vascularized adipose tissue, with a statistically significant difference between mature adipocytes and purified fat, suggesting the positive role of the extracellular matrix in fat grafts, which plays the role of a natural scaffolding. The positive role of scaffolding was further confirmed in the cell/scaffold groups, in which the cell survival rate was statistically greater. The difference between the free-cell grafts (adipocytes and ASCs) and the scaffold-containing groups in terms of remaining weights was significant (Tables 2, 3). The positive role of the collagen scaffold for the survival of adipose cells and the transformation of ASCs to adipocytes was confirmed by the clinical and histologic studies (Table 3).

It must be underscored that specimen weights were used as an objective parameter of the amount of tissue before implantation and after explantation. This parameter could characterize the evolution of the amount of grafted preparation but not the tissue in it. Histologic analysis was used to confirm the existence of newly formed fat tissue.

The primary cellular component for adipose tissue is a collection of lipid cells known as adipocytes, held in place by collagen fibers [12]. Fat tissue contains other cellular types such as vascular smooth muscle cells, blood cells, fibroblasts, and inflammatory cells [12].

Our aim was not to detect and characterize their existence in the newly formed fat tissue but rather to observe and provide evidence of the formation and existence of this fat tissue (if any) from ASC-seeded scaffolds in relation to free-cell grafts. In this sense, the formation of this fat tissue certainly is not due to any inflammatory or immune response, although we cannot completely remove the possibility of an inflammatory reaction induced by the foreign proteins of the scaffold. We do not consider that newly formed fat tissue and differentiated adipocytes, proved by the Oil-Red-O staining, is due to any inflammatory reaction. Furthermore, although not the ideal animal model, the nude mouse is immunodeficient, which significantly diminishes the chance of any immune response. In addition, it is reported that ASCs not only enhance angiogenesis but also minimize the inflammatory response [12]. In this sense, fat tissue should contribute to a decreased inflammatory response and not increase it. However, further investigation is required to clearly elucidate and provide scientific evidence on these questions.

The resorption rates of collagen scaffolds were not studied extensively. Our aim was to evaluate the quantity of fat tissue formed in the scaffolds and the cellular component rather than the resorption of the scaffold. However, the decrease in weight shown by all the groups was due to some collagen scaffold resorption, which was presumed to be identical in the various groups.

The fact that collagen scaffolds, unlike the ASC/scaffold groups, did not present any fat tissue colonization suggests the role of the ASCs for the formation of fat tissue in the scaffold. Although previous studies compared different scaffolds [33], we could not find any reports that compared the behavior of free-cell grafts and cell/scaffold components using in vivo models.

The current study suggests that scaffolding is important for the differentiation and proliferation of adipose cells in vivo. A similar suggestion was made by von Heimburg et al. [33]. We hypothesized that free ASC grafts would proliferate and create adipose tissue in the nude mouse because of their high proliferation potential [5, 18]. Actually, very little adipose tissue with poor vascularization was formed in contrast to the ASC-seeded scaffolds. Rubin et al. [26] also reported stable proliferation and high viability of cells in in vitro seeded microbeeds over several weeks of culture.

Because the basic aim of this experiment was to observe the amount of fat tissue that resulted after free-fat cell grafting compared with the ASC/scaffold combination (natural scaffold in purified adipose tissue or collagen scaffold in ASC-seeded collagen scaffolds), we did not perform a specific histologic or other evaluation (labeling for endothelial cells) of the vasculature within the adipose constructs. Because the superficial vascular network is clinically and macroscopically visible, we may draw the conclusion that at least the surface of the construct is well vascularized via a rich vascular network. However, this cannot reflect the status of the vascular network within the construct, which requires further investigation.

We could not reach a conclusion as to the origin of the extracellular matrix. Although ASCs were shown to synthesize the extracellular matrix [22], the techniques used in this study were not sufficient to determine its exact origin. A previous study showed that the thickening of the dermis after fat grafting was due to the increased amount of collagen with a murine origin, attributable to adipose-stimulated collagen synthesis [21]. The study period of 8 weeks exceeded the period of healing (3–4 weeks), thus avoiding confusion between wound healing and adipogenesis [32].

The current results show the early recipient-site response rather than any long-term tissue changes, which will require further investigations under the same conditions. Collagen scaffolds seem to provide optimal conditions for ASC differentiation and proliferation and for new extracellular matrix synthesis. This is not the case with the host conditions, as suggested by the results of free-cell grafting, because the cellular type in both the cell/scaffold and free-cell groups was identical. Similar observations were reported by von Heimburg et al. [34] and Hemmrich

et al. [13]. Although the nude mouse is not the ideal animal model (important skin laxity), it is one of the animal models that can tolerate cells of human origin [14].

Other combinations and preparations that could be evaluated were not considered in the current study. Mature adipocytes cultured on collagen scaffolds, fresh ASCs with growth factors, a mixture of ASCs and mature adipocytes, and fat grafts supplemented with ASCs all could be studied as well. This would increase the number of studied groups and render the study too complicated. Our aim was to study the survival of free-cell grafts compared with seeded scaffolds to evaluate the role of the scaffolding for the cellular survival in vivo.

The collagen scaffold used in this study was composed of types 1 and 3 bovine collagen (72%), glycosaminoglycan (8%), and cross-linked chitosan (20%) with no angiogenic and cytostimulatory agents before implantation. That is why we excluded any influence of angiogenic and cytostimulatory agents imported via the collagen scaffold itself. The fact that many growth factors adhere to the extracellular matrix probably is one of the possible explanations why ASCs do better within than out of scaffolds.

Recent reports demonstrate that ASCs produce several angiogenic and anti-apoptotic factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and bFGF, as well as VEGF and insulin-like growth factor-1 (IGF-1). This suggests that ASC would promote angiogenesis and prevention of apoptosis, which could be beneficial in the creation of fat tissue constructs [27]. The growth factors secreted by ASCs themselves probably are attached better in the presence of scaffold to influence their development. However, this study did not provide sufficient data to confirm this hypothesis.

Adipose-derived stem cells have demonstrated a capacity for differentiating into a variety of lineages depending on the inducing stimuli and growth factors [32, 39]. Dexamethasone, T3 thyronine, and insulin were shown to stimulate adipogenic differentiation of fat cell precursors [16]. We hypothesized that these bioactive factors would further direct, stimulate, or improve the transformation of ASCs into fat tissue.

Previous studies showed that no fat formation occurred in constructs without adipogenic precultivation. Weiser et al. [35] showed that implantation of mature fat pads (after 35 days of adipogenic precultivation) resulted in adiponecrosis within the constructs, whereas implants with an immature phenotype at the time of implantation (2 and 9 days) gave rise to vascularized, mature adipose tissue in vivo. In this sense, our results are in line with the results of Weiser et al. [35] because the application of adipogenic factors did not improve the survival, differentiation, or proliferation of adipose cells. However, we used only 2 days for adipogenic precultivation, which is not enough time for adequate adipogenic precultivation, as shown also by Weiser et al. [35].

The results of this study suggest that fat grafts and ASC-seeded scaffolds provide the same rate of graft survival. Then why should we use engineered fat constructs instead of fat grafts? First, fat grafting really is the technique of choice when indicated, but in some slender patients with a scarce subcutaneous fat layer, it is difficult to harvest sufficient quantities of fat tissue. For these patients, the engineered fat constructs would provide an alternative.

The results of this study suggest that extracellular matrix, natural (as in fat grafts) or imported (as in engineered fat constructs), seems essential for the survival and proliferation of ASCs and adipocytes. Thus, providing a matrix for support seems to augment the survival of fat cells and thus ameliorate the results.

A complete understanding about the mechanisms of interactions among adipose stem cells, growth factors, and biomaterials in tissue engineering still is lacking [29]. Three-dimensional scaffolding seems essential in tissue engineering strategies, and this study aimed to clarify its role, but further investigation is needed.

Study Limitations

Several limitations in the design of this study can be identified. The vascularization of the newly formed fat tissue was not specifically studied. Thus, conclusions as to the vascularization of the entire scaffold with fat tissue could not be drawn. The role of any potential inflammatory reactions was not extensively studied, and further investigations will additionally clarify these processes. Phenotype analysis of the various subpopulations of ASCs would further enhance the obtained results. The conclusions should be interpreted with these limitations in mind.

Conclusions

A 3D collagen scaffold seems to improve fat tissue survival in vivo compared with free-cell grafting over an 8-week period. Fat grafts show smaller resorption rates than free-cell grafts, confirming the positive role of 3D scaffolding. Differentiation and proliferation of cultured ASCs appear to be under local control without the influence of exogenous bioactive factors.

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