Autologous *In Vivo* Adipose Tissue Engineering in Hyaluronan-Based Gels—a Pilot Study

Karsten Hemmrich, M.D.,*,1 Karlien Van de Sijpe, M.D.,† Nicholas P. Rhodes, Ph.D.,‡ John A. Hunt, Ph.D.,‡ Chiara Di Bartolo, M.Sc.,§ Norbert Pallua, M.D., Ph.D.,* Phillip Blondeel, M.D., Ph.D.,† and Dennis von Heimburg, M.D., Ph.D.*

Department of Plastic Surgery and Hand Surgery–Burn Center, University Hospital of the Aachen University of Technology RWTH, Aachen, Germany; †Department of Plastic and Reconstructive Surgery, UZG University Hospital Gent, Gent, Belgium; ‡UK Center for Tissue Engineering, UKBioTEC Laboratories, Department of Clinical Engineering, University of Liverpool, Liverpool, United Kingdom; and §Fidia Advanced Biopolymers S.r.l., Abano Terme, Italy

Background. There is a major clinical need for strategies for adequately reconstructing the soft tissue defects found after deep burns, tumor resection, or trauma. A promising solution is adipose tissue engineering with preadipocytes, stem-cell derived precursors of the adipose tissue, implanted within biomaterials. This pilot study evaluated hyaluronan gels mixed with autologous undifferentiated preadipocytes in a pig model for their potency to generate new fat.

Materials and methods. Preadipocytes were isolated from intra-abdominal pig fat by collagenase digestion, plated on fibronectin-coated culture dishes in Dulbecco's Modified Eagle Medium/Ham's F12 (Biochrom, Berlin, Germany) combined with 10% pig serum, expanded, and mixed with hyaluronan gel. Two types of gels with varying degrees of amidation of the carboxyl groups were tested (HYADD3, HYADD4). Cell-loaded gels and unseeded controls were injected subcutaneously into the ears of three pigs, explanted at 6 wk, and analyzed histologically.

Results. Both cell-loaded specimens were detected macroscopically. They demonstrated a slight volume effect with limited stability after 6 wk. Unloaded HYADD3 and HYADD4 controls could not be identified at the time of explantation. Histology of HYADD3 revealed islets of mature adipocytes and vessels embedded in fat tissue surrounded by gel. In contrast, no fat formation was found in HYADD4 gels when implanted in the ear.

Conclusions. Histological findings demonstrate that HYADD3 is a promising gel for generating adipose tissue. Even though HYADD3 might be a potential material for the reconstruction of small tissue defects, the question remains as to whether the adipose tissue within the gel is attributable to preadipocyte maturation or ingrowth from neighboring tissue. © 2007 Elsevier Inc. All rights reserved.

Key Words: preadipocytes; adipose tissue engineering; hyaluronic gel.

INTRODUCTION

Adipose tissue engineering aims to generate autologous, vascularized adipose tissue for the reconstruction of soft tissue in human clinical practice [1, 2]. The potential clinical applications for engineered adipose constructs are extensive, ranging from the correction of simple contour incongruencies to large volume tissue defects following mastectomy or trauma. Current possible approaches to reconstruct small tissue defects are collagen injections, dermal fat grafts, synthetic materials, and the transfer of free adipose tissue grafts. Unfortunately, all of these techniques have serious disadvantages, especially shrinkage and foreign body reactions [3]. Reduction of adipose volume is thought to be partly related to insufficient vascularization of grafted fat tissue [4]. Fat tissue is highly vascularized with extensive capillary networks surrounding each adipocyte, and fat tissue itself has angiogenic properties [5, 6]. Adequate vascularization of the tissue engineered fat is still the most important problem in preventing more extensive use of this tissue. Free adipose tissue grafts are largely absorbed or replaced by fibrous

[1] To whom correspondence and reprint requests should be addressed at Department of Plastic Surgery and Hand Surgery–Burn Center, University Hospital of the RWTH Aachen University, Pauwelsstr. 30, 52074 Aachen, Germany. E-mail: hemmrich@gmx.de.

tissue and oil cysts [7], even though some reports demonstrate increasing survival of the transplanted tissue by careful handling [8, 9].

In contrast, cell based tissue engineering approaches represent a novel and promising field of science. The stromal-vascular fraction (SVF) of adipose tissue harbors pluripotent mesenchymal progenitor cells [10, 11] that have a high proliferation and differentiation capacity and are, therefore, ideal candidates for tissue engineering applications. Furthermore, adipogenic progenitors have lower oxygen consumption than mature fat cells [12]. This tolerance of ischemia is helpful for survival of the time period until grafts are sufficiently vascularized. To use preadipocytes for tissue engineering purposes, cells are isolated from adult human adipose tissue, cultured, and seeded onto matrices to facilitate their pervasiveness at the site of transplantation. Materials used for adipose tissue engineering may either be solid scaffolds or injectable materials, such as cell-loaded hydrogels. Structural biopolymer carriers such as sponges or nonwoven meshes provide a permissive microenvironment in which seeded preadipocytes proliferate and differentiate. In the absence of seeded cells, common scaffold materials alone have not been demonstrated as having appreciable adipogenic activity [13, 14].

Polylactide glycolic acid (PLGA), polyglycolic acid (PGA), hyaluronan, and collagen-I in the form of nonwoven meshes or sponges have been evaluated as three-dimensional carriers for ex vivo expanded preadipocytes with varying success [13–16]. The *in vivo* efficacy of constructs in generating mature adipose tissue has been clearly correlated to the *in vitro* cell inoculation of the scaffolds and on the structure and type of biopolymer used [17]. In previous studies, we have demonstrated that open hyaluronan sponges with interconnecting pores of 50 to 340 μm had deeper penetration of adipocytes than either collagen sponges or nonwoven hyaluronan meshes [16]. Even larger scaffold pores were shown to allow better cell penetration, although maturation of the progenitor cells was compromised [18].

In contrast to rigid biopolymer carriers, biological hydrogels such as fibrin and Matrigel (Becton Dickinson, Heidelberg, Germany) lack of structural rigidity and deform easily. Matrigel has been demonstrated to effectively support angiogenesis and preadipocyte maturation both *in vitro* and *in vivo* [19–22]. However, its use is restricted to experimental purposes because its origin is from a murine sarcoma. Therefore, it would be highly desirable if alternative gels with adipogenic characteristics were available for human clinical use. It would be advantageous to have an implantable biomaterial that would both support the growth of adipocytes and actively induce neovascularization of the graft. It is well known that hyaluronic acid and its derivatives are actively angiogenic [23]. A major stumbling block, however, is that naturally occurring hyaluronan is a viscous fluid with limited retention as a consequence of quick degradation. This pilot study therefore evaluated two forms of amidated hyaluronan, which are both viscous gels, in unseeded and seeded forms, in a pig model using autologous undifferentiated preadipocytes. Preadipocytes were isolated from intra-abdominal pig fat by collagenase digestion, expanded in cell culture, and mixed with modified hyaluronan gel. Gels were injected subcutaneously into the ears of three pigs and explanted after 6 wk for histological analysis.

MATERIALS AND METHODS

Reagents

Hyaluronan, which had been modified by intermediate and long chain amidation of the carboxyl groups (HYADD3 (dodecyl-amide) and HYADD4 (hexadecylamide), respectively) was manufactured by Fidia Advanced Biopolymers, Abano Terme, Italy. The degree of amidation was 6% to 7% for HYADD3 and 3% to 4% for HYADD4 (Fig.1). For gel preparation details see patent WO0001733 (amides of hyaluronic acid and the derivatives thereof and a process for their preparation). Collagenase solution Type 1, M199, Dulbecco's modified Eagle medium (DMEM), Ham's F12 (F12), and porcine serum were purchased from Biochrom, Berlin, Germany. Trypsin/EDTA was purchased from PAA Laboratories, Cölbe, Germany. Basic fibroblast growth factor (bFGF) was obtained from Tebu, Frankfurt, Germany. The 250 μm nylon sieve was from Verseidag Techfab GmbH, Geldern, Germany.

Isolation and Culturing of Porcine Preadipocytes

The pig was brought under general anesthesia and a sterile field was created around the inguinal and abdominal region. Adipose tissue was harvested by excision of subcutaneous fat from the inguinal region and some intra-abdominal fat. In total, 80 to 100 g clean adipose tissue was collected. This fat was cut into small pieces by sterile scissors and afterward digested for 4 h with 0.2% (wt/vol) collagenase Type 1 CLS under conditions described for human preadipocyte isolation [18] (1 mL of collagenase buffered solution/g of fat, shaking at 37°C). Cells were then seeded onto fibronectin-coated culture flasks in proliferation medium (DMEM/F12 supplemented with 1 nM bFGF, 100 U/mL penicillin and 100 μg/mL streptomycin) with a seeding density of 3×10^4 cells/cm². Fibronectin-coating was achieved by incubating culture flasks with 125 μL of 0.2 mg/mL fibronectin solution for 2 min, then drying for 1 h after emptying. The culture medium was supplemented with 2.5% porcine serum 24 h after cell seeding.

Preparation of Cell-Loaded HYADD Gels and *In Vivo* Experimental Pig Model

Preadipocytes were expanded in 2D culture without differentiation. 1 mL of each HYADD gel (40 mg/mL) was mixed homogenously with 10^6 preadipocytes at passage 2. Homogeneous dispersion of the cells within the gel was ensured by microscopical *in vitro* analyses. Gels were injected subcutaneously into the back of the ear through an 18 G needle. Two cell-loaded and two control HYADD3 gels were injected into the right ear of each pig while HYADD4 samples were implanted contralaterally. The distance between cell seeded samples was 4 cm and the distance between seeded and unseeded samples was 12 cm. A total of three pigs with four HYADD3 and four HYADD4 samples each were used in the study. All animal experiments were performed according to Belgian animal protection laws following ethical committee approval.

Microscopical Examination of Specimens

Twelve cell-loaded (6 × HYADD3, 6 × HYADD4) and 12 control samples (6 × HYADD3, 6 × HYADD4) were harvested at 6 wks, sectioned, and fixed overnight in Lidi's 4% formalin (Merck, Darmstadt, Germany). After removal of formalin by extensive washing with water, the probes were dehydrated by increasing concentrations of isopropanol, embedded into paraplast, and cut into 15 μm sections. Paraplast was removed from sections using xylol followed by staining with hematoxylin and eosin or Giemsa using standard histological procedures. Microscopical analyses of representative areas in the samples were performed by light and electron microscopy.

RESULTS

Macroscopical Analyses of Explanted HYADD Gels

After the gel had been injected, there was a clear plug underneath the skin that remained stable in size for 3 to 4 wk. This subcutaneous prominence then continuously decreased in size, in part due to active movement of the ears of the pig. At the time of explantation at 6 wk, small lumps were found at the injection site where cell-seeded HYADD3 and HYADD4 gels had been injected. The remaining lumps were explanted and photographed. HYADD3 samples were larger than the HYADD4 explants. No control gels or any remnants could be identified through the skin at the time of explantation. The tissue that was consecutively explanted at the spots where the control gels had been injected did not reveal any HYADD material at all, neither HYADD3 nor HYADD4.

Microscopical Analyses of Explanted HYADD Gels

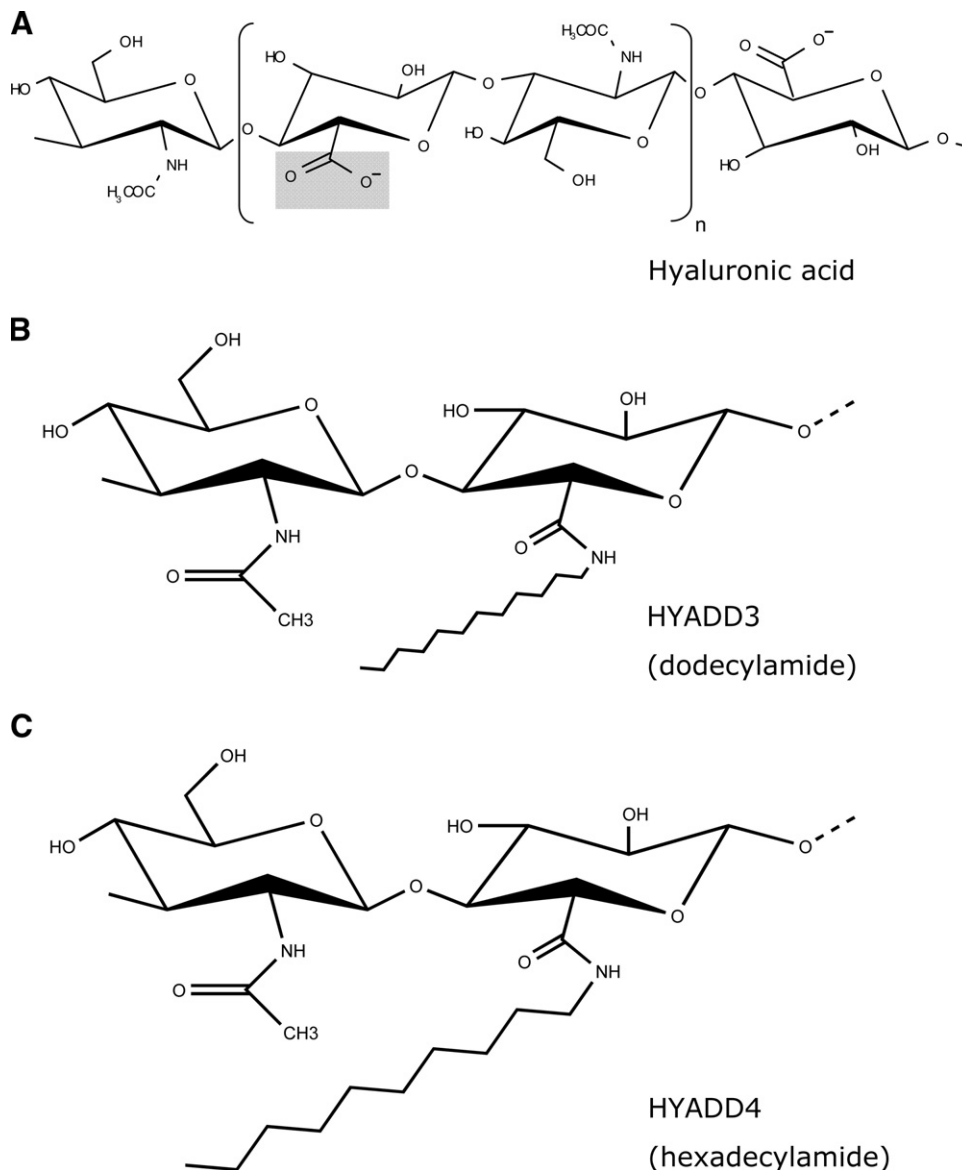Microscopical analyses showed in cell-loaded HYADD3 samples the formation of highly vascularized adipose tis-

FIG. 1. Chemical structure of (A) hyaluronic acid; (B) HYADD3 (dodecylamide), and (C) HYADD4 (hexadecylamide). Highlighted in (A) is the carboxyl group, which is modified by amidation to obtain HYADD3 and HYADD4.
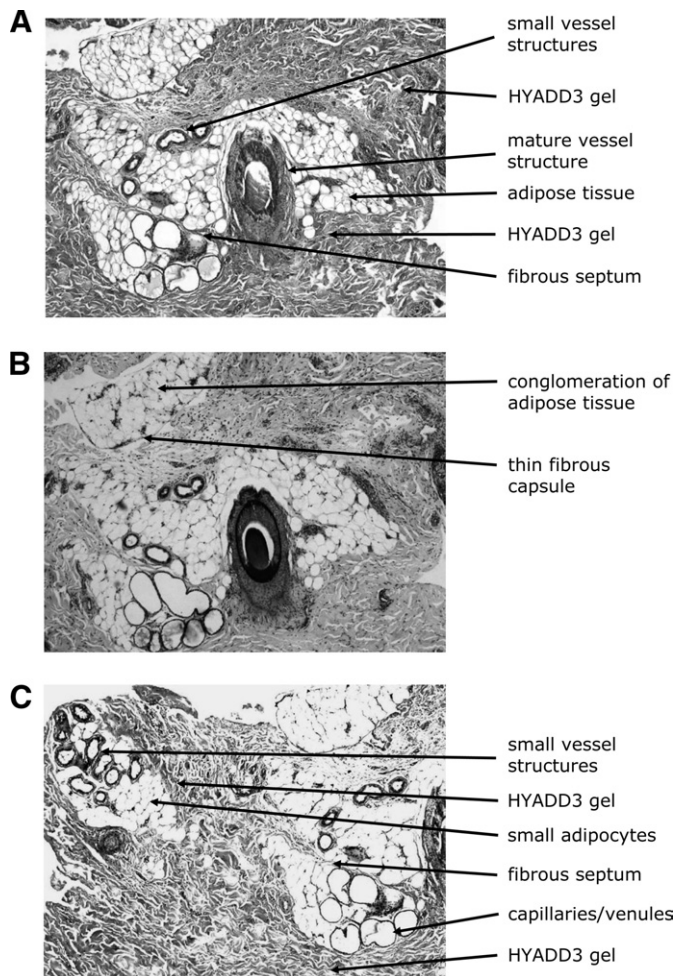
FIG. 2. Microscopical analyses of HYADD3. Representative picture of preadipocyte-seeded HYADD3 gel after 6 wk subcutaneous implantation in the pig (magnification ×50). (A) Hematoxylin and eosin staining. (B) Giemsa staining. (C) Giemsa staining of outer portion.

sue surrounded by gel. As shown in Fig. 2A, the adipose tissue was found around mature vessels and also around newly formed small vascular structures. There was minor inflammation but no substantial foreign body cell infiltration. Figure 2C, which continues Fig. 2A to the left, reveals the same tissue architecture shown in Fig. 2A, i.e., fat tissue consisting of small adipocytes and disseminated vascular structures. In the right part of the picture, the adipocyte conglomeration contains a group of capillaries or venules whereas the fat cells on the left side surround several small arteries. The upper left corners of Fig. 2A and B also illustrate the architecture of small adipocytes forming a conglomeration and being surrounded by a thin capsule. The adipose tissue accumulations are even sometimes crossed by thin septums that run through the fat cells (Fig. 2).

In HYADD4 gels, in contrast, no fat formation was observed in any specimen implanted subcutaneously in the ear combined with higher infiltrating cellularity than

in the HYADD3 samples. As can be seen from Fig. 3, there is substantial granulocyte infiltration into the gel. Furthermore, there is very little tissue formation within the HYADD4 gel compared to HYADD3, including around large vessels (bottom left in Fig. 3A and B).

In electron microscopy, HYADD4 and HYADD3 explants revealed foreign body cell infiltrations after 6 wk (Fig. 4). However, this was much more intense and evident in HYADD4 than in HYADD3 samples, confirming light microscopy findings. There was a significant accumulation of macrophages and giant cells. Figure 4A and B illustrate overlapping cell membranes of these giant cells and macrophages (filled triangle), incorporating the gel (open triangle), and digesting it in lysosomes (filled arrow).

DISCUSSION

Transplantation of autologous fat grafts has been the classical method of soft tissue reconstruction in plastic surgery for many years. However, the most significant drawback is the unpredictable resorption of the transplanted fat tissue since most of it is absorbed or replaced by fibrous tissue and oil cysts [24, 25]. The reduction in adipose volume due to resorption is thought to be partly related to insufficient vascularization of grafted fat tissue. To overcome the problems of volume reduction and tissue necrosis, tissue engineering strategies have begun to emerge that focus on a
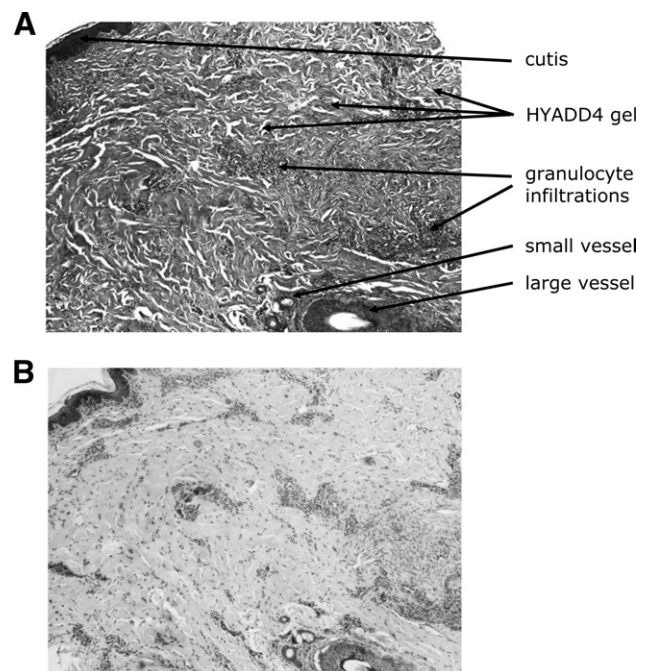

FIG. 3. Microscopical analyses of HYADD4. Cell-loaded HYADD4 gel after 6 wk subcutaneous implantation in the pig (magnification ×50). Shown is a representative image in (A) (hematoxylin and eosin staining) and (B) (Giemsa staining).
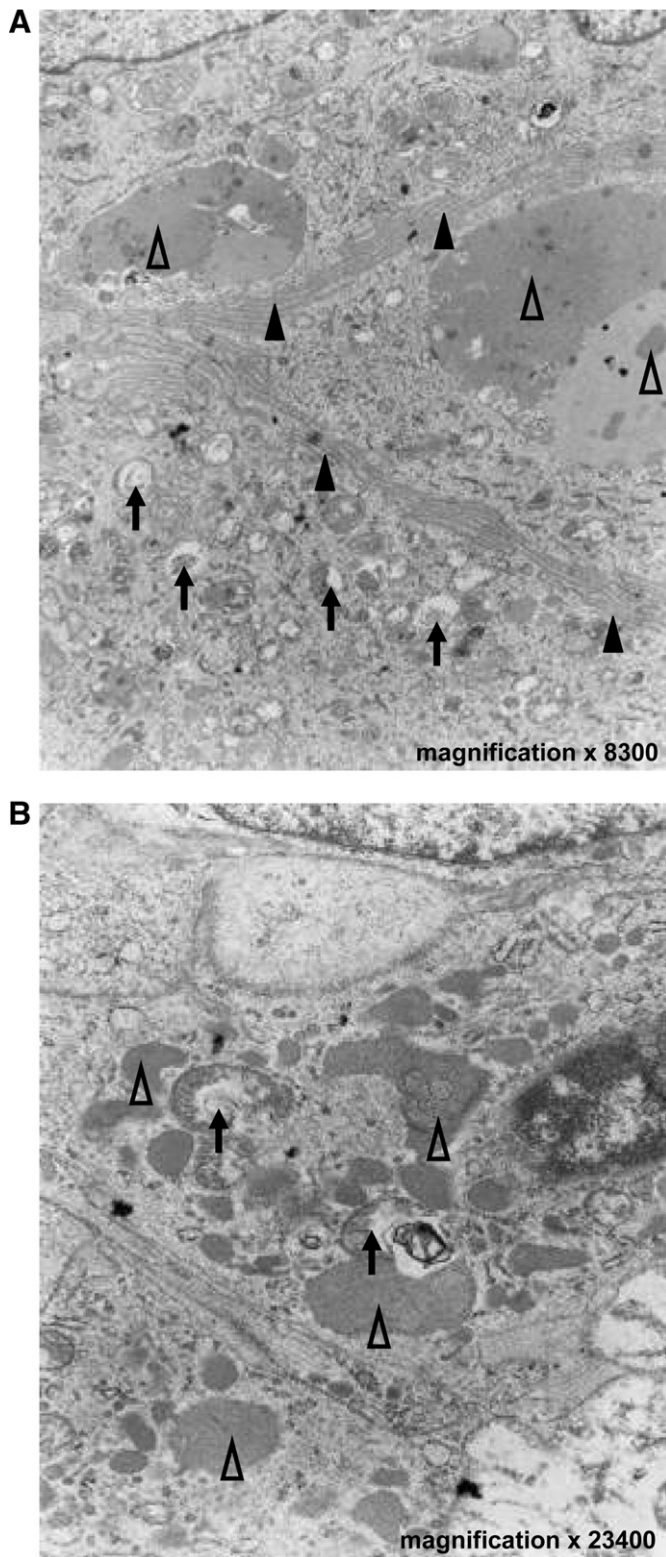
FIG. 4. Electron microscopy of HYADD samples after explantation. Cell-loaded HYADD samples after 6 wk implantation in the pig. (A) Representative picture of cell-loaded HYADD3 sample (magnification ×8300). (B) Representative picture of cell-loaded HYADD4 sample (magnification ×23,400). (filled arrow) lysosomes; (filled triangle) outer cell membranes; (open triangle) incorporated HYADD gel.

combination of a cellular and a material component. The stromal-vascular fraction (SVF) of adipose tissue harbors a progenitor fraction that shows high proliferation and differentiation capacity [26, 27] with a lower oxygen consumption and higher tolerance of ischemia than found in mature fat cells [26]. These progenitor cells, often termed preadipocytes, have been the center of attention in adipose tissue engineering for several years. However, autologous *in vivo* tissue engineering models applying preadipocytes in a large animal model have not been described in the literature thus far. This study focuses on the reconstruction of small defects requiring small portions of adipose tissue. Injectable hyaluronic acid-based gels mixed with undifferentiated pig preadipocytes were evaluated in an autologous pig model to analyze their suitability as filler for soft tissue reconstruction. Two types of gels with varying degrees of amidation were tested (Fig. 1). HYADD3 and HYADD4 are linear derivatives of hyaluronic acid in which some of the carboxyl groups have been modified with linear amines to form activated complexes. In contrast to plain hyaluronic acid, the HYADD products obtained are barely soluble in aqueous solution and are characterized by a high degree of viscosity and long-lasting stability, due to the fact that the amide bond is not readily degradable. Both the degree of amidation and the length of the amide chain contribute to the rheological behavior of the gel. A degree of amidation of 3% to 4% für HYADD4 and 6% to 7% for HYADD3 applying dodecyl- and hexadecyl-carbon chains, respectively, revealed optimal properties for obtaining a true gel. Cell-loaded gels (1 mL) and untreated controls (1 mL) were injected subcutaneously into the ears of three pigs. Unmodified hyaluronan was not applied as a control since preceding *in vivo* experiments with and without cells had revealed a rapid degradation and loss of integrity of the biomaterial (data not shown).

Our results demonstrated pronounced degradation in the explanted cell-loaded samples at 6 wk, especially in HYADD4 (Table 1). The degradation rate, however, was decreased in comparison with unseeded control gels, which could not be found at all at 6 wk by microscopical analysis. This could be explained by a reduction in the rate of digestion due to the presence of the seeded cells, or delayed digestion due to a reduced phagocyte infiltration because of volume effects.

Microscopical examination of explanted tissue samples revealed the formation of highly vascularized adipose tissue surrounded by gel in cell-loaded HYADD3 (Fig. 2). The small size of the adipocytes supports the belief that these fat cells are newly formed, differentiated (pre)adipocytes. The adipose tissue was found around mature vessels in addition to newly formed small vascular structures. These findings are in complete accordance with the literature, where a close association between adipogenesis and angiogenesis is

TABLE 1

Study Design and Histological Findings

Gel	Cells	Number of implanted gels	Number of explanted gels	Histological findings
HYADD3	+	6	6	Adipose tissue, vessels, gel
HYADD3	−	6	Not detectable	−
HYADD4	+	6	6	Connective tissue, inflammatory foci, gel
HYADD4	−	6	Not detectable	−

stated [28]. In HYADD4, in contrast to HYADD3, no fat formation was observed in any specimen, and there was higher cellularity than in the HYADD3 samples. Using electron microscopy, HYADD4 and HYADD3 explants revealed foreign body cell infiltration after 6 wk (Fig. 4). However, the infiltration of macrophages and giant cells was much more intense and evident in HYADD4 than in HYADD3 samples, confirming light microscopy findings (Figs. 2 and 3). A possible explanation for the inflammatory reaction is the amidation of the hyaluronic acid activating immune reactions; however, this will need to be confirmed in additional experiments.

The nature of the inflammation caused as a result of the host response to implantation of any biomaterial will control the remodeling events that occur during and subsequent to its phagocytosis. It is known that differentiation of preadipocytes to mature adipocytes is inhibited by various inflammatory mediators [29] and, additionally, that phagocytic functions of preadipocytes are enhanced within inflammatory environments in lean animals [30]. Thus, small changes in implantation-associated inflammatory response are likely to lead to positive feedback enhanced inhibition of mature adipocyte formation linked to increased degradation of the gel. We believe that this process is observed in the case of HYADD4. It may be possible to reduce such an effect and dramatically enhance adipose tissue formation in both cases by anti-inflammatory treatments such as troglitazone.

In summary, our findings demonstrate that HYADD3 is applicable for generating adipose tissue in gels, displaying adipogenic as well as angiogenic properties, and strongly indicate its use as a matrix material for use as an injectable strategy for soft tissue reconstruction. Granulocyte infiltration was minimal in HYADD3 with only a minor foreign body cell reaction. To further improve the generation of adipose tissue, the addition of growth factors mixed with biomaterials might be a promising perspective. FGF-2 and insulin-like growth factor-1 (IGF-1) have been investigated, bound to gelatin or PLGA/polyethylene glycol microspheres, for delivery in a sustained and predictable manner [20, 31, 32]. Since our results show improved angiogenic and adipogenic potential, hyaluronic acid-based gels using such a strategy could represent a promising next step toward the clinical application of tissue engineering for soft tissue reconstruction.

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