Gelatin-Based Microcarriers as Embryonic Stem Cell Delivery System in Bone Tissue Engineering: An in-Vitro Study

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Mouse embryonic stem cells were cultured on commercially available biodegradable macroporous microcarriers. A culture period of 1−2 weeks was needed to colonize the microcarriers. Embryonic stem cells retained their pluripotency for up to 14 days when cultured in medium supplemented with leukemia inhibitory factor. Replacing this medium by differentiation medium for 2 weeks initiated osteogenic differentiation. Encapsulation of the cell-loaded microcarriers in photopolymerizable polymers (methacrylate-endcapped poly-D,L-lactide-*co*-caprolactone), triacetin/hydroxyethylmethacrylate (HEMA) as solvent and with/without gelatin as porogen, resulted in a homogeneous distribution of the microcarriers in the polymer. As observed by transmission electron microscopy, viability of the cells was optimal when gelatin was omitted and when using triacetin instead of HEMA.

Introduction

Congenital or acquired bone defect is a major problem in orthopedic surgery. Commonly used bone-grafting materials include both autologous and banked allogenic bone grafts.[1] Autologous bone grafting is widely used for graft procedures because of its superior efficacy and capability of avoiding transmission of infections.[2] The iliac crest is most frequently chosen as the donor tissue site because of the quality and amount of bone available. However, some drawbacks include extra surgery to remove bone from the patient's body, need for multiple operations, high cost, complications such as chronic pain at the donor site, and the availability especially in children.[3−5] Other alternatives are allogenic bone grafts and natural and synthetic materials. The increased availability of banked, donated bone tissue has made the fabrication of customized forms possible. Drawbacks of allografts include their high variability and less satisfactory clinical results compared to autografts and the possibility of disease transfer and immunogenic reaction. In the past decade, several materials (metals, ceramics, polymers, etc.) have been developed to create bone substitutes. In general, they restore the mechanical properties of the affected bone, however, they often do not restore its functional properties. Tissue engineering is rather a new discipline, aiming for the substitution of damaged tissue by cell/material constructs. The general principle of tissue engineering involves combining living cells with a natural or synthetic support or scaffold to produce a three-dimensional living tissue, constructed in such a way that it is functionally, structurally, and mechanically equal (if not superior) to the lost tissue. The challenge is to combine all these elements to create stable, complex three-dimensional (3D) constructs in sizes which are clinically useful for in-vivo application.

To restore damaged bone defects, not only suitable biomaterials, integrating at the defect sites are important, but often also additional sources of potential bone-forming cells are needed. These cells should be able to replace the degraded biomaterials simultaneously by new bone. In this regard, the most promising biomaterials for bone tissue engineering are in-situ forming materials (e.g., moldable calcium phosphate cements, injectable polymers hardening by redox- or photo-cross-linking, etc.), capable of filling irregular defects. These well-integrating materials, however, are mostly inconvenient to be mixed directly with cells. The use of cell delivery systems which can be colonized by appropriate cells and in which such cells are protected during the polymerization process of the biomaterial scaffolds could be advantageous.

There are several sources of cells that could be used for bone repair and regeneration, but one of the most promising involves the use of stem cells. In 1981, embryonic stem (ES) cells were first isolated from the inner cell mass (ICM) of preimplantation mammalian embryos.[6,7] ES cells are characterized by an unlimited proliferative potential, the ability for self-renewal, and the capability of generating derivatives of all three germ layers (endoderm, mesoderm, and ectoderm).[8−10] Long-term culture conditions for maintaining undifferentiated ES cells can be achieved by culture upon mitotically inactivated mouse embryonic fibroblasts (MEFs) or upon gelatin-coated dishes in the presence of the interleukin-6 family member cytokine leukemia inhibitory factor (LIF). Without these conditions, ES cells differentiate spontaneously.[12−14]

The aim of the present work is to evaluate the use of gelatin-based microcarriers (Cultispher S) as a cell delivery system for ES cells. The maintenance of pluripotency and the differentiation potential into osteoblast-like cells is evaluated. Preliminary results using these microcarriers in combination with an in-situ cross-linkable polymer (in-situ photopolymerizable polyesters based on D,L-lactide and ϵ-caprolactone) are discussed.

Materials and Methods

Culture of Undifferentiated ES Cells and MC3T3 Cells. The ES cells used in this research were obtained from 3.5-day-old blastocysts

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of the B6D2 mouse strain.[15] ES cells were maintained in an undifferentiated state on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) in an ES medium containing Dulbecco's modified minimum essential medium (DMEM) supplemented with 10^{-4} M β-mercaptoethanol, 10 (v/v) % knock-out serum replacement (Gibco BRL, Life technologies, Merelbeke, Belgium), 10^{-4} M nonessential amino acids (Gibco BRL, Life technologies, Merelbeke, Belgium), and 1000 IU/mL LIF (leukemia inhibitory factor) (ESGRO, Chemicon International, Asse-Relegem, Belgium). The medium was changed daily and the cells growing in colonies were split every 2 days and were replated onto dishes with fresh mitomycin C-inactivated MEFs.

MC3T3 cells (ATCC) were routinely grown in monolayer culture in α-MEM medium containing α-MEM with 10% fetal bovine serum (FBS). No antibiotics were used in any of these cultures.

Microcarrier Seeding, Expansion, and Differentiation. Macroporous Cultispher S microcarrier beads (gelatin-based) (Percell Biolytica), diameter 130−380 μm, were prepared according to the manufacturer's instructions. Briefly, 0.09 g of microcarriers/vial was hydrated prior to use in phosphate-buffered saline (PBS) and was autoclaved (15 min, 121 °C). The microcarriers were rinsed in PBS and twice with DMEM medium (37 °C). The microcarriers were transferred and divided over four wells of a 12-well tissue culture dish (for suspension culture). One million ES- or MC3T3 cells were seeded/0.09 g Cultispher S in ES- and α-MEM medium, respectively. After 48 h, the microcarriers were transferred to a 50-mL spinner flask (working volume 5 mL, end concentration 18 mg CultiSpher S/mL), and the stirring speed was set at 55 rpm. The cultures were maintained at 37 °C in 5% CO_2 for 14 days.

To induce differentiation of ES cells loaded onto the microcarriers into the osteogenic lineage, ES medium was replaced by differentiation medium: α-MEM with 10% FBS (Gibco BRL, Life technologies, Merelbeke, Belgium) and 10^{-4} M ascorbic acid (Sigma-Aldrich NV/SA, Bornem, Belgium). Cells were incubated under these conditions for an additional 14 days and the culture medium was replaced every 2 days. The same batch of FBS was used in all the experiments.

Cell Viability. MTT analysis was used to monitor cell viability. In this test, a tetrazolium salt is converted to a colored formazan by mitochondrial dehydrogenase activity in living cells. On the specific time points, 4-mg samples from spinner microcarrier cultures were taken and transferred to Eppendorf tubes, and the medium was replaced by MTT containing medium (0.5 mg/mL). After 4 h incubation, the medium was withdrawn, the microcarriers were rinsed twice with Ringer solution, and 0.5 mL of 1% Triton X-100 in isopropanol/0.4 N HCl was added. The formazan was dissolved by shaking at 37 °C for 30 min. Three hundred microliters was pipetted in a 96-well tissue culture dish and the absorbance was measured at 580 nm (Universal Microplate Reader EL 800, BIO-TEK instruments INC.).

Encapsulation of ES Cell-Seeded Microcarriers into the Polymer Scaffold. Methacrylate-endcapped poly(D,L-lactide-*co*-caprolactone) was used to encapsulate the cell-loaded microcarriers. The synthesis and characterization of the polymers was performed at the Polymer Material Research group and is described elsewhere.[16,17] The in-situ formation of a solid, 3D polymer network was obtained by visible-light irradiation in the presence of d,l-camphorquinone/ethyl 4-diaminobenzoate initiator system. By adding leachable particulates (porogens), a porous 3D network was obtained. A fast degrading gelatin was used as porogen. The gelatin was sieved with ASTM E11-70 standard testing sieves with openings of 250 μm and 355 μm and was placed on the Retsch AS 200 sieved shaker until particulates with a defined particle size (205−355 μm) were obtained.

Methacrylate-endcapped oligomers (LA_{50}-CL_{50}-$HXD_{20/1}$-bismethacrylate), 15 wt % 2-hydroxyethylmethacrylate (HEMA) or triacetin, catalyst (0.6 wt % D,L-camphorquinone and 0.7 wt % ethyl 4-diaminobenzoate), and gelatin (particle size 205−355 μm) were sterilized by ethylene oxide (12 h at 60 °C, 48 h air). HEMA or triacetin was added to the catalyst until dissolved. The (HEMA or triacetin)/catalyst solution followed by gelatin was added to the viscous prepolymer and was mixed thoroughly. Before encapsulation, the medium of the ES cell-seeded microcarriers was removed. The microcarriers were added to the catalyst/(HEMA or triacetin)/polymer/gelatin paste and were carefully mixed. The viscous paste was then brought into a cylindrical Teflon mould (diameter 5 mm, height 3 mm), and the prepolymer was photopolymerized for 20 s at one side with the dental lamp curing unit (500 mW/cm^2) (3M Unitek, Ortholux XT) (3M Dental products). After polymerization, the scaffolds were removed from the mold and were sectioned to 1.5-mm height by means of a scalpel. The scaffolds were placed in α-MEM medium and were incubated at 37 °C for a culture period of 7 days. The medium was replaced after 3−7 h and during the following days to remove excess gelatin.

The typical experimental procedure was as follows. Scaffolds with apparent porosity of 68 were prepared with 50 wt % microcarriers versus polymer. Scaffolds with 50 wt % microcarriers initially contained 0.355 g polymer, 0.64833 g gelatin, and 0.18 g microcarriers. In an additional experiment, the addition of the porogen gelatin was omitted.

Oct-4, Cbfa-1 Immunohistochemical Staining. Nonspecific staining was blocked with PBS containing 2% BSA, 0.2% Tween, and 2% normal rabbit serum for 30 min. The slides were incubated for 2 h with the primary mouse monoclonal antibody (1:50) raised against a recombinant protein corresponding to amino acid 1-134 of human Oct-4 (C10) (sc-5279) (Santa Cruz Biotechnology, Santa Cruz, CA) and with the primary goat polyclonal antibody (1:50) raised against cbfa1 (PEBP2αA) (C-19) (sc-8566) (Santa Cruz Biotechnology, Santa Cruz, CA). After extensive washing, the slides were exposed to biotinylated rabbit antimouse secondary antibody and rabbit antigoat secondary antibody (1:200), respectively. After reaction with the horseradish peroxidase (HRP) labeled streptavidin, localization of antigens was visualized by diaminobenzidine (DAB).

Osteocalcin Expression. Osteocalcin levels were measured using the mouse osteocalcin EIA kit (Biomedical Technologies, Inc). Forty-eight hours prior to assaying, microcarriers seeded with ES- and MC3T3 cells were placed into 5-mL serum-free osteogenic medium containing 10^{-9} M 1,25-dihydroxyvitamin D_3. After 48 h, the medium was harvested and analyzed. Osteocalcin was measured at 450 nm according to the company's instructions.

Histology. Light microscopy was performed on paraffin sections obtained from cell-loaded microcarriers, before and after polymerization in the in-situ forming polymer.

ES- and MC3T3 loaded microcarriers were fixed at 2, 7, 14, 21, and 35 days of culture. Encapsulated microcarriers were fixed immediately after cross-linking. Fixation was performed in 4% formaldehyde buffered with 10 mM phosphate (pH 6.9) (4 °C, 24 h). After dehydration in a graded alcohol series, 5-μm-thick paraffin sections were made and stained with Mayer's Haematoxylin/eosin Y or Masson's Trichrome.

Electron Microscopy. Transmission electron microscopy (TEM) was carried out to analyze the viability of the cells within the microcarriers and encapsulated in the polymer.

The microcarriers and the polymer were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h and were post-fixed with 2% OsO_4 for 1 h. After dehydration, the samples were embedded in Spurr resin and were sectioned (60 nm). The sections were routinely stained with uranyl acetate and lead citrate. The samples were analyzed using a JEOL 1200 EX II TEM operating at 80 KeV.

Results

Microcarrier Loading with MC3T3 and ES Cells. Different seeding procedures were performed to obtain an optimal loading and colonization of the microcarriers. Different combinations of well and cell numbers and media and culture times were performed.

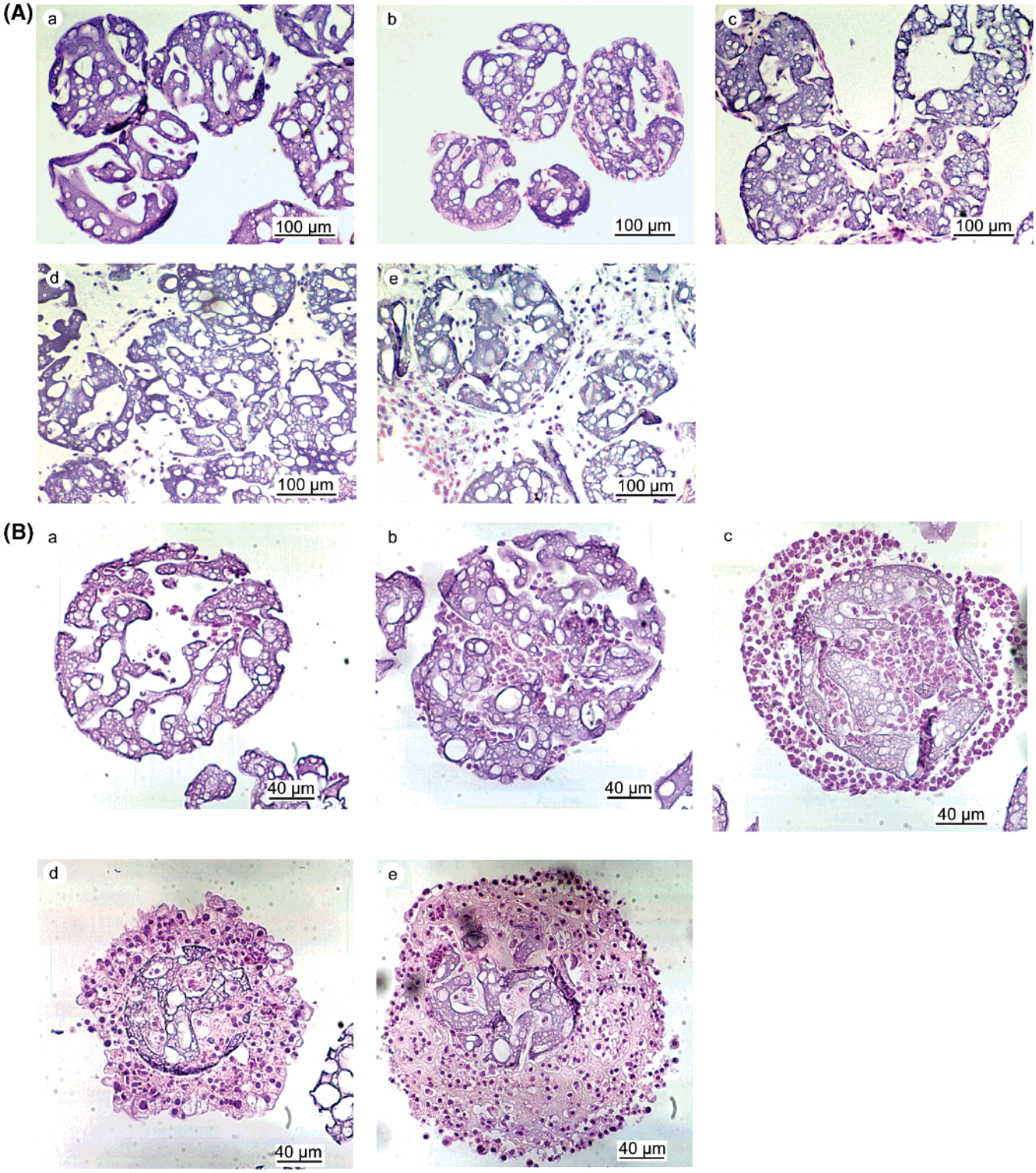

Figure 1. LM pictures of cell-loaded microcarriers (5-μm sections, HE staining) of MC3T3 cells (Figure 1A) and ES cells (Figure 1B) at day 2 (a), 7 (b), 14 (c), 21 (d), and 35 (e) A period of 7−14 days is needed to colonize the microcarriers. A longer culture period results in the formation of cellular bridges between the microcarriers. Some of the microcarriers remain unloaded.

In summary, the use of 0.9 g microcarriers divided over 4 wells of a 12-well plate resulted in a complete covering of the bottom of the wells without superposition of the carriers. In this way, a maximal contact between cells and microcarrier surface was achieved and cells were prevented to attach to the bottom of the well. To optimize the colonization of the microcarriers, different cell densities (5×10^5, 1×10^6, and 2×10^6) and culture times were assessed.

An initial static culture period of 2 days was used to allow the cells to attach to the microcarriers. From then on, extended cultures were performed under dynamic conditions. Light microscopic analyses were performed after 2, 7, 14, 21, and 35 days of culture (Figure 1). A density of 1×10^6 cells/0.9 g was finally used as cell concentration. A culture period of 1−2 weeks resulted in a good colonization of the microcarriers. From then on, cells were overgrowing the microcarriers (especially obvious

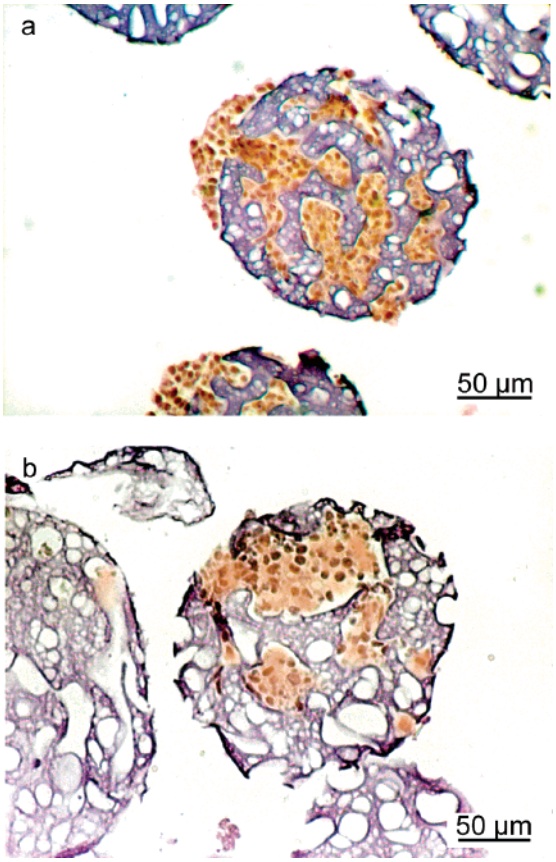

Figure 2. Oct-4 staining on ES cells cultured on Cultispher S microcarriers. ES cultured in the presence of LIF for 7 days (a) and 14 days (b). Cells are positive for Oct-4 indicating their pluripotency.

for the ES cells), with the formation of cellular bridges between different carriers. After 3 weeks, necrotic areas in the center of the carriers were noticed. Some ES cells did not attach to the microcarriers but formed embryoid bodies (EBs). Although the above-described conditions gave the best results concerning the colonization of the microcarriers, some carriers remained empty.

ES Cells Retain Their Oct-4 Expression on Cultispher S Microcarriers. When ES cells were cultured on the microcarriers with medium supplemented with LIF, which was changed daily, ES cells retained their Oct-4 expression until 14 days in culture (Figure 2).

Bone Differentiation of ES on the Cultispher S Microcarriers. To promote differentiation of the ES cells into the osteogenic lineage, cultures were performed in differentiation medium. Different time points for switching the ES medium into the differentiation medium were analyzed. An initial culture period of 1 week in LIF containing medium was necessary for a good colonization of the carriers. The use of the differentiation medium from the start of the loading experiments showed a clear lower infiltration capacity of the cells into the carriers.

Optimal colonization was achieved by culturing the ES cells for 2 weeks in ES medium. Replacing the ES medium by differentiation medium resulted in cells expressing the early osteogenic marker cbfa1 after 2 weeks (Figure 3a).

A Trichrome Masson staining showed the formation of a collagen-containing extracellular matrix (Figure 3b). As observed by TEM, the collagen fibrils showed a typical 67-nm periodicity, characteristic for collagen I (Figure 4).

Cell Viability before and after Encapsulation of the Cultispher S Microcarriers (MTT, LM, and TEM). Cell viability on the microcarriers before encapsulation was assayed

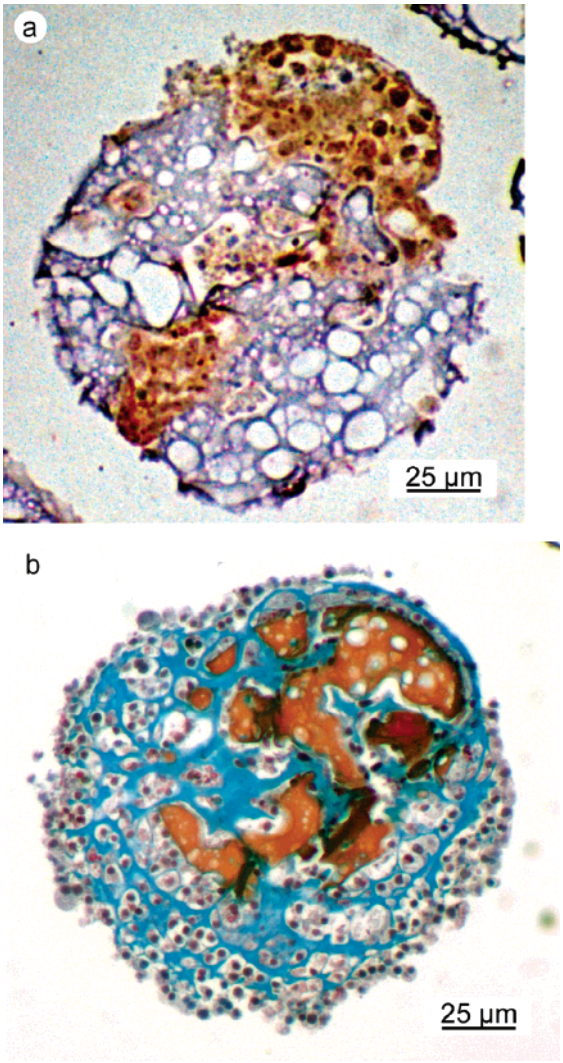

Figure 3. LM pictures of ES cells grown for 14 days on cultispher S in a differentiation medium. a: Immunostaining: ES cells are positive for cbfa1, an early marker for osteoblast differentiation. b: Trichrome Masson's staining. The formation of a collagen containing extracellular matrix is shown. Blue: newly formed collageneous extracellular matrix, red: microcarrier.

by the MTT viability test. In addition, to evaluate the distribution of the microcarriers and viability of the cells after encapsulation, LM and TEM were performed.

Data obtained by analyzing 4 mg of cell-seeded microcarriers over a period of 5 weeks are presented in Figure 5. Four independent experiments were performed. For the MC3T3 cells, a plateau was reached after 14 days in culture. For the ES cells, a plateau was reached after 7 days.

No significant differences were noticed between the curves of the two different cell types.

As previously published by our group, light microscopic examination of encapsulated microcarriers immediately after the cross-linking showed that the microcarriers were homogenously distributed throughout the scaffold. After 7 h, the gelatin was dissolved and leached out, but the microcarriers retained their original position. The polymer and gelatin seemed to infiltrate into the microcarriers with the resulting loss of several cells.[18] In the present paper, ultrastructural analysis was performed to evaluate the time point at which cell viability decreased and to compare the different polymerization conditions. Consequently,

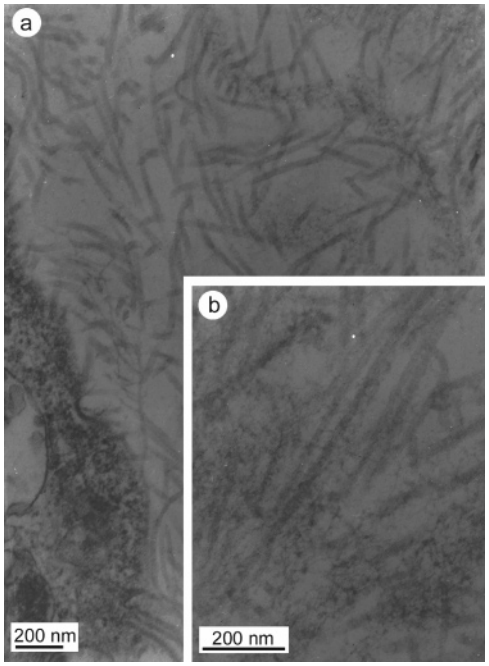

Figure 4. TEM picture of a 60-nm section of the extracellular compartment of ES cells grown on Cultispher S. Collagen fibers with a clear banding, typical for collagen I, are present.

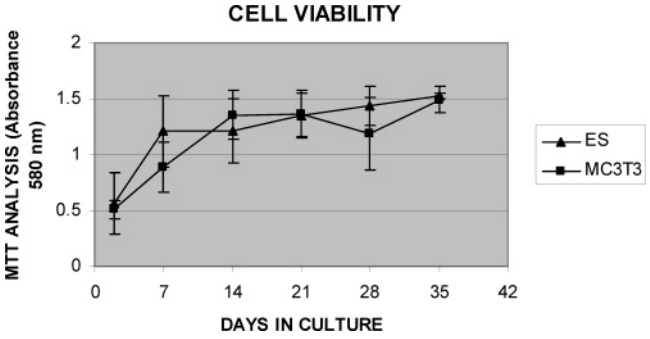

Figure 5. Cell viability (proliferation curves) of ES and MC3T3 cells grown on Cultispher S microcarriers. On specific time points, the amount of viable cells on 4 mg of cell-seeded microcarriers was quantified by MTT assay. Mean and standard deviation (SD) of four independent experiments in duplicate.

cell-seeded microcarriers were evaluated by TEM before and after cross-linking into the polymer with and without addition of gelatin.

Before encapsulation, viable cells showing euchromatic nuclei often containing a prominent nucleolus were observed. No swelling of the mitochondria, indicative for loss of viability, was observed. Cells were attached to the inner surface of the microcarriers (Figure 6).

After encapsulation, differences correlated with the type of in-situ forming material were observed. When HEMA (with or without gelatin) or triacetin with gelatin was used to encapsulate the microcarriers, already immediately after the cross-linking procedure, no viable cells could be found in the carriers. Cellular debris and cells with clumped chromatin were present in the pores of the carriers. Often, noncellular material was formed between the cells and the Cultispher surface, confirming the impression obtained by LM of material infiltrating the microcarrier (Figure 7a−f). It seemed that the polymer as well as the gelatin infiltrated into the microcarriers. In contrast, when using triacetin without gelatin during the polymerization, cells

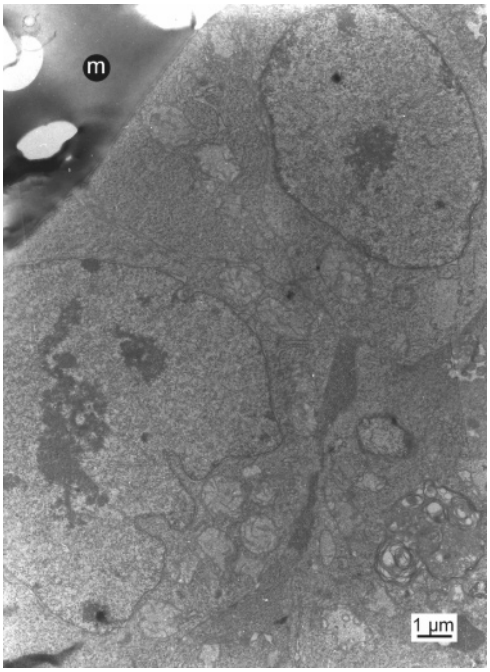

Figure 6. TEM pictures of a cell-loaded microcarrier before encapsulation. Viable cells with euchromatic nuclei well attached to the internal surface of the microcarrier are present.

remained viable after cross-linking: euchromatic nuclei were present, and no blebbing or dilatation of the nuclear or plasma membrane were seen. In the cytoplasm, an abundant network of RER indicative for a high-protein synthesis was present. Cells seemed to be firmly attached to the inner surface of the microcarrier (Figure 7g, h).

Discussion

Bone repair is thought to be one of the first major applications for TE. Once bone is damaged, the defects resulting from trauma, disease, aging, and tumor often do not heal spontaneously. Conventional approaches in bone repair have involved biological grafts such as autogenous bone or autotografts and xenografts. However, the limited availability of autografts and risks of donor site morbidity have fueled great interest in the development of alternative approaches to bone repair.

In the present study, we investigated a new approach for 3D bone tissue generation with ES cells and biodegradable macroporous microcarriers on the basis of cross-linked gelatin. Different parameters were analyzed in this study. First of all, the conditions to maximize the colonization of the microcarriers by ES- and MC3T3-cells, an osteoprogenitor cell line, were compared. Second, the ability of ES cells to retain their pluripotency as well as their capacity to differentiate into bone-forming cells was assessed. Finally, the encapsulation of the cell-seeded microcarriers in in-situ forming polymers was analyzed.

Embryonic stem cells have generated significant excitement in many areas of biomedical research, including tissue engineering and regenerative medicine.

The use of ES cells as a cell source for bone tissue engineering is advantageous compared to other cell sources such as mature (nonstem) cells and adult stem cells.[19] The supply of autologous tissues and compatible bone marrow derived grafts is limited, while ES cells have the capacity of unlimited proliferating potential, the ability for self-renewal, and the

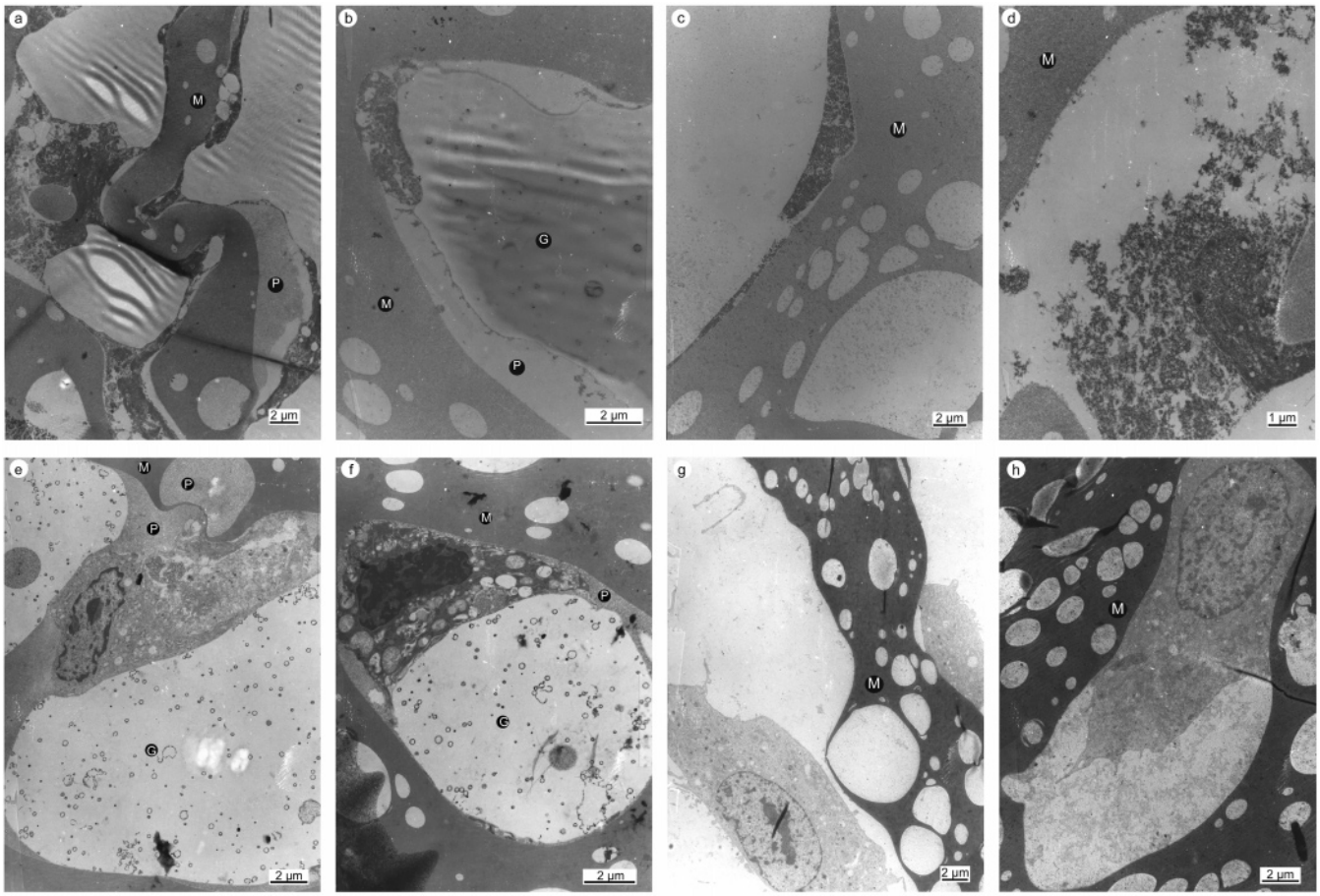

Figure 7. TEM pictures of a cell-loaded microcarrier after encapsulation. (a, b): Encapsulated in polymer combined with HEMA/gelatin; (c, d) HEMA without gelatin; (e, f): TA/gelatin. Cellular debris or disintegrating cells are present. A cell with a pyknotic nucleus and disintegrating cytoplasm is shown. The microcarrier is indicated by (M). Between the cell and the microcarrier surface, material, probably representing the polymer (P), is present. The rest of the pore is filled with gelatin (G). After encapsulation using triacetin without gelatin (g, h), cells remained viable. They are firmly attached to the inner surface of the microcarrier. Euchromatic nuclei and an abundant network of RER are noticed.

capacity to contribute to multiple lineage (pluripotency). More specifically, it has been shown that ES cells can differentiate into osteogenic cells under selective culture conditions.[20]

Routine propagation of mouse ES cells in an undifferentiated state can be achieved by culture upon mitotically inactivated mouse embryonic fibroblasts (MEFs).[12−14] Challenges in expansion of undifferentiated stem cells for clinical applications include the removal of feeder layers and nondefined components in the culture medium. Previous studies have already described the application of ES cells using in-vivo experimental models with induced tissue damage.[21−23] Undifferentiated ES cells are delivered into the animal by simple injection of cells in suspension. It is assumed that the cells are not only attracted to the site of injury but also differentiate in the damaged area into the phenotype of the original tissue by local environmental stimuli. However, recent papers focus on the need to search for new strategies to optimize cell delivery of ES cells.[24−26] We describe the use of gelatin-based, commercially available microcarriers as ES cell delivery system for bone tissue engineering. Using a spinner flask culture system in combination with LIF supplemented medium, cell proliferation was enhanced with the maintenance of the ES cell phenotype. Although we considered this condition as optimal to load the carriers, dependent on the experiment, a variable amount of microcarriers remained empty. Methods to select the loaded from the unloaded carriers are under investigation. In the case ES cells are used for in-vivo application, current opinion is still unclear as to whether cells have to be delivered in situ in their undifferentiated state or if pre-differentiation in vitro is preferable. Both possibilities were evaluated on the Cultispher microcarriers. Oct-4 staining performed on sections of cell-seeded microcarriers and cultured in the presence of LIF showed that ES retained their pluripotency upon 14 days in culture. Replacing the LIF containing medium by the differentiation medium showed the onset of differentiation of the ES cells into the osteogenic lineage. After 2 weeks of culture in this differentiation medium, the cells were positive for cbfa1 and collagen I, both early markers of osteoblast differentiation. In contrast to MC3T3 cells, osteocalcin expression, a final marker for osteoblast differentiation, was negative (data not shown). This is in accordance with our previous observation concerning osteoblast differentiation in different osteogenic media reflecting that the culture on the microcarrier substrate and under dynamic conditions does not inhibit the differentiation potential. Probably, longer culture periods are needed to express the late osteoblastic markers.

As described above, the gelatin nature and macroporosity and commercial availability of Cultispher S make these microcarriers potential cell delivery systems for ES cells. Moreover, there are indications that these carriers could give protection for the cells against high fluid shear forces.[27]

In this paper, we wanted to investigate if Cultispher microcarriers offered enough protection for the cells during the encapsulation and polymerization of an in-situ forming polymer. Biodegradable polymeric networks and hydrogels have received

much attention in medicine as implant materials in tissue engineering.[28] The functionalization of resorbable oligomers such as those based on poly(ethylene glycol), L- or D,L-lactide, and ϵ-caprolactone with unsaturated groups and subsequent radical polymerization and cross-linking has extensively been studied.[29−31] Especially, photoinitiated cross-linking is interesting as it is usually rapid, effective, and well-controlled and can be carried out at low temperature.[30,32,33] If the networks are formed in situ, or if the networks are not extracted prior to their application, the toxicity of the compounds involved in the cross-linking may be an important issue.

The in-situ forming material (methacrylate-endcapped D,L-lactide-*co*-ϵ-caprolactone with camphorquinone and gelatin as porogen) used in this study was previously tested for cytotoxicity and cell infiltration potential.[34] The polymer material is biodegradable and biocompatible, and bone marrow derived cells are able to attach and differentiate into the material. Preliminary results using rat osteosarcoma cells (UMR-106) grown on Cultispher S and encapsulated in the LA/CL scaffolds were also published. Cultures were analyzed until 7 days after cross-linking.[18] The dental lamp, used to harden the polymer, was not toxic for cultured cells. The gelatin leached out but encapsulated microcarriers retained their original position. No clear-cut conclusions on cell viability after encapsulation were drawn. Although the in-vitro viability and differentiation of rat bone marrow cells was similar on preformed scaffolds of polymer systems with HEMA or triacetin as solvent,[34] we found a significant difference in the viability of encapsulated cells in both systems. Promising results are obtained using methacrylate-endcapped poly (D,L-lactide-*co*-ϵ-caprolactone) with triacetin as solvent for the catalyst. It seems that the replacement of HEMA by triacetin has a positive effect on the viability of the encapsulated cells. Triacetin (glycerol triacetate) is approved by the Federal Drug Administration and is known to have a very low toxicity. In contrast, when using HEMA as a solvent, the presence of the methacrylate groups on HEMA increases the probability of the cells for being in the near vicinity of radical reactions during the polymerization process.

However, not only the presence of radical reactions can cause a decrease of the cell viability; also, mechanical damage of cells when encapsulating cell-loaded microcarriers in polymer systems has to be taken into account.

Triacetin, often used as a plasticizer, was added to the polymer, so decreasing the viscosity of the polymer. The use of a polymer with lower viscocity (polymer/triacetin) clearly has a positive influence on the cell viability in comparison with polymer/HEMA.

Nevertheless, this positive effect of the reduced viscocity is lost when gelatin is added as a porogen. Although gelatin is not toxic and is often used in vitro as well as in vivo, in the present experiment, addition of gelatin as a porogen, seemed to have a negative influence on cell viability. Cells on the microcarriers probably suffer from shear stress when they are mixed with the polymer/gelatin paste. Second, immediately after cross-linking and incubating the scaffolds in culture medium at 37 °C, the gelatin starts to miquify, leading to a very high concentration of gelatin in the culture dish, possibly leading to a lower viability of the cells.

Conclusion

This study demonstrates the use of gelatin microcarriers as a substrate for 3D cell culture of ES cells and their suitability for bone tissue engineering purposes. The protocol concerning the colonization and proliferation efficiency of ES cells onto the microcarriers was improved. The microcarrier spinner culture system provides an efficient method to culture a large number of viable undifferentiated ES cells. When cultured in a differentiation medium, the ES cells on the microcarriers expressed osteospecific markers such as cbfa-1 and collagen I. ES cell-seeded gelatin microcarriers are potentially useful as delivery system in bone tissue engineering. To elucidate their use in combination with the proposed polymers, in-vivo experiments using a goat model are under investigation.

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