Chapter 8

Fat Tissue Engineering

D. von Heimburg*, G. Serov, T. Oepen and N. Pallua

Summary

Background: To date no adequate implant material for the correction of soft tissue defects such as those caused by extensive deep burns, tumor resection and congenital defects is available. A biohybrid composed of viable adipocyte-precursor cells and an optimised matrix could thus help finding a solution. After grafting, preadipocytes require an appropriate environment to differentiate into mature adipocytes (cell diameter up to 120 µm).

Aims: The aim of this study was to evaluate the survival and differentiation of dedifferentiated preadipocytes seeded into hyaluronan matrices in the immunodeficient mouse model.

Materials and methods: Isolated and cultured human preadipocytes were seeded onto hyaluronan scaffolds (HYAFF 11 sponges, HYAFF 11 non-woven carrier and ACP sponges) sized 7.5x7.5x5 mm and implanted into 42 immunodeficient mice. The transplanted scaffolds without cells were used as controls. After 3 and 8 weeks (2 and 4 weeks in ACP) the grafts were excised. Macroscopical appearance, weight, thickness, histology, immunohistochemistry (scaffold structure, cellularity, penetration depth of the seeded cells) and ultrastructure were assessed after 24 hours in vitro and after explantation at 3 and 8 weeks.

Results: Three and 8 weeks after grafting, macroscopical layers of adipose tissue that were vascularized to some extent, were seen in all preadipocyte-seeded HYAFF 11 hyaluronan carriers. Control grafts appeared unchanged and showed no blood vessels. The ACP sponges were present two weeks after implantation, but not after four weeks. Histological analysis of the preadipocyte/hyaluronan carriers showed the presence of adipose tissue and rich vascularisation in the upper layers of the grafts, but there was no homogeneous blood vessel distribution. The controls contained only a few cells and fibrous capsule but no adipose tissue. Human-vimentin positive cells were found in all preadipocyte/hyaluronan grafts but not in controls, at a maximal depth of 2.2µm±0.7µm (in sponges after 8 weeks). Ultrastructural analysis showed complete in vivo differentiation of viable adipocytes in the sponges seeded with preadipocytes.

Conclusions: Transplantation of human preadipocytes within a standardised hyaluronan matrix resulted in formation of well-vascularised adipose-like tissue. It appears that the HYAFF 11 sponge structure is superior to the HYAFF 11 non-woven carrier as preadipocytes enlarge during differentiation due to incorporation of lipids.

Keywords: Adipose Tissue, Hyaluronan, Preadipocytes, Fat Tissue Engineering

*Correspondence to: D. von Heimburg, Praxisklinik Kaiserplatz, Kaiserstr. 14, D-60311 Frankfurt, Germany.
E-mail: dvheimburg@praxisklinik-kaiserplatz.de

Introduction

The reconstruction of soft tissue defects is a problem; an ideal filler material for the correction of congenital deformities, cancer defects has still to be found. Autologous mature adipose tissue has been used as free grafts for the reconstruction of soft tissue defects for more than 100 years (1) and is still in use because of the lack of a better alternative, although the results are poor and unpredictable (2). The transplants are largely absorbed and replaced by fibrous tissue and oil cysts (3). The poor results of free fat autotransplantation are thought to be due to the low tolerance of mature fat cells' to ischemia and the slow rate of revascularisation of the grafts (4).

Using tissue-engineering approach, cultured preadipocytes were transplanted instead of mature cells to test the hypothesis that these cells would display better survival. Preadipocytes can undergo differentiation and dedifferentiation *in vitro* under specific culture conditions (5) and are a potential material for soft tissue engineering (6) due to their ability to proliferate and differentiate into adipose tissue after transplantation. In our recent study we have achieved good results by grafting human preadipocytes (7) attached to collagen sponges. It was demonstrated that preadipocytes need optimal biodegradable carriers with specific properties in terms of type of material and structure which allow these cells to invade and differentiate after transplantation. Mechanical stability of the carrier is important and it should not be resorbed too quickly after transplantation.

Hyaluronic acid (HA) is present in the extracellular matrix of many tissues and abundant in mesenchymal tissues in the foetus (8) and it is assumed that HA-based biomaterials are supportive for progenitor cell development and can facilitate tissue repair (9). Hyaluronan-based scaffolds were thus examined in the study presented here as new carriers in tissue engineering.

Materials and Methods

Carriers

Three types of carriers were used as examples: HYAFF 11 sponges (HS), HYAFF 11 nonwoven (HV), and ACP sponges (AS). All the carriers (HS, HV, and AS) were generously supplied by Fidia Advanced Biopolymers (Abano T., Italy). All materials were cut into pieces of equal size (7.5 mm x 7.5 mm x 5mm).

Sponges were made of HYAFF 11, a linear derivative of hyaluronic acid (HA) modified by complete esterification of the carboxylic function of the glucuronic acid with benzyl groups (10). The structure of these sponges shows open interconnecting pores. The pore size varies between 50 to 340 µm and the porosity is over 90%. HV is composed of nonwoven fibers (10 to 20 µm thick) of hyaluronan benzyl ester with an interfiber distance of 100-300 µm and a specific weight of 100 g/m². ACP are made of crosslinked hyaluronan derivatives recently obtained (11). ACP sponges have pores of 10-300 µm and a porosity over 85%.

Preadipocyte Isolation and Culture

Out of freshly excised human subcutaneous adipose tissue of young adults (18-29 years old) who underwent elective operations, preadipocytes were isolated. This was approved by the Ethical Committee of the Aachen University of Technology. Using dissecting microscope, vessels and fibrous tissue were removed, the adipose tissue was minced and digested by collagenase 0.1 U/ml/dispase 0.8 U/ml (Boehringer Mannheim, Mannheim, Germany) at 37°C for 60 min. Digestion was stopped by adding Dulbecco´s modified Eagle medium (DMEM) containing 15% foetal calf serum (FCS, Biochrom, Germany) and incubated in erythrocyte lysis buffer (154 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, 1 mmol/l EDTA, 10 minutes). The cell suspension was centrifuged (200xg at 17°C for 10 minutes) and the cells were seeded on tissue culture dishes (63.6 cm², Greiner, Germany) with DMEM 15% FCS (added 100 U/ml penicillin, 100µg/ml streptomycin) with a seeding density of $3x10^4$ cells/cm². The cells were cultured at 37°C at 10% CO_2 and 95% humidity (Fig. 1). Medium was changed on day 2 and supplemented with EGF (epidermal growth factor, 10ng/ml, Sigma, USA).

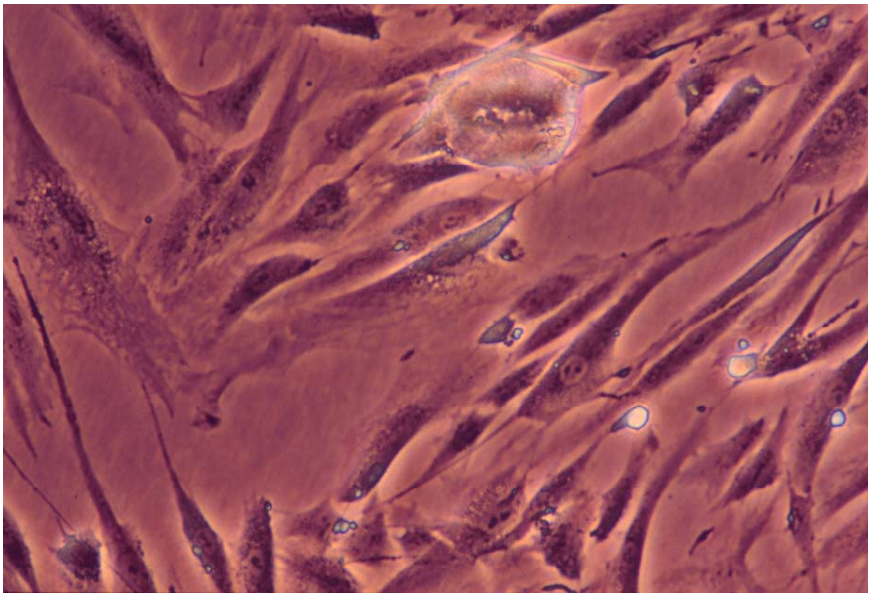

Fig. 1. Primary human preadipocytes 10 days after isolation shown as a monolayer (2-D culture). Medium is supplemented with EGF (epidermal growth factor) to inhibit differentiation.

Implants in Vitro and in Vivo

The carriers were coated with undiluted FCS overnight at 37°C before seeding the cells. A suspension of 100 µl containing $10^6 \pm 5x10^4$ preadipocytes (after trypsinisation and resuspension) was seeded on the upper surface of the carriers and incubated for 24 hours to allow cell attachment.

Fourty-two preadipocyte/carrier constructs (HS+, HV+, AS+) and 42 negative controls (HS-, HV-, AS-, carriers without cells, soaked in DMEM for 24 hrs.) were implanted into NMRI nu/nu, eight-week-old, athymic mice (n=42). Each animal received one construct implanted subcutaneously in the left scapular area and its corresponding control in the contralateral side through separate incisions. The base of the constructs was placed on the muscle fascia. Approval for the animal experiments was obtained and experiments were performed according to German regulations for the care and use of laboratory animals.

The HS and HV grafts were excised after three weeks (*n*=14: 7 HS+ and HS-, 7 HV+ and HV-) and eight weeks (*n*=14: 7 HS+ and HS-, 7 HV+ and HV-), the AS grafts were excised after 2 weeks (*n*=7: 7 AS+ and 7 AS-) and 4 weeks (*n*=7: 7 AS+ and 7 AS-). The explanted grafts were analysed macroscopically (for colour, vessels, tissue/cell ingrowth) and microscopically. Each carrier was weighed after 24 hours *in vitro* before transplantation and after explantation.

Histology and Immunohistochemistry

Half of the vertically "corner-to-opposite-corner" bisected specimen was cryofixed. The other half was fixed in 4% buffered formaldehyde solution and embedded in paraffin. Six µm thick cross parrafin sections were prepared and stained with hematoxylin-eosin and Giemsa. The cryofixed specimens were stained with oil-red for identification of lipid vacuoles. Paraffin sections of the seeded matrices and unseeded controls were stained with monoclonal antibodies specific for human vimentin (mahv, clone V9, Code Nr. M 0725 Lot 057, DAKO, Glostrup, Denmark) at a dilution of 1:10. Three blinded examiners independently assessed all sections. When there were differences between the assessments, the mean value was calculated.

Non-specific cellularity (donor and host) was assessed by counting all Giemsa stained cell nuclei in five defined microscopic fields of the sections at 200-fold magnification.

Specific cellularity (donor=human) was evaluated by counting all human-vimentin positive cells in five defined microscopic fields at 200-fold magnification.

Penetration depth of donor cells was measured in three defined microscopic fields at 200-fold magnification by using intraocular micrometer (Zeiss, Jena, Germany).

Vascularisation of the grafts was estimated in the cross-sections. The score was "-" when no vessels were seen, "+" when vessels were seen in one or more superficial regions, "++" when vessels were present in the central region and, "+++" when there was a homogenous distribution of vessels within the graft.

Statistical Evaluation

Weight and thickness of the grafts, the overall and specific cellularity in the grafts and the penetration depth of the seeded human preadipocytes were expressed as mean value and ± standard deviation. Differences between the implantation periods at three and eight weeks and between the preadipocyte/scaffold constructs and the controls were assessed. Statistical analysis was performed using Wilcoxon signed rank test to compare paired data (preadipocyte construct and negative control in the same animal, median values) and using Mann-Whitney rank sum test to compare non-paired data (different scaffolds, different time points, median values). Significance level was accepted at $p < 0.05$. No correction was made for multiple testing because of the small sample size.

Results

Macroscopy

Three and eight weeks after subcutaneous implantation, the HS and HV carriers were still easily identified (Fig. 2 and 3). In contrast, the AS carriers were only detected two weeks after implantation (Fig. 4), but were not present at 4 weeks. The gross shape of the HS+ carriers was almost unchanged whereas that of HV and AS grafts has changed. The control sponges taken from the same animals revealed almost no change and showed no vessels. All preadipocyte/scaffold contructs were covered by tightly adherent layers of macroscopically yellow tissue and new vessels on the top. The control grafts appeared white and almost avascular.

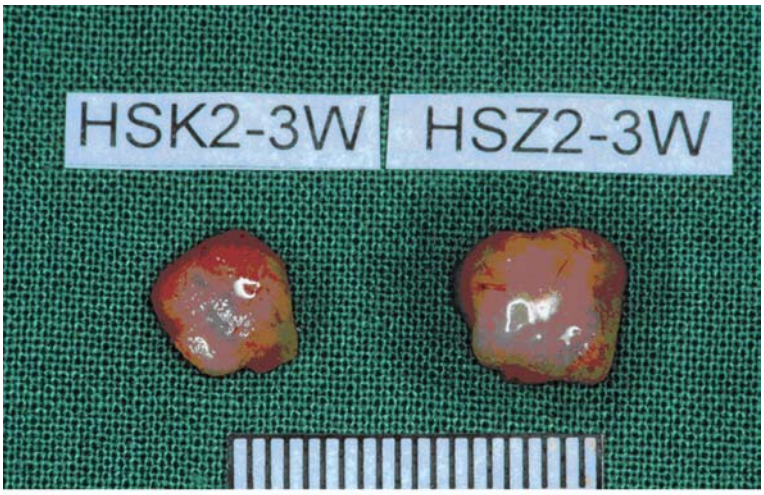

Fig. 2. Explanted grafts after 3 weeks in vivo (HS=HYAFF 11 sponge, Z=carrying preadipocytes HS+, K= control HS-, scale in mm). HS+ specimens showed rounded edges and looked smaller than HS-. Yellow tissue formation presented on all preadipocyte grafts (right).

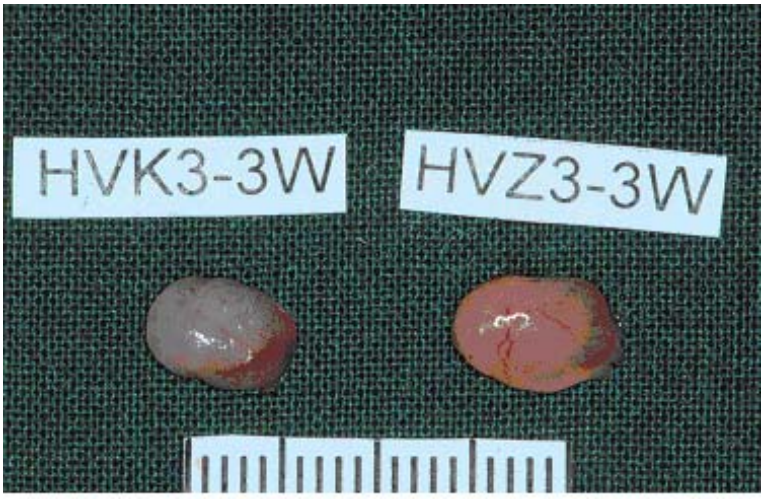

Fig. 3. HV carriers (HYAFF 11 non woven) after 3 weeks in vivo showed deformation, shrinkage and a remarkable variation in morphology between the specimens. Adipose-like tissue was present in the preadipocyte grafts with new vessel formation (HV+, right).

Fig. 4. AS+ carriers (ACP sponges with preadipocytes right) showed stable structure after two weeks and appeared yellowish and firm. The AS- control specimens (left) on the other hand were amorphous and soft and looked avascular.

Weight

HS+ and HS- weights

After three weeks, implantation in nude mice there was an increase in the weight of HS+ while there was a significant decrease in the weight of control HS- (HS+: 0.240g±0.041g, HS-: 0.133g±0.017g, p=0.008). After eight weeks, HS+ still had a significantly higher weight than the controls (HS+: 0.232g±0.047g, HS-: 0.171g±0.07g, p=0.016).

HV+ and HV- weights: After 3 weeks, the nonwoven matrix with preadipocytes showed higher weight than the controls (HV+: 0.103g±0.011g, HV-: 0.072g±0.007g, p=0.008).

AS+ and AS- weights: After two weeks, ACP sponges with preadipocytes had a significantly higher weight as compared to their weight at the time of implantation (AS+: 0.283g±0.018g, AS+: 0.336g±0.08g).

Comparing the three scaffolds (HS, HV and AS) with each other, HV showed the lowest dry weight of the scaffolds examined and the greatest variation between the specimens after explantation. The seeded grafts (+) had a higher weight at all times, a difference which was significant for HS+, HV+ and AS+. A significant weight gain from the time of implantation to the time of explantation was only observed with HS+ preadipocytes constantly. This weight gain corresponded to the amount of adipose-like tissue which developed in these scaffolds.

Histology

Carriers in vitro

The cells adhered to the scaffolds 24 hours after the seeding process, just before transplantation (Fig. 5). At this time cells were only observed in the superfacial areas of the carriers. The adipose precursors showed multiple cytoplasmic vacuoles and rounded shape as signs of early fat cell differentiation as indicated by histological and ultrastructural analysis (not shown).

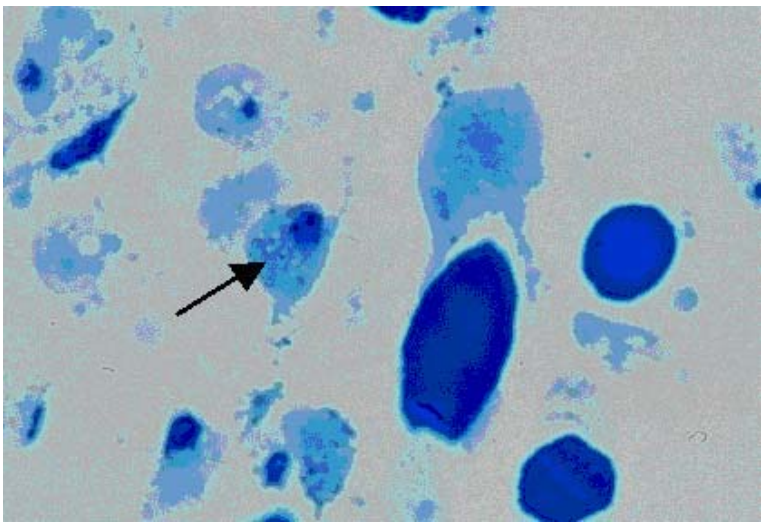

Fig. 5 Human preadipocytes stained with toluidine-blue 24 hours after being seeded on HYAFF 11 non-woven 3-D scaffolds. Cells are closely attached to fibres. Cytoplasmic lipid vacuoles (arrow) increase in number and size and show morphological signs of differentiation (x700).

Overview

Every specimen was surrounded by a thin fibrous capsule, which separated the scaffold with the newly formed adipose-like tissue from surrounding host tissue. Microscopical examination of the preadipocyte/carrier constructs demonstrated viable adipose tissue located on the surfaces of the carriers under the capsule. Mature adipocytes presenting signet ring appearance were seen in the areas beneath the surface.

Differentiated adipocytes were only observed in the central region of HS(+) but not in HV(+) or AS(+). Many vessels were present in the newly formed adipose tissue (Fig. 7). In the control grafts no adipocytes were found at all. The pores appeared to have collapsed in HV (swollen fibers and narrowed pores). Only in HS the porous structure was still present. In these areas, adipose tissue was composed of clusters of adipocytes (Fig. 6 and 7).

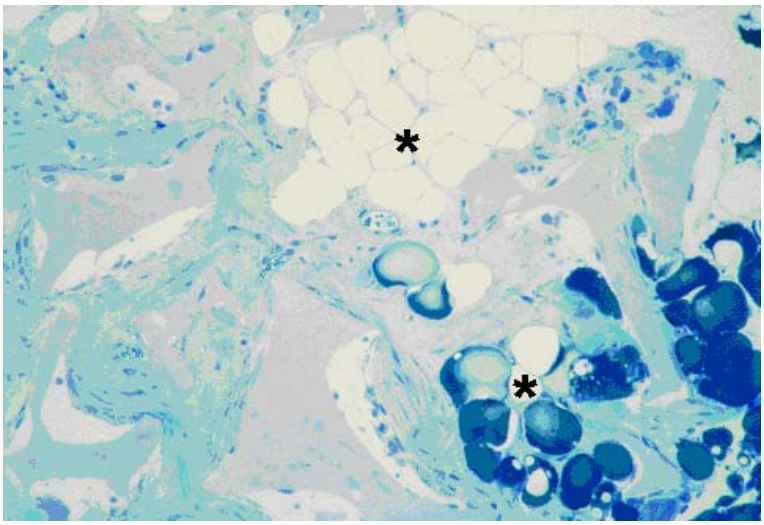

Fig. 6. Microscopical view of the HS+ section after 3 weeks *in vivo*. Clusters of differentiated adipocytes (*) in open pores at the centre (1800 µm) of the sponge (toluidine-blue, x200).

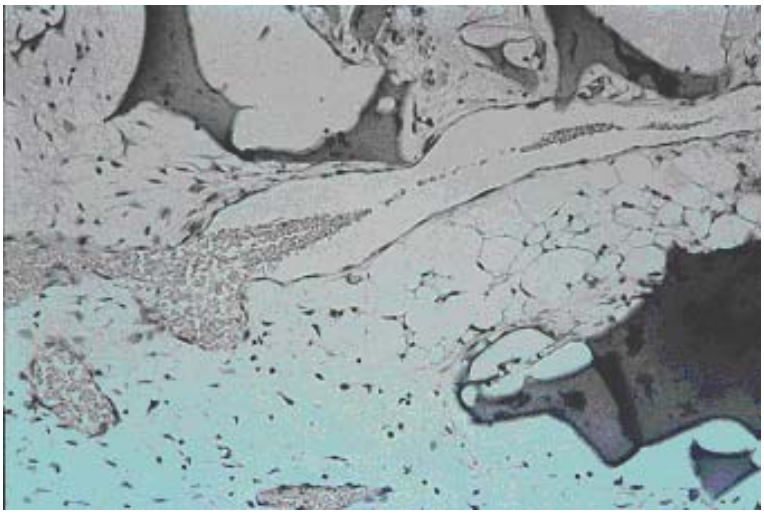

Fig. 6. Newly-formed capillaries (+) filled with erythrocytes are soon in the HS+ centre in close vicinity to engineered mature adipose tissue (*) (hematoxylin-eosin, x200).

Cellularity

In the HS and HV specimens there was a higher non-specific (donor and host) cellularity in the control grafts (HS- and HV-) than in the preadipocyte-carrying specimens (HS+ and HV+) as indicated by analysis of giemsa positive nuclei. Between week 3 and week 8 there was a slight decrease of the non-specific cellularity in HS+, HV+ and HV- (data not shown).
Staining for human-vimentin indicated that cells in HS+, HV+ and AS+ specimens were strongly positive demonstrating human origin. There was no positive staining in the HS-, HV- or AS- specimens. This specific (donor) cellularity showed different results in the three different types of scaffolds. In HS+ there was no difference between week 3 and week 8 (52±27 cells/field vs. 53±26 cells/field); non-specific cellularity was higher than specific cellularity.

In HV+ there was a decrease ($p<0.001$) in donor cell number between week 3 and week 8. The highest donor cell number of all scaffolds was observed in HV+ at week 3 (115±56 cells/field). Eight weeks after implantation the number of human cells was reduced in HV+ to the lowest specific cellularity level of all scaffolds (27±22 cells/field).

Depth of Penetration

The depth of penetration of human adipose precursor cells into the scaffolds after three weeks *in vivo* was different for each type of scaffold. Deepest penetration was found in HS+ (3 weeks: 1727±1196 μm, 8 weeks: 2227±706 μm). Less penetration was seen with HV+ (3 weeks: 1460±447 μm, 8 weeks: 1253±677 μm).

Mature adipocytes were found only in some areas. Mature adipocytes had penetrated 1.8 mm into HS+ scaffolds (Fig. 6), whereas the depth of penetration was only 280 μm in HV+ scaffolds. No adipocytes were found in any of the control grafts.

Neovascularisation

There was a better vascularisation in preadipocyte/carrier (+) constructs as compared to the negative controls (-) for all types of scaffolds. Highest neovascularization (++) was seen in the open interconnecting pores of HS (Fig. 7). None of the explanted grafts showed a homogenous vessel distribution (+++).

Discussion

Tissue-engineered adipose tissue offers a new solution to correct congenital or traumatic soft tissue defects without creating a major donor defect. The present study examined three different carrier materials and demonstrated the feasibility of engineering of adipose-like tissue by transplanting cultured autologous preadipocytes which can differentiate after implantation.

In three week and eight week *in vivo* studies, hyaluronan benzyl ester (HYAFF 11) sponges proved to be better scaffolds than HYAFF 11 non-woven carriers or (ACP) sponges in respect to constant maintained weight and homogenous distribution of surviving donor precursor cells that resulted in the highest amount of differentiated adipose tissue.

It is likely that the collapse of the nonwoven carriers (swollen fibers and decreased interfiber space) has resulted in reduced the space available for the preadipocytes to occupy and to differentiate. In contrast, the porous structure was still present after eight weeks in hyaluronan benzyl ester sponges (HS+). In these areas adipose tissue that was composed of clusters of adipocytes was found. Another explanation for the lower proportion of differentiated adipocytes in carriers with smaller interfiber space, i.e. HV+, could be that the mechanical pressure of the degrading carrier may have led to a delipidation of mature adipocytes. As seen in a previous studiy mechanical compression of adipose tissue between tissue expanders and skin leads to delipidation of the adipocytes which finally resemble fibroblasts (12).

Migration of cells into a carrier is an important parameter for scaffold/cell interaction analysis in tissue engineering (13,14). In the study presented here human cells were still found in HYAFF 11 sponges and non-woven carriers eight weeks after implantation in nude mice. The HYAFF 11 sponges showed the best preadipocyte penetration *in vivo* in HS+ and the best mature adipocyte penetration. This carrier had the largest pore size.

All explants containing human preadipocytes appeared vascular, compared to control scaffolds that contained no cells. Increased vascularisation was probably induced by the new extracellular matrix, of which a large amount is produced by preadipocytes as found by Kuri-Harcuch *et al.* (15). Kawaguchi *et al.* found that extracellular matrix (ECM) is a potent inducer of neovascularisation (16). ECM of the loose connective tissue of the native adipose tissue interconnects the adipocytes and induces fat cell clustering *in vivo* (17). These authors suggested that Hyaluronan which is present in all the connective tissues (18) may positively influence adipogenesis *in vivo*. Apparently the material of the scaffold itself is as important as its three dimensional structure.

The use of autologous preadipocytes has several advantages over the use of mature adipocytes because mature adipocytes are fragile from a mechanical point of view and have a low tolerance to ischemia. Furthermore, being fully differentiated, they do not proliferate (19,20). Preadipocytes on the other hand are mechanically stable, tolerate hypoxic environments better and are highly proliferative (7).

Conclusions

All together our work indicates that the ideal scaffold should have large (>120 μm) interconnected pores and the material should not swell. The HYAFF 11 sponge proved to be superior to the HYAFF 11 nonwoven and the ACP sponge in the present experiments. Engineered adipose tissue could be used for soft tissue augmentation such as in small contour defects and in larger defects for breast reconstruction following mastectomy. Evaluation of the influence of hyaluronan scaffolds coated with different ECM substrates on *in vitro* and *in vivo* adipocyte differentiation will be the next step towards the development of a living adipose tissue substitute.

Acknowledgements

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