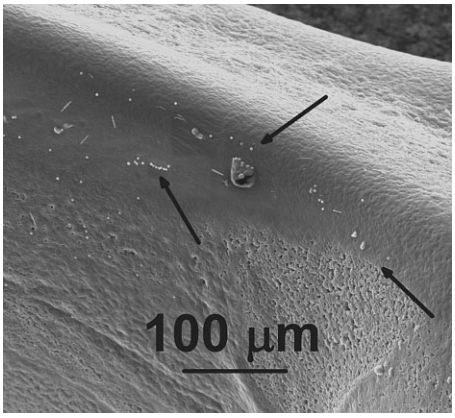
The Use of Poly(L-lactide-*co*-caprolactone) as a Scaffold for Adipose Stem Cells in Bone Tissue Engineering: Application in a Spinal Fusion Model

Pieter-Paul A. Vergroesen,[a] Robert-Jan Kroeze,[a] Marco N. Helder, Theodoor H. Smit*

Since the early 1990s, tissue engineering has been heralded as a strategy that may solve problems associated with bone grafting procedures. The original concept of growing bone in the laboratory, however, has proven illusive due to biological, logistic, and regulatory problems. Fat-derived stem cells and synthetic polymers open new, more practicable routes for bone tissue engineering. In this paper, we highlight the potential of poly(L-lactide-*co*-caprolactone) (PLCL) to serve as a radiolucent scaffold in bone tissue engineering. It appears that PLCL quickly and preferentially binds adipose stem cells (ASCs), which proliferate rapidly and eventually differentiate into the osteogenic phenotype. An in vivo spinal fusion study in a goat model provides a preclinical proof-of-concept for a one-step surgical procedure with ASCs in bone tissue engineering.



Introduction: Bone Tissue Engineering

In orthopaedics, there is an unmet need for graft material to provide support, fill voids and enhance biologic repair of bone defects. It is a large and rapidly growing segment of the orthopaedic market: today, bone replacement is required in about half of the musculoskeletal procedures performed, over 2.2 million annually.[1,2] Historically, donor bone from the patient (autograft) has been the standard of care as the procedure is well established, safe, relatively cheap and it has excellent long-term results. Autograft has strong osteogenic potential because the cells within the donor bone are actively involved in the process of bone remodelling. Harvesting autograft, however, requires additional surgery at the donor site that is related to a high level of morbidity such as chronic pain, infection, incisional hernias, vascular injuries and iliac wing fractures.[3–5] Furthermore, the quantity of bone tissue that can be harvested is limited.[6] Allograft (bone from other patients stored in a bone bank) is a reasonable alternative, but despite the criteria set by the American Association of Tissue Banks (AATB) and European Association of Musculo Skeletal Transplantation (EAMST), abnormal histopatholo-

P.-P. A. Vergroesen, R.-J. Kroeze, M. N. Helder, T. H. Smit
Department of Orthopedic Research, VU University Medical Centre, Amsterdam, The Netherlands
Fax: +31-20-4442357; E-mail: th.smit@vumc.nl
R.-J. Kroeze, M. N. Helder, T. H. Smit
Research Institute MOVE/Skeletal Tissue Engineering Group, Amsterdam, The Netherlands

[a] Both the authors contributed equally to this paper.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

DOI: 10.1002/mabi.201000433

Early View Publication; these are NOT the final page numbers, use DOI for citation !!

gical findings were found, including highly suspicious low-grade B-cell lymphoma, monoclonal plasmacytosis and other non-specific inflammation of bone marrow.[7,8] In addition, due to the lack of living cells, allografts contain a lower osteogenic potential compared to autologous bone as well as inferior revascularisation, which prolongs healing time.[6,9] Finally, stricter regulations to increase safety will further raise the costs of allografting in the near future.

Bioceramics

The limitations of bone grafting have induced the quest for alternative materials.[9,10] The main function of such a material is to allow new bone to grow into the defect. Biomaterials do not contain cells, but function as a scaffold on which cells can migrate, attach, differentiate and make new bone (osteoconductivity). Bioceramics, based on calcium phosphates, calcium sulphates and/or hydroxyapatite (HA), do exhibit this property because they have a close resemblance to the mineral component of bone. Cells like osteoblasts and their precursors easily attach to their surface and form bone. Remodelling is also supported because osteoclasts are able to degrade this type of scaffold and induce osteoblastic activity.[11,12] A drawback of bioceramics is that close proximity to the host bone is necessary to achieve osteoconduction.[13] Furthermore, bioceramics are rather brittle and their strength is limited unless a considerable amount of HA is added. However, this makes the material stiffer and more difficult to degrade, which may slow down or even inhibit bone growth.[14,15] Brittleness and hardness also make ceramics difficult to process, particularly during surgery. Another practical disadvantage of bioceramics is that they eclipse the healing area on radiographs: it is hard to see whether new bone has been formed and whether the defect is healed or not.

Biopolymers

Some of the drawbacks of bioceramics can be countered by degradable polymers. Polymers have great design flexibility because their structure, composition and (thus) properties can be tailored to specific needs.[16,17] Biodegradability can be imparted into the material by molecular design: some polymers undergo hydrolysis, others can be degraded by enzymatic pathways. Polymers are much more ductile than ceramics and some can — in their solid form — reach mechanical compression strength close to cortical bone.[18] On the other hand, tensile and bending strength are generally much less than bone. Another advantage is that polymers are radiolucent, so that the bone healing process can be followed radiographically (Figure 1). A drawback of degradable polymers is that they are

Pieter-Paul A. Vergroesen is a medical student at the faculty of the VU University Medical Centre in Amsterdam, The Netherlands. After finishing his BSc in medicine in 2009 he worked for a year in the VU medical centre orthopedics department as a PhD assistant. After continuing his MSc in medicine in 2009 he is currently working in a Doctorantenstelle at the Centre for Translational Regenerative Medicine in Leipzig, Germany. He is projected to graduate as an MSc in medicine in the fall of 2012.

Robert Jan Kroeze holds a medical degree from the VU University Medical Centre, The Netherlands (2004) and is finishing his PhD at the Department of Orthopaedic Surgery in conjuction with the Skeletal Tissue Engineering Group Amsterdam. His thesis is focused on the analyses of a biodegradable polymer as a cage filler for spinal fusion in the context of bone tissue engineering using a one-step surgical procedure. As part of his training for orthopaedic surgery, he started his residency in general surgery at the Kennemer Gasthuis, Haarlem, The Netherlands in 2010. In 2012 he will continue his orthopaedic surgery training at the Medisch Centrum Alkmaar, Alkmaar, The Netherlands.

Marco N. Helder undertook a PhD in 1994 on the roles of bone morphogenetic proteins during the development of skeletal and extraskeletal tissues. Until 1997 he worked as a post-doc at RU Leiden on the biochemical isolation and functional characterisation of a nonsteroidal hormone from the follicular fluid of ovaries. He then moved to Groningen, where he was involved in developing antisense and other, virus-mediated gene therapeutic strategies against cervical cancer. Since 2002 he has been an assistant professor at the Department of Orthopaedic Surgery at the Vrije Universiteit medical centre in Amsterdam, focusing on oncology and the clinical implementation of regenerative medicines for musculoskeletal, cardiac, and connective tissue disorders.

Theodoor H. Smit holds an MSc in mechanical engineering at the Technical University Delft, Netherlands, and a PhD in biomechanical engineering at the Technical University Hamburg-Harburg, Germany. Since his graduation, he has been affiliated with the VU University Medical Centre in Amsterdam, currently as associate professor in Skeletal Physics and Tissue Engineering within the department of Orthopedic Surgery. Dr. Smit is founding director and current chair of the Skeletal Tissue Engineering Group Amsterdam, a research foundation working on translational regenerative medicine.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

↖ Early View Publication; these are NOT the final page numbers, use DOI for citation !!

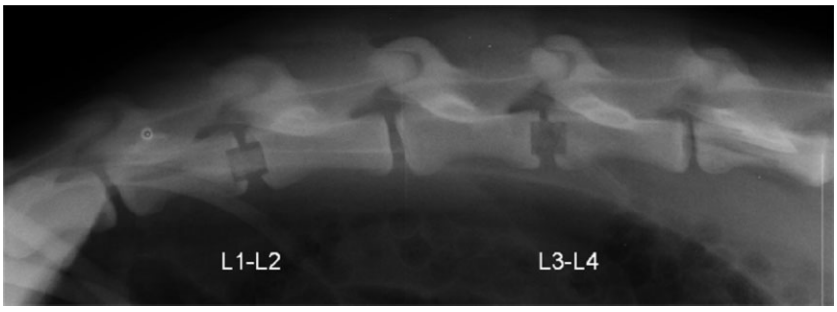


Figure 1. Post-surgical lateral radiograph of a goat spine undergoing a split-level spinal fusion. L1-L2: PEEK cage filled with autologous bone, L3-L4: PEEK cage filled with PLCL scaffold.

complex materials and their properties can differ, even for the same composition, as a function of manufacturing, history of temperature and loading, sterilisation, local environment, and design geometry.[19] Furthermore, most polymers, including the often used polyesters, are not osteoconductive: the response elicited in vivo is usually encapsulation by a persistent fibrous layer of fibroblasts, collagen, and inflammatory cells.[20,21] Surface roughness and hydrophobicity play an important role here: cells are less likely to attach and differentiate on hydrophobic surfaces and show more fibrous tissue formation.[20] This is the case for practically all polyesters (polylactides, polyglycolydes, polycaprolactone, and their co-polymers), which form the most popular class of polymers in bone tissue engineering. A logical and well explored route in materials science is to combine the properties of bioceramics and polymers; after all, bone also is a composite of natural polymers and minerals. It is indeed clearly observed that composites can be made osteoconductive like bioceramics, but lack their brittleness.[22] However, this comes at the cost of loss of mechanical strength and an increased resorption rate.

Growth Factors

Despite the considerable progress booked in the field of bone scaffolds over the last decades in terms of understanding cell–material interactions and enhancing their physical properties, their clinical performance still lags behind those with autografts. This is due to the fact that the cells and the extra-cellular matrix components in the graft play an active role in the regenerative remodelling process. Many of the bioactive molecules like tissue growth factors (TGFs) have been identified and have been shown to enhance bone formation when added to synthetic bone scaffolds.[23,24] Some factors, in particular BMP-2, have been commercially successful as well. However, despite their potency and relative ease of use, there are some serious concerns with this type of stimulation. Dosages needed for effective treatment are three to six orders of magnitude larger than needed for osteogenic induction.[25] This is not only expensive, but also may induce excessive bone formation.[26] Furthermore, the physiological role of BMP-2 and the many other growth factors is not well understood and sometimes may end up counterproductive: due to strong bone resorption shortly after surgery, implants may migrate and stabilisation may get lost instead of consolidated.[27,28]

Cell Therapy

Another approach to enrich synthetic bone scaffolds is the seeding of stem cells. One of the most common sources is autologous bone marrow, but also other tissues contain stem cells.[29] Adult stem cells are used in tissue engineering to produce autologous tissues and to avoid ethical issues and immune responses. Bone marrow consists of haematopoietic and mesenchymal stem cells (MSCs), which can differentiate into various cell types including endothelial cells for vascularisation and osteoblasts for osteogenesis.[29] Bone marrow stem cells have shown great regenerative potential in the laboratory and in animal experiments, but their clinical application is as yet limited. The most obvious reason is that the number of stem cells per volume bone marrow is rather limited: about 1 in 100 000 nucleated cells.[30] Also the volume of bone marrow that can be harvested is limited. The low number of stem cells necessitates in vitro culture expansion to obtain sufficient numbers of cells for clinical application. This is unattractive as it costs time and money and runs into massive regulatory restrictions.[31]

Mesenchymal stem cells (MSCs) derived from adipose tissue were first identified by Zuk et al.[32] Adipose tissue is home to various cells, including endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells and preadipocytes. The incidence of MSCs in adipose tissue, however, is estimated to be about 1 per 1 000–10 000 nucleated cells, which is 2–3 magnitudes higher than the number of MSCs in bone marrow.[33] Yet their biological properties are not compromised: differentiation potential has been shown into adipogenic, myogenic, chondrogenic, osteogenic, endothelial, cardiomyogenic and neurogenic, phenotypes.[32,33] But the most important features are expandability and accessibility: adipose tissue can be obtained in substantial quantities and is accessible at most surgical sites, neutralising the need for a separate harvest site and its concomitant morbidity. Adipose tissue thus is a promising source of stem cells for tissue engineering, and the adipose-derived stem cells have enormous clinical potential for the regeneration of tissues.[34,35]

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

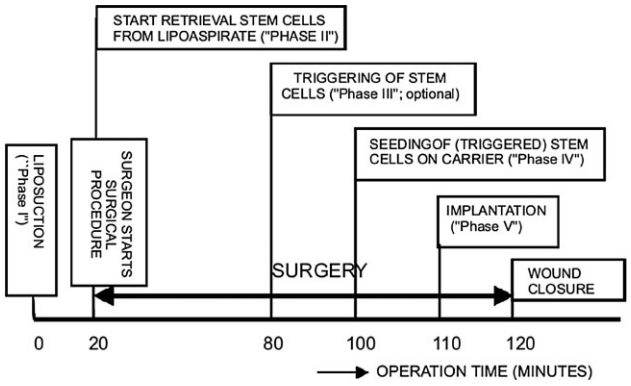


Figure 2. Concept of a one-step surgical procedure. The surgery starts with harvesting of the adipose tissue followed by a split procedure. The surgeon continues the surgery, while the tissue engineer isolates the stem cell-containing cell population from the adipose tissue, treats the cells to initiate differentiation into the proper phenotype, and seeds the stimulated cells on the scaffold. The surgeon then implants the scaffold containing the stem cells and finishes the surgery. The whole procedure takes 2–2.5 h.[35]

Based on the above, Helder et al. formulated the concept of a one-step surgical procedure for tissue engineering (Figure 2).[35] The proposed concept uses off-the-shelf bioresorbable materials and easily accessible autologous MSCs from adipose tissue, harvested with minimally invasive techniques and in a clinically relevant yield. This approach allows restoring damaged tissues using tissue-engineering concepts without any foreign materials remaining in the patient on the long term. This concept is not only cost effective as compared to cell expansion in vitro, it is also beneficial to the patient, because the use of expensive recombinant growth factor(s) can be reduced to a minimum, a second surgical intervention is avoided, and expensive contamination-sensitive stem cell expansion in specialised laboratories is not needed. Prolonged culturing may result in increased risk of contamination, loss of multipotency, loss of cell quality due to accumulating DNA damage and increased senescence rates due to shortening of telomeres.

Purpose

Stem cells are the corner stone of tissue engineering because they have the capacity to vitalise synthetic degradable biomaterials for tissue engineering. At the same time, the feasibility of a one-step surgical procedure depends on the ability of the adipose tissue derived stem cells (ASCs) to attach to a scaffold material not only in sufficient quantities, but also within the short time frame of the surgical procedure. Furthermore, the attached cells should be able to proliferate and to differentiate along the osteogenic lineage in order to enhance the clinical outcome. In earlier studies, we showed that poly(L-lactide-*co*-caprolactone) (PLCL) has such properties and would be a strong candidate to serve as a radiolucent, degradable scaffold in bone tissue engineering.[37] In this paper, we present the application of the PLCL-ASC construct in an in vivo spinal fusion goat model as a surgical proof of concept. The biocompatibility of this cell-scaffold combination is established in a 1-month follow-up pilot study. Furthermore, we present preliminary radiological data of a larger goat study with three and 6 months follow-up, in which substantial bone growth is observed. Although efficacy of the cell-seeded scaffold yet has to be established, the observation that spinal fusion was obtained in some goats may be interpreted as a preclinical in vivo proof of concept.

Experimental Part

Tissue Sampling and Processing

Isolation of the stromal vascular fraction (SVF) from goat subcutaneous adipose tissue was performed as described previously.[36] The animal care and use committee of the Vrije Universiteit Amsterdam approved the use of goats in these experiments. In short, harvested peri-renal adipose tissue was enzymatically dissociated with collagenase, passed through a 100 μm-mesh filter, pelleted and washed several times with phosphate buffered saline (PBS) to obtain the final SVF preparation.

SVF Attachment, Proliferation, and Differentiation in PLCL Scaffolds

In earlier in vitro studies, the co-polymer was tested for ASC attachment from SVF. From the data presented in these studies it was concluded that (i) attachment of cells from the SVF to PLCL was very rapid (≈10 min; Figure 3A), (ii) stem cell-like cells preferentially adhered to the PLCL scaffold, as demonstrated by depletion of the colony forming unit (CFU)-capable cells from the SVF (Figure 3B) and (iii) cells in the scaffold were able to proliferate (Figure 4a) and differentiate (Figure 4b) towards the osteogenic lineage.[37,38]

Biodegradable Polymer

The material used for this study is a 70/30 poly(L-lactide-*co*-ε-caprolactone). The co-polymer was chosen as it is a combination of L-lactic acid and ε-caprolactone, both of which, either alone or combined as a co-polymer, are approved by the Food and Drug Administration and suitable for bone and cartilage regeneration.[39,40] The polymer had an average molecular weight of ≈200 000 $g \cdot mol^{-1}$. The inherent viscosity ranges from 1.2 to 1.8 $dL \cdot g^{-1}$ with a nominal value of ≈1.5 $dL \cdot g^{-1}$. The L-lactide content is 67–73 mol-% and the ε-caprolactone content is 33–27 mol-%. Glass transition temperature is ≈18–20 °C. The material will degrade by hydrolysis in ≈1 year.

Scaffold Design

Not only the micro-architecture, but also the macro-design of the scaffold plays a pivotal role in tissue engineering. In bone tissue

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Early View Publication; these are NOT the final page numbers, use DOI for citation !!

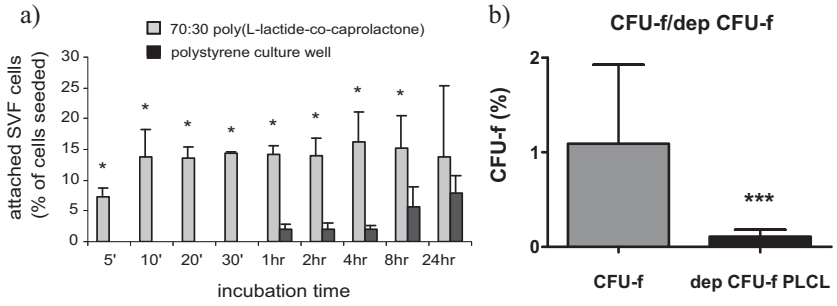


Figure 3. Attachment of cells from the SVF of adipose tissue. (a) After only 10 min, 10–15% of the cells attached to PLCL, a number that did not increase with longer incubation time. Note that the attachment to culture plastic (polystyrene) took a day to obtain a substantial number of cells (*, $p < 0.05$). (b) Pre-seeding and post-seeding (depletion) CFU-f assays. A treatment over control ratio (T/C) was applied to the CFU-f assays (depletion CFU-f/pre-seeding CFU-f). A significant decrease in CFU-f ratio can be seen when compared to the pre-seeding CFU-f (***, $p < 0.001$).

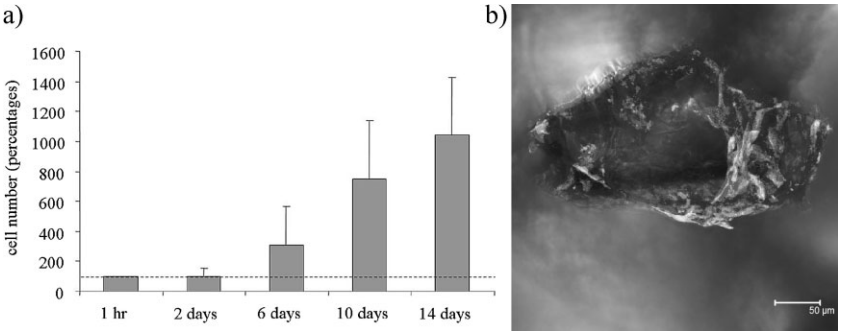


Figure 4. Proliferation and differentiation of cells attached to the PLCL scaffold. (a) After 2 weeks, a tenfold increase of cell number was observed. (b) ASC differentiation towards the osteogenic lineage is shown by the expression of osteonectin after three weeks of culture in an osteogenic medium.

Sterilisation

When focusing on the clinical application of biomaterials, sterilisation is a mandatory step. Standard hospital steam sterilisation would exceed the melting temperature of PLCL, and therefore three other types of sterilisation were evaluated: e-Beam sterilisation, ethylene oxide sterilisation (EtO) and argon glow discharge (aGD).[44] PLCL specimens sterilised with either method were compared in surface roughness, contact angle (wettability), cell proliferation and alkaline phosphatase (ALP) activity. Significantly higher values for surface roughness (EO > aGD > e-beam) and significant differences in contact angles (EO > e-beam > aGD) and surface energies (aGD > e-beam > EO) were observed. Increased cell attachment and proliferation rates were observed with lower contact angles. Although specimens treated with aGD showed the highest cell proliferation and the smallest contact angle, regular EtO showed a significantly higher increase in ALP activity. As higher ALP activity favours bone tissue engineering, and EtO has higher cost-effectiveness and better off-the-shelf potential, it was favoured as a sterilisation method for PLCL scaffold for spinal fusion.

Spinal Fusion Animal Model

To test bone tissue engineering concepts in vivo, spinal fusion is an attractive model because it aims to bridge a well-defined critical size defect between two vertebral bodies under dynamic loading conditions. The surgery in goats is relatively easy, mildly stressing to the goats and relevant to the clinical situation.[45,46] In the pilot study with 1 month follow-up, seven mature female Dutch milk goats received twin level special fusion. Three cages were filled with the PLCL scaffold only, and 3 with SVF cells (see below). Four other cages were filled with autologous bone and served as control (golden standard). Furthermore, we show preliminary results from 32 goats in a 3- and 6-months follow-up study (to be published elsewhere) receiving

engineering it is repeatedly noted that a porosity of at least 80–90%[41] and a pore size >250–300 µm[42,43] favours bone growth. Therefore, it was tried to design our scaffold according to these specifications. The PLCL was laser cut in sheets by Proxy Biomedical (Gallway, Ireland). Ten of these sheets were stacked to form a rectangular three-dimensional scaffold of 7 mm × 10 mm × 15 mm with aligned pores of 280 µm (Figure 5). Eventually, the porosity of the stacked construct was calculated to be 71%.

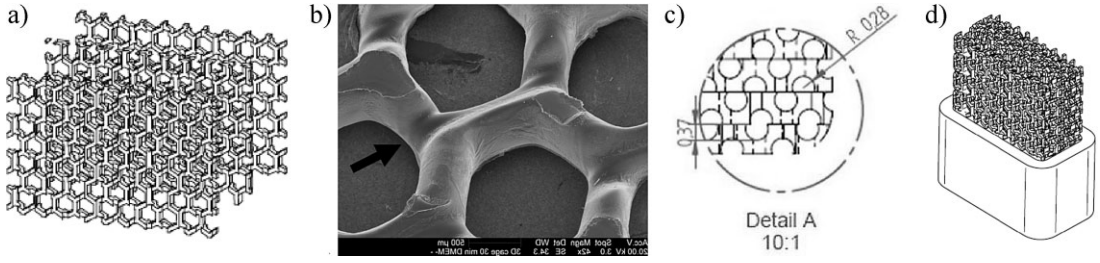


Figure 5. 3D polymer scaffold for bone tissue engineering. (a) Exploded view of the stacked sheets of PLCL. (b) Scanning electron micrograph of the scaffold showing a honey comb motif and a groove allowing vertical bone ingrowth (arrow). (c) Detail of the stacked sheets showing the canals created by the grooves. (d) Stacked sheets inserted in a cage device designed for spinal fusion in the goat model.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

the same surgery. At the 3 month-time point, 9 cages each received PLCL scaffold or autologous bone, and 11 cages received SVF-loaded scaffolds. In the goats sacrificed after 6 months, 12 cages were filled with PLCL scaffold, 11 received autologous bone and 10 received SVF.

The surgical procedure has been described in detail elsewhere.[47,48] Briefly, through a left retroperitoneal approach, the L1-L2 and L3-L4 intervertebral discs were identified. Under fluoroscopic guidance, a 2-mm guide wire was centered transversely in the IVD. An 8-mm drill was placed over the guide wire and a hole was created through the IVD and the adjacent endplates. A 10 mm × 10 mm box gouge was placed over the drill and used to punch a transverse rectangular defect through the intervertebral disc and ≈2 mm of endplates and subchondral bone of both adjacent vertebral bodies (Figure 1). Spinal cages (10 mm × 10 mm × 18 mm) were custom made of poly-ether-ether-ketone (PEEK), a material routinely used in clinical practice nowadays. The cages were filled either with autologous bone from the iliac crest, or with a stack of lasered PLCL sheets as described above (Figure 5d). The sheets in some cages were seeded with 5×10^6 nucleated cells from the SVF concentrated from peri-renal fat, that was harvested during the approach.[35] The sheets in other cages were only pre-wetted with NaCl before implantation. After 1 week, when wound healing was completed, the animals were moved to a large indoor and outdoor environment without restrictions. Health status, eating habits and ambulatory activities were monitored daily. At the designated time (1, 3 or 6 months after surgery), the goats were sedated and euthanised with an overdose (20 mg · kg^{-1}) of pentobarbital. Spinal columns were collected and analysed as described previously.[48] Briefly, parasagittal sections (3–5 mm) were made with a water-cooled band saw. Lateral macroscopic images and radiographs of the 5-mm sections were used to evaluate fusion within the cages. Subsequently, samples were decalcified and embedded in paraffin, 5 μm sections were cut and histological staining with haematoxylin-eosin. To determine vasculogenesis, rabbit affinity purified anti-mouse laminin (Abcam:, UK, cat 11575) 1:50 concentration was used as a primary antibody and a goat–anti-rabbit (Vectorlabs, Burlingame, CA, USA) as a secondary antibody. The differentiation of cells towards osteoblasts was determined by staining for proteins expressed by osteoblast precursors. Mouse anti-rat osteopontin (monoclonal MPIIIB10. Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) in 1:200 concentration was used as a primary antibody and horse-anti-mouse was used as a secondary antibody (Vectorlabs, Burlingame, CA, USA).

Results and Discussion

The One-Step Surgical Procedure in a Goat Model

All goats included in this study recovered uneventfully from the surgical procedure and no complications were observed during the follow-up period. Normal ambulatory activities were resumed on the second-postoperative day. CFU assays showed clinically relevant numbers of ASCs in all SVF samples. In all operated goats sufficient amounts of peri-renal adipose tissue could be harvested through the primary incision. Therefore, a second incision was not necessary. The work-up times for adipose tissue towards stromal fractional fraction were as projected by Helder et al.[35] However, an average delay of 20 min was experienced because the spinal surgery went a little faster than planned. In human spinal fusion surgery this will not be an issue because the stand alone model used in our experiment is not common practice in humans. For less extensive human surgery requiring the use of SVF, the time delay could be countered by using well established methods of liposuction either through the primary incision or in the conventional way which is known to have little or no complications. We can conclude that adding cells from SVF, containing sufficient amounts of MSCs[37,49] to any tissue engineering construct is feasible in a one-step surgical procedure.

One Month Follow-Up

The most important finding on the PLCL scaffolds is the decrease in porosity observed in all samples. The mean porosity was 57% with a standard deviation of 4.50, which is significantly lower than the 71% porosity it was originally designed with ($p < 0.01$). This suggests that the scaffold swells when inserted into the body. In line with this, minor bulging of the scaffold outside the cage was observed in some sections (Figure 6a).

After 1 month, no bone ingrowth was observed in any of the PLCL filled cages. However, extensive bone deposition was found at the interface between the scaffold and the bone bed of the vertebral body (Figure 6). Vasculogenesis, required for osteogenesis to start, was well developed in both, the empty and the stem cell seeded PLCL scaffolds (Figure 7). This contrasts sharply with earlier studies in which biphasic calcium phosphates were used as cage filler[35] and suggests that PLCL is more favourable for

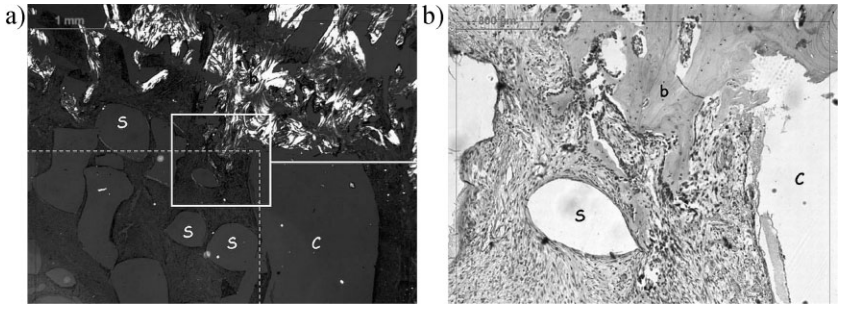


Figure 6. (a) Polarised light view of section of a cage (c) filled with PLCL scaffold not containing any ASCs (s). To the left of the box, scaffold material (s) can be observed to bulge beyond the cage perimeter (dashed lines). (b) Enlargement of Figure 6a. Shows a lot of OPN positive cells (brown stain) around the scaffold (s) near the vertebral bone (b).

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Early View Publication; these are NOT the final page numbers, use DOI for citation !!

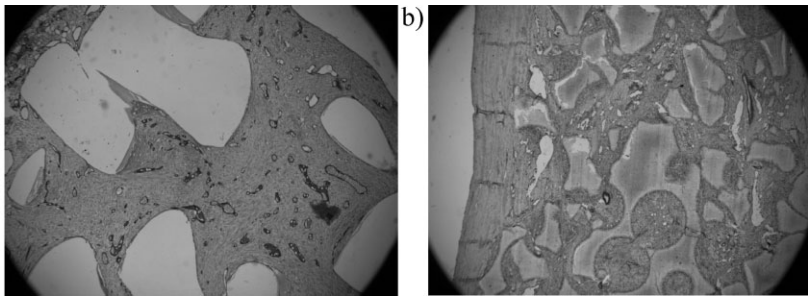


Figure 7. Laminin staining showing newly formed blood vessels within cages filled with PLCL (a) and tricalcium phosphate (b). Note that vascularisation is much stronger in PLCL than in tricalcium phosphate. Sample b is from a separate study on the use of ASCs in spinal fusion in a goat model.[35]

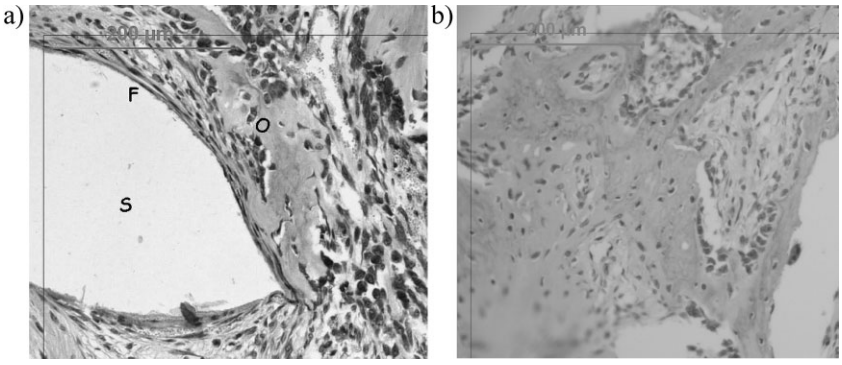


Figure 8. Further enlargement of Figure 6, showing a thin layer of fibrous tissue (F) around the unseeded PLCL scaffold (S). O indicates osteopontin positive cells depositing woven bone. Right: negative control for osteopontin staining.

vascularisation than biphasic calcium phosphate. The total area of new blood vessels was similar for scaffolds with and without SVF (4.9 and 4.4%, respectively). Interestingly, the number of blood vessels appeared higher in the empty scaffolds (72.5 mm^{-2} vs. 56.5 mm^{-2}), but in contrast, the blood vessel cross-sectional area was found to be higher in the SVF-seeded scaffolds (859 μm^2 versus 667 μm^2). Unfortunately, the low number of samples in this study did not allow proper statistical evaluation, and further studies are warranted to substantiate these findings. Nevertheless, this could indicate that the stem cell-seeded scaffold produced a more mature vasculature, but also that blood vessels may have merged to create larger lumens. The diameter of the blood vessels (mean 30 μm) is well above the size of capillaries (5–10 μm) and can therefore be considered sufficient for bone formation. At still higher magnification, a mild foreign body reaction can be seen consisting of a 2–3 cell layer around the PLCL scaffold (Figure 8). This indicates that the surface of the scaffold was suboptimal for the deposition of bone. On the other hand, the number of multi-nucleated giant cells (MNGCs) was marginal in both, the empty and the ASC-seeded PLCL scaffolds (4.2 and 3.2 cells·mm^{-2}, respectively). This is comparable to previous studies of our group with biphasic calcium phosphate[14] and indicates an excellent biocompatibility.

Three and Six Months Results—Radiography

Of the goats that were sacrificed after 3 and 6 months follow-up, only preliminary results are available in the form of radiographs (Figure 9). In general, it must be emphasised that the results were heterogeneous: only the best results in each group are shown. These provide an in vivo proof of principle for bone tissue engineering with PLCL scaffolds (Figure 9e), and suggest a beneficial effect of ASCs over the scaffold only (Figure 9f). Furthermore, it can be appreciated that bone ingrowth actually can be observed radiographically, a feature of the radiolucent polymer that should be considered a practical clinical advantage over non-radiolucent bioceramics.

Using a fusion scoring system, which considers full interbody fusion as well as full bone bridging outside the cages itself as fused segments — common in human clinical practice as well — we found that fusion rates were 50% for the PLCL scaffold only, 60% for the SVF-loaded PLCL-scaffolds and 73% for fusions using autologous bone. If only considering interbody fusion, some of the cages showed moderate or even poor bone ingrowth. Although this can be interpreted as a bad result, we feel that this may be due to a lack of initial stability of the fused spinal segment. To reduce surgical stress to the goats, we applied the cages in a stand-alone situation, i.e., without additional instrumentation to further stabilise the segment. Earlier in vitro studies have shown that the insertion of a stand-alone cage does not stabilise the spinal segment[50] and that additional stabilisation with an internal fixator does enhance spinal fusion.[48] In the current study, also the autograft group shows a fusion rate of only 73% (8/11) after 6 months. In previous studies, we achieved solid fusion in 80% of the cases.[51,52] We believe that this decrease in achieving fusion is due to the use of older animals (>3.5 year) in this study, compared to the ±2 year old animals in previous studies. The results of the study thus may have been affected by the lack of initial stability in the operated segments, and by the use of relatively old animals. While the latter could be indicative for the clinical situation, the former is not, because stand-alone application of spinal cages is seldom performed in the lumbar spine area.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

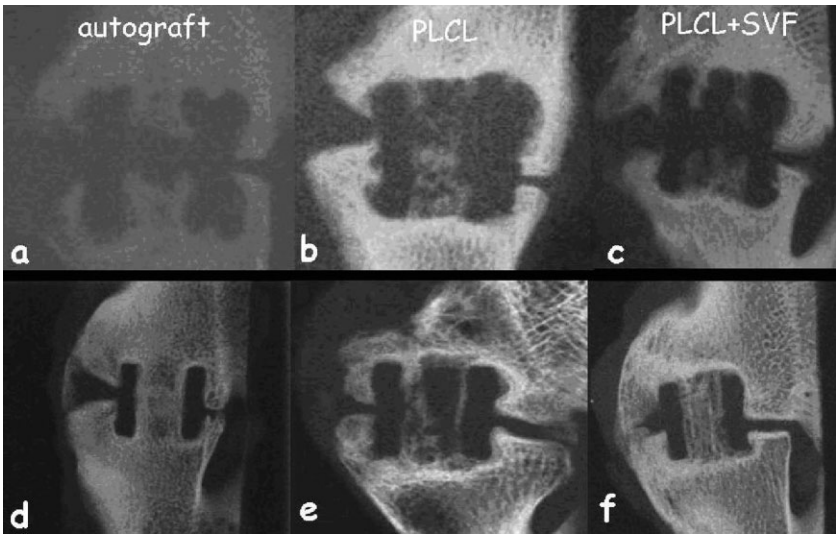


Figure 9. Radiographs of the spinal segments after 3 (a–c) and 6 months follow-up (d–f). The left column (a,d) shows the golden standard in spinal fusion, a cage filled with autograft. Fusion is better than with the empty PLCL scaffold (b,e), although bone ingrowth is clearly observed. The addition of ASCs appears to enhance fusion (c,f).

Conclusion

Bone tissue engineering aims to avoid problems associated with bone grafting by using degradable scaffolds enriched with stem cells and/or growth factors. PLCL can function as such a scaffold because it is biocompatible, it facilitates vasculogenesis and it favours the rapid attachment of ASCs, thus allowing their application in a one-step surgical procedure. An additional practical advantage of PLCL over bioceramics is its radiolucency, which allows the surgeon to actually see bone ingrowth into the scaffold on post-surgical radiographs. The current study provides a proof of principle for this approach and indicates a beneficial effect of ASCs. However, initial instability may frustrate the fusion process and end in non-fusion. Histology shows that the surface of the PLCL scaffold is covered by a thin layer of fibrous tissues, which is suboptimal for bone formation. Surface functionalisation may solve this issue.[53–56]

Acknowledgements: The authors acknowledge Dr. *P. Gingras* and *P. Mulrooney* for their help in designing and manufacturing the PLCL sheets used in this study; Dr. *A. L. J. J. (Ton) Bronckers*, Academic Centre of Dentistry Amsterdam (ACTA-VU/UvA) for his advise on histological staining and *Klaas-Walter Meyer* and *Paul Sinnige*, University Animal Laboratory, VU University Amsterdam, for their help with the animal experiments. The work of R. J. K. has been supported by the *Dutch Program for Tissue Engineering* (Grant No. BGT 6734).

Received: November 1, 2010; Revised: December 30, 2010; Published online: DOI: 10.1002/mabi.201000433

Keywords: adipose stem cells; bone; composites; degradable polymers; one-step surgical procedures; surfaces; tissue engineering

[1] Espicom Business Intelligence (2009) Market Report 2008 ISBN: 978 1 85822 308 7.
[2] P. V. Giannoudis, H. Dinopoulos, E. Tsiridis, *Injury* **2005**, *36* (Suppl 3), S20.
[3] E. M. Younger, M. W. Chapman, *J. Orthop. Trauma* **1989**, *3*, 192.
[4] E. D. Arrington, W. J. Smith, H. G. Chambers, A. L. Bucknell, N. A. Davino, *Clin. Orthop. Relat. Res.* **1996**, 300.
[5] B. N. Summers, S. M. Eisenstein, *J. Bone Joint Surg. Br.* **1989**, *71*, 677.
[6] M. Miyazaki, H. Tsumura, J. C. Wang, A. Alanay, *Eur. Spine J.* **2009**, *18*, 783.
[7] S. Sugihara, A. D. van Ginkel, T. U. Jiya, B. J. Van Royen, P. J. van Diest, P. I. Wuisman, *J. Bone Joint Surg. Br.* **1999**, *81*, 336.
[8] E. W. Zwitser, G. A. De, M. J. Basie, F. J. van Kemenade, B. J. Van Royen, *BMC Musculoskelet. Disord.* **2009**, *10*, 53.
[9] J. M. Kanczler, R. O. Oreffo, *Eur. Cell Mater.* **2008**, *15*, 100.
[10] C. Laurencin, Y. Khan, S. F. El-Amin, *Expert. Rev. Med. Devices* **2006**, *3*, 49.
[11] G. Spence, N. Patel, R. Brooks, W. Bonfield, N. Rushton, *J. Biomed. Mater. Res. A* **2010**, *92*, 1292.
[12] G. Spence, N. Patel, R. Brooks, N. Rushton, *J. Biomed. Mater. Res. A* **2009**, *90*, 217.
[13] A. El-Ghannam, *Expert. Rev. Med. Devices* **2005**, *2*, 87.
[14] M. R. Krijnen, T. H. Smit, v. Everts, P. I. Wuisman, *J. Biomed. Mater. Res., Part B: Appl. Biomater.* **2009**, *89*, 9.
[15] M. Kamitakahara, C. Ohtsuki, T. Miyazaki, *J. Biomater. Appl.* **2008**, *23*, 197.
[16] J. C. Middleton, A. J. Tipton, *Biomaterials* **2000**, *21*, 2335.
[17] X. Liu, P. X. Ma, *Ann. Biomed. Eng.* **2004**, *32*, 477.
[18] M. van Dijk, T. H. Smit, M. F. Arnoe, E. H. Burger, P. I. Wuisman, *Eur. Spine J.* **2003**, *12*, 34.
[19] P. I. Wuisman, T. H. Smit, *Eur. Spine J.* **2006**, *15*, 133.
[20] K. James, H. Levene, J. R. Parsons, J. Kohn, *Biomaterials* **1999**, *20*, 2203.
[21] J. Hunt, "Foreign Body Response", in: *Encyclopedia of Biomaterials and Biomechanical Engineering*, Marcel Dekker, New York 2004, p. 641.
[22] K. E. Tanner, "Composites based on Degradable Polymers", in: *Degradable Polymers for Skeletal Implants*, P. I. Wuisman, T. H. Smit, Eds., Nova Science Publishers, Inc., New York 2009, p. 73.
[23] F. R. Rose, Q. Hou, R. O. Oreffo, *J. Pharm. Pharmacol.* **2004**, *56*, 415.
[24] A. H. Simpson, L. Mills, B. Noble, *J. Bone Joint Surg. Br.* **2006**, *88*, 701.
[25] M. Knippenberg, M. N. Helder, B. Z. Doulabi, R. A. Bank, P. I. Wuisman, J. Klein-Nulend, *Tissue Eng. Part A* **2009**, *13*, 1799.
[26] T. E. Mroz, J. C. Wang, R. Hashimoto, D. C. Norvell, *Spine (PhilaPa 1976)* **2010**, *35*, S86.
[27] R. Vaidya, J. Carp, A. Sethi, S. Bartol, J. Craig, C. M. Les, *Eur. Spine J.* **2007**, *16*, 1257.
[28] B. B. Pradhan, H. W. Bae, E. G. Dawson, V. V. Patel, R. B. Delamarter, *Spine (PhilaPa 1976)* **2006**, *31*, E277.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

↖ Early View Publication; these are NOT the final page numbers, use DOI for citation !!

[29] A. Vats, R. C. Bielby, N. S. Tolley, R. Nerem, J. M. Polak, *Lancet* **2005**, *366*, 592.
[30] H. Castro-Malaspina, W. Ebell, S. Wang, *Prog. Clin. Biol. Res.* **1984**, *154*, 209.
[31] A. Parson, *Cell* **2006**, *125*, 9.
[32] P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim, M. H. Hedrick, *Mol. Biol. Cell* **2002**, *13*, 4279.
[33] P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz, M. H. Hedrick, *Tissue Eng.* **2001**, *7*, 211.
[34] P. A. Zuk, *Science* **2001**, *293*, 211.
[35] M. N. Helder, M. Knippenberg, J. Klein-Nulend, P. I. Wuisman, *Tissue Eng.* **2007**, *13*, 1799.
[36] M. J. Oedayrajsingh-Varma, S. M. van Ham, M. Knippenberg, M. N. Helder, J. Klein-Nulend, T. E. Schouten, M. J. Ritt, F. J. van Milligen, *Cytotherapy* **2006**, *8*, 166.
[37] W. J. Jurgens, R. J. Kroeze, R. A. Bank, M. J. Ritt, M. N. Helder, *J. Orthop. Res.* **2011**, DOI: 10.1002/jor.21314.
[38] R. J. Hoogendoorn, Z. F. Lu, R. J. Kroeze, R. A. Bank, P. I. Wuisman, M. N. Helder, *J. Cell Mol. Med.* **2008**, *12*, 2205.
[39] J. Xie, M. Ihara, Y. Jung, I. K. Kwon, S. H. Kim, Y. H. Kim, T. Matsuda, *Tissue Eng.* **2006**, *12*, 449.
[40] M. Honda, T. Yada, M. Ueda, K. Kimata, *J. Oral Maxillofac. Surg.* **2000**, *58*, 767.
[41] T. D. Roy, J. L. Simon, J. L. Ricci, E. D. Rekow, V. P. Thompson, J. R. Parsons, *J. Biomed. Mater. Res., Part A* **2003**, *66*, 283.
[42] V. Karageorgiou, D. Kaplan, *Biomaterials* **2005**, *26*, 5474.
[43] M. W. Laschke, Y. Harder, M. Amon, I. Martin, J. Farhadi, A. Ring, N. Torio-Padron, R. Schramm, M. Rucker, D. Junker, J. M. Haufel, C. Carvalho, M. Heberer, G. Germann, B. Vollmar, M. D. Menger, *Tissue Eng.* **2006**, *12*, 2093.
[44] R. J. Kroeze, M. N. Helder, W. H. Roos, G. J. Wuite, R. A. Bank, T. H. Smit, *Acta Biomater.* **2010**, *6*, 2060.
[45] T. H. Smit, *Eur. Spine J.* **2002**, *11*, 137.
[46] T. H. Smit, M. R. Krijnen, D. M. van, P. I. Wuisman, *J. Mater. Sci. : Mater. Med.* **2006**, *17*, 1237.
[47] M. van Dijk, T. H. Smit, E. H. Burger, P. I. Wuisman, *Spine* **2002**, *27*, 2706.
[48] M. R. Krijnen, M. G. Mullender, T. H. Smit, v. Everts, P. I. Wuisman, *Spine* **2006**, *31*, 1559.
[49] M. J. Varma, R. G. Breuls, T. E. Schouten, W. J. Jurgens, H. J. Bontkes, G. J. Schuurhuis, S. M. van Ham, F. J. van Milligen, *Stem Cells Dev.* **2007**, *16*, 91.
[50] M. R. Krijnen, D. Mensch, J. H. van Dieen, P. I. Wuisman, T. H. Smit, *Acta Orthop.* **2006**, *77*, 454.
[51] M. Van Dijk, T. H. Smit, E. H. Burger, P. I. Wuisman, *Spine* **2002**, *27*, 2706.
[52] M. Van Dijk, T. H. Smit, S. Sugihara, E. H. Burger, P. I. Wuisman, *Spine* **2002**, *27*, 682.
[53] M. Nakagawa, F. Teraoka, S. Fujimoto, Y. Hamada, H. Kibayashi, J. Takahashi, *J. Biomed. Mater. Res., Part A* **2006**, *77*, 112.
[54] F. Teraoka, M. Nakagawa, M. Hara, *Dent. Mater. J.* **2006**, *25*, 560.
[55] W. L. Murphy, D. J. Mooney, *J. Am. Chem. Soc.* **2002**, *124*, 1910.
[56] W. L. Murphy, D. H. Kohn, D. J. Mooney, *J. Biomed. Mater. Res.* **2000**, *50*, 50.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim