Freeze-Dried Human Serum Albumin Improves the Adherence and Proliferation of Mesenchymal Stem Cells on Mineralized Human Bone Allografts

Miklós Weszl,[1] Gábor Skaliczki,[2] Attila Cselenyák,[1] Levente Kiss,[1] Tibor Major,[1] Károly Schandl,[1] Eszter Bognár,[3] Guido Stadler,[4] Anja Peterbauer,[4] Lajos Csönge,[5] Zsombor Lacza[1,2]

[1]Tissue Engineering Laboratory, Department of Human Physiology and Clinical Experimental Research, Semmelweis University, 1094 Budapest Tu"zoltó utca 37-47, Budapest 1094, Hungary, [2]Department of Orthopedics, Semmelweis University, Karolina út 27, 1113 Budapest, Hungary, [3]Department of Materials Science and Engineering, Budapest University of Technology and Economics, Bertalan L. u. 7., H-1111 Budapest, Hungary, [4]Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Donaueschingenstr 13, A-1200 Vienna, Austria, [5]Petz Aladár Teaching Hospital, West-Hungarian Regional Tissue Bank, Vasvári Pál utca 2-4, H-9023, Gyo"r, Hungary

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ABSTRACT: Mineralized scaffolds are widely used as bone grafts with the assumption that bone marrow derived cells colonize and remodel them. This process is slow and often unreliable so we aimed to improve the biocompatibility of bone grafts by pre-seeding them with human mesenchymal stem cells from either bone marrow or dental pulp. Under standard cell culture conditions very low number of seeded cells remained on the surface of freeze-dried human or bovine bone graft or hydroxyapatite. Coating the scaffolds with fibronectin or collagen improved seeding efficiency but the cells failed to grow on the surface until the 18th day. In contrast, human albumin was a very potent facilitator of both seeding and proliferation on allografts which was further improved by culturing in a rotating bioreactor. Electron microscopy revealed that cells do not form a monolayer but span the pores, emphasizing the importance of pore size and microstructure. Albumin coated bone chips were able to unite a rat femoral segmental defect, while uncoated ones did not. Micro-hardness measurements confirmed that albumin coating does not influence the physical characteristics of the scaffold, so it is possible to introduce albumin coating into the manufacturing process of lyophilized bone allografts. J Orthop Res 30:489–496, 2012

Keywords: albumin; lyophilization; freeze-drying; bone graft

A serious limitation of the clinical use of bone grafts is their often unreliable incorporation.[1] Mineralized implants such as hydroxyapatite cubes can turn into live bone in dental or orthopedic settings, however, sometimes the graft is resorbed or fails to connect to the host tissue with no foreseeable reason.[2,3] One explanation can be that colonization of the graft by stem cells is too slow and falls below the threshold for bone remodeling.[4–6]

The ideal bone graft can be characterized by good mechanical strength, osteoinductive, osteoconductive, and osteogenic capabilities and only the autologous human bone graft possesses all of the mentioned properties.[7] The unique bone healing potential of autologous bone graft stems from its three well-defined features: (a) osteoconductivity is provided by the structure of native bone, (b) osteoinductivity is given by morphogenetic proteins and other growth factors, and (c) osteogenicity is provided by cells, like osteoblasts, osteoclasts, and mesenchymal stem cells (MSCs) which are on the surface of a freshly harvested autologous bone graft. However, the complications associating with the harvest of autologous bone, like chronic pain at the donor site and its limited availability often impede the use of autograft.[8] Unfortunately, autograft donor site morbidity and persistent complications can be as high as 25.3% even exceeding the complication rate of the grafting itself.[9]

Allogeneic bone graft is usually the 2nd choice for clinical bone replacement.[10] The off-the-shelf availability and lack of donor site morbidity are undisputed advantages of the allograft.[11,12] Fresh, frozen, and freeze-dried allografts are seemed to be the most popular, however, there is not a well-established protocol for their manufacture.[11–13] For the patient safety the allografts are supposed to be subjected to desinfection so as to avoid the transfer of contagious agents from the donor to the recipient.[14,15] Freeze-drying technique allows the profound desinfection of allogeneic bone grafts with chemicals, like acids and ethylene-oxide because these agents are eliminating from the allograft during the freeze-drying. As the disadvantageous effect of such a desinfection the osteogenic cells are killed on the allograft and most of the osteoinductive proteins become denatured, which impairs the biological value of the allograft.[15,16] Thus, the lege artis manufactured freeze-dried allograft can be characterized by a good osteoconductivity but a low osteoinductive and osteogenic capability that ultimately result in its unreliable incorporation. Replacement of the protein structure may improve the cell adhesion properties of freeze-dried allograft. Several proteins are used in cell culture for increasing attachment, among them bone structure proteins such as fibronectin and collagen I. It is common practice to soak plastic dishes in fibronectin solution which then significantly increases the seeding efficiency of added cells.[17] Although albumin

Correspondence to: Miklós Weszl (T: +36-70-512-5658; F: +36-1-334-3162; E-mail: miklos.weszl@gmail.com).

acts as an anti-attachment protein in plastic surfaces, it is also the main component of culture media which is required for proliferation.[18,19] In order to improve the biological value of freeze-dried allgraft, an optimized protein structure should be provided which allows the fast attachment and proliferation of bone forming cells after implantation of the graft. The aim of the present study was to investigate the effects of bone structure proteins or serum components on the colonization of freeze-dried allografts and other bone grafts by MSCs.

MATERIALS AND METHODS

Cell Culture

All procedures were approved by the ethical committee of Semmelweis University. MSCs were isolated from human bone marrow (BMSCs) and from dental pulp (DPSCs). The bone marrow samples were obtained from young patients (aged 2–20 years) during standard orthopedic surgical procedures with the informed consent of the patients or their parents under approved ethical guidelines set by the Ethical Committee of the Hungarian Medical Research Council. Only such tissues were used that otherwise would have been discarded. Bone marrow was taken into T75 flasks and diluted with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 10 μg/ml streptomycin, 2 mM L-glutamine and 1 g/L glucose. The flasks were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 for 3 days. After the incubation period the BMSCs adhered to the surface of the flasks and the remaining components of bone marrow were eliminated by washing with PBS. BMSCs were between one and five passages in the experiments.

The protocol for culturing DPSCs is based on the procedure described by Gronthos et al.[20] with modifications. Human impacted third molars were collected from adults (18–26 years of age). The tooth was cut around the cemento-enamel junction by sterile dental fissure burs to expose the pulp chamber. The pulp tissue had been removed from the crown and the roots then digested in a solution of collagenase type I (3 mg/ml) and dispase (4 mg/ml) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer and were seeded into six-well plates with alpha modification of Eagle's medium (α-MEM) supplemented with 20% FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, then grown under standard cell culture conditions.

Characterization of Cells

The identity of cells was confirmed by the presence of lineage-specific cell surface markers with flow cytometry (BD® FacsCalibur, Becton Dickinson, NJ). Hematopoietic linage-specific surface markers (CD34, CD45) and mesenchymal surface markers (CD73, CD90, CD105, and CD166) were investigated.

Scaffolds

Freeze-dried autolysed antigene-extracted allogeneic bone grafts, lyophilized bovine spongious bone graft (Bio-Oss, Geistlich Pharma AG, Wolhusen, Switzerland) and porous hydroxyapatite (META BIOMED, Chungbuk, Korea) were used as scaffolds. The freeze-dried bone allografts were provided by the West Hungarian Regional Tissue Bank.[15] The scaffolds were cut into 0.3–0.5 cm^3 pieces with an orthopedic saw under sterile conditions. The preparation of the scaffolds before seeding was performed by incubating in protein solution at +4°C overnight. The experimental groups were fibronectin (20 μg/ml Sigma–Aldrich, Budapest, Hungary), albumin of human serum origin (200 g/1,000 ml, BIOTEST, Hungary), and 1.5% porcine type I collagen (1.5%, Biom' up, St. Priest, France). Coated scaffolds had been initially prepared by the same protocol as described above then they were freeze-dried at 32°C, at 1 Pa for 24 h. We used a fixed volume and percentage of protein solutions in each vial throughout the study, which ensured that the total amount of protein in each bone graft was the same.

Seeding of Cells on Coated and Uncoated Bone Scaffolds under Standard Conditions

Just before seeding BMSCs were labeled with the fluorescent membrane dye Vybrant DiD (excitation/emission: 644/665 nm, Molecular Probes, Invitrogen, Eugene, OR) for 30 min at 37°C in monolayer. The DiD-labeled BMSCs had been trypsinized and suspended in culture medium then applied with pipette to the surface of the coated and uncoated control allografts (100,000 BMSCs per scaffold). After seeding BMSCs were expanded on the allografts under standard cell culture conditions for 18 days and their proliferation was investigated at the 3rd and 18th days.

Dental pulp-derived cells were used in the same manner as BMSCs. The DPSCs did not take up the Vybrant DiD dye to allow uniform staining thus their proliferation was followed up with Alamar Blue assay (Biosource, Invitrogen).

Seeding of Cells on Albumin Coated and Uncoated Human Allografts under Dynamic Conditions

We built a rotating bioreactor based on Terai's and Hannouche's Rational Oxygen-Permeable Bioreactor System.[21] The bioreactor was designed to provide sufficient oxygen tension to cell culture media under dynamic cell culture conditions. A thin coating of silicone polymer was applied inside a 50-ml polypropylene centrifuge tube, which is permeable for gas through a window and does not support cell attachment. The filled tubes with the stem cell suspension and scaffolds were placed into a rotator apparatus in an incubator maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air. The rotating speed was set to 8 rpm to allow the motion of scaffolds inside the tube.

First, 100,000 cells per scaffold were seeded on the surface of freeze-dried albumin coated bone allografts and stored under standard culture conditions for 24 h. Following the incubation period the bone grafts were placed into a bioreactor tube, which had been filled with 25 ml cell culture medium comprising 1.5 million MSCs in suspension. The bone grafts had been incubated in bioreactor under dynamic cell culture conditions for 24 h then the cells were further expanded on the surface of bone grafts under standard culture conditions for 18 days. The viability and quantity of attached MSCs on the surface was investigated after 3 and 18 days of incubation.

Assessment of the Proliferation of BMSCs and DPSCs

The seeded DiD-labeled BMSC's proliferation was observed with confocal microscopy (LSM 510 META, Zeiss, Germany) on the surface of uncoated control and on the coated lyophilized allografts. Three individual view fields were randomly

selected on the grafts where we measured the quantity of pixels belonging to the Vybrant DiD labeled BMSCs. The proliferation of the cells was investigated 3 and 18 days after the seeding.

We used Alamar Blue to track the proliferation of DPSCs on the surface of bone grafts. Cell proliferation was investigated at the 3rd and 18th days. After 4 h of Alamar Blue incubation the absorbance of supernatant was measured at 570 and 600 nm wavelengths (BIOTEK Powerwave XS, Houston, TX). The absorbance and the quantity of cells were correlated.

Scanning Electron Microscopy

An argentiferous adhesive was applied on the bottom of the samples which were coated with an electrically conductive gold layer using a vacuum-pulverization method. The procedure was then performed in vacuum. Electronmicrographs were taken in the secunder electron (SE) mode with 15 kV accelerating voltage (Philips XL 30, Zagreb, Croatia).[22] The whole surface of the samples were examined and representative fields were photographed. One sample was used from each experimental group and in total nine different samples were inspected. From each sample, a 50×, 200×, and 1,000× magnified photograph was taken.

In Vivo Experiments

All animal procedures were approved by the Scientific Research Committee of the Semmelweis University. Adult male Wistar rats a 2 mm mid-diaphyseal osteoperiosteal defect was created in the femur under halothane anesthesia. After fixing the bone with plate and screws, a 2 mm thick bone cement spacer was interposed into the defect to block normal bone healing. After 4 weeks the spacer was removed and uncoated or albumin-coated bone graft was introduced ($n = 3$ in both groups) for 4 more weeks. Bone healing was investigated with μCT.

Micro-Hardness Measurements

This method makes it possible to study the basic parameters of porous samples, thus eliminating the errors caused by the random distribution of small holes under the measuring tool. We measured micro-hardness of cortical phase of albumin coated and uncoated mineralized allografts.[23] The HV Vickers-hardness measurement is performed with a 136° angle of the vertex and square based diamond-pyramid. In this case, it was pressed on an even and flat surface of the sample with 50 g load weight for a period of 5 s. On each sample at least five measurements were carried out, during which the two diagonals of the impression and the micro-hardness values were measured and averaged. The numeric value of the Vickers-hardness can be determined; that the load force (F) explicit in N is divided by the surface (A) of the impression in mm^2, and then the result has to be multiplied with a constant, the value of which was 0.102.

Statistical Analysis

Repeated measures one-way ANOVA analysis was performed (Tukey's post hoc test) to compare the quantity of MSCs on the scaffolds. One-way ANOVA analysis was used (Tukey's post hoc test) to compare the effect of dynamic and standard conditions on the seeding efficiency of MSCs. A p value <0.05 was considered significant.

RESULTS

The majority of cells which were used for the study exhibited mesenchymal lineage specific cell surface markers (CD73, CD90, CD105, CD166) and lacked hematopoietic markers (CD34, CD45) (Fig. 1). Although these cells temporarily adhered to the surface of bare freeze-dried human bone allografts, their quantity was decreasing gradually during the experiment and they completely diminished at day 18 (mean of pixels at the

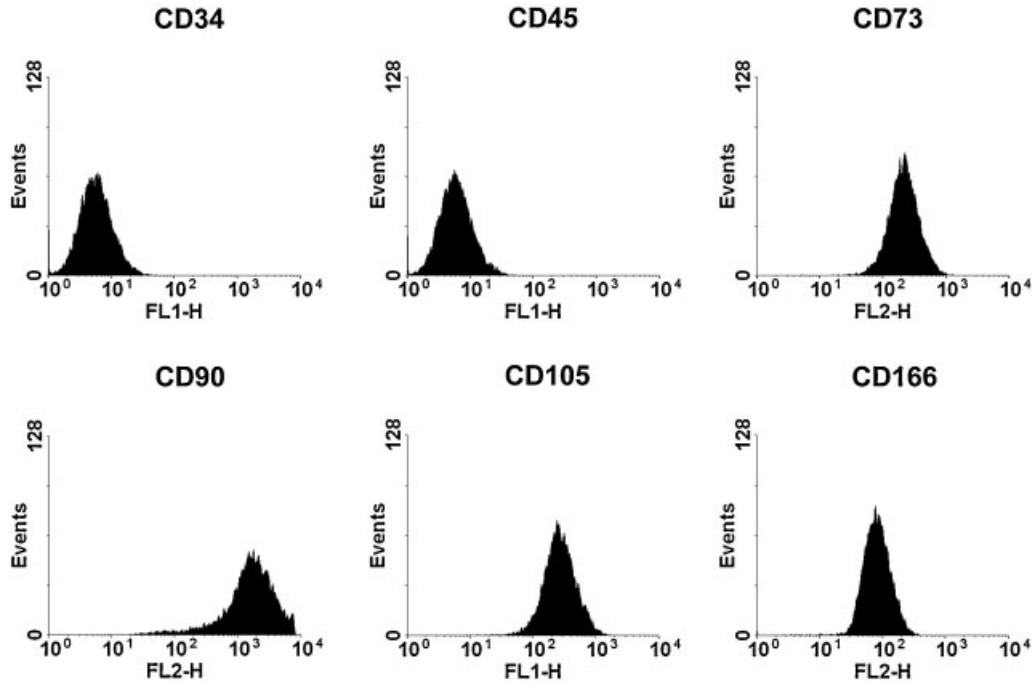

Figure 1. Flow cytometry analysis of cell surface markers. Our cell preparation is positive for MSC lineage-specific markers $CD73^+$, $CD90^+$, $CD105^+$, and $CD\ 166^+$ and negative for hematopoietic markers $CD34^-$ and $CD45^-$.

3rd day: 183 ± 34; at the 18th day: 0 ± 0) (Fig. 2). The coating of freeze-dried allografts with aqueous collagen type I or fibronectin moderately increased the initial attachment of BMSCs but failed to support proliferation until the 18th day (Fig. 2A). Pre-incubation of freeze-dried allografts with aqueous human serum albumin markedly improved the short-term adherence of BMSCs, however, they also disappeared from the surface at day 18th (mean of pixels at the 3rd day: 2,373 ± 142; at the 18th day: 0 ± 0) (Fig. 2A). Freeze-drying of serum albumin onto the surface of allografts reversed the tendency, so the initially attached BMSCs

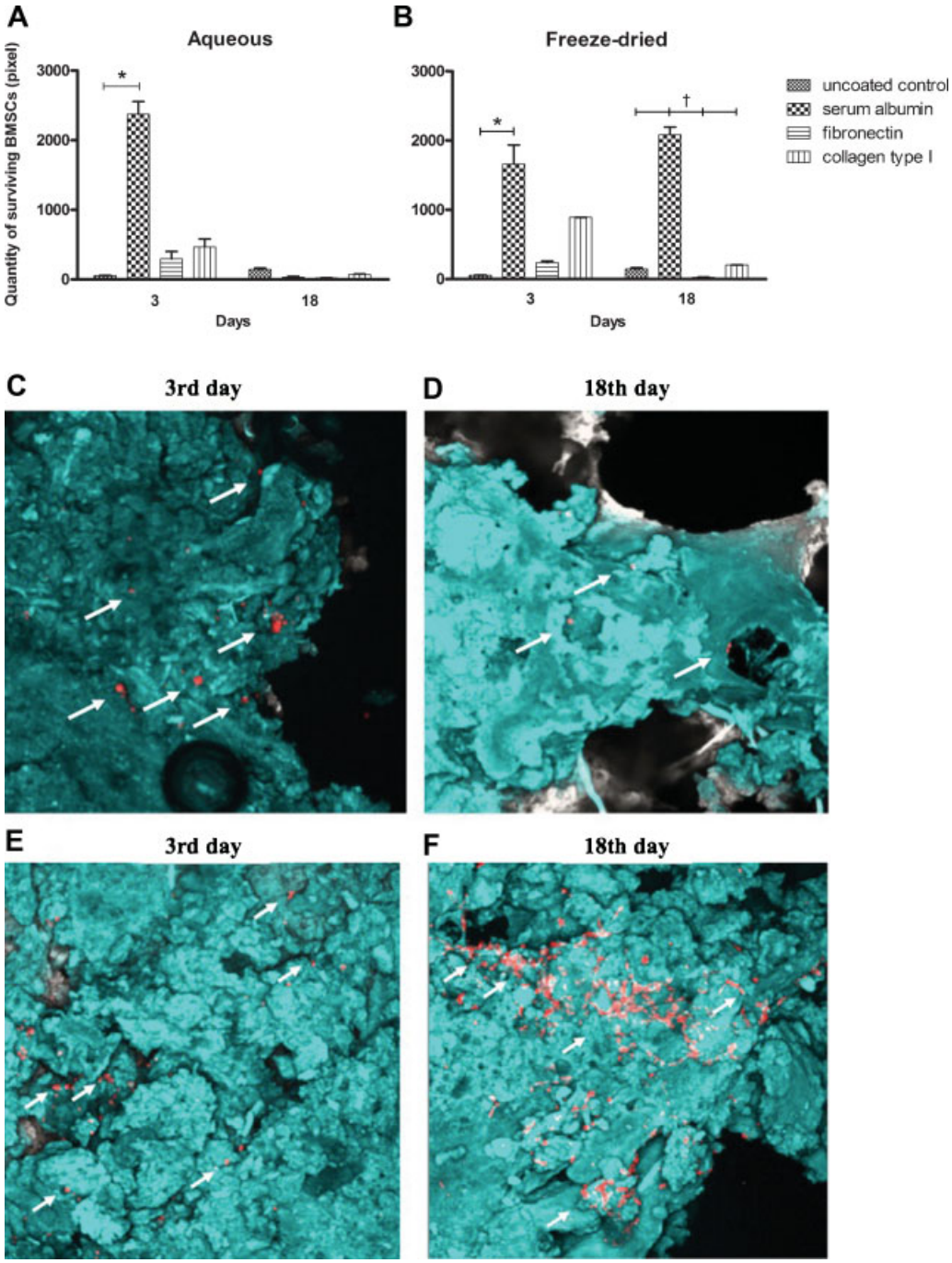

Figure 2. Attachment and proliferation of BMSCs on the surface of mineralized human bone allografts coated with aqueous and freeze-dried proteins. (A) Pre-incubation of the allografts with albumin resulted in high cell density at day 3 ($p^* < 0.05$), whereas fibronectin or collagen has a much lower effect. Regardless of the aqueous coating materials very few cells can be detected on the surface at day 18. (B) Freeze-drying of albumin onto the surface of bone allografts does not affect the cell number at day 3 but markedly increases the proliferation ($p(\dagger) < 0.05$). In contrast, although collagen slightly increases the cell number at day 3, the proliferation drops at day 18. (C,D) Representative confocal fluorescent images of uncoated allografts (blue) with BMSCs labeled with DiD (red). The few cells observed on the surface at day 3 diminished even more at day 18. (E,F) Representative images of freeze-dried albumin coated bone. The cells showed significant proliferation on the graft surface at day 18. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jor]

retained their capability of proliferating (mean of pixels at the 3rd day: 1,658 ± 278; mean of pixels at the 18th day: 2,082 ± 110; $p < 0.05$) (Fig. 2B). Freeze-drying of proteins only improved proliferation of cells in case of albumin and not fibronectin or collagen.

Dynamic culture conditions further increased the quantity of initially attached BMSCs on the surface of the freeze-dried albumin coated allografts compared to the standard culture conditions and the agitation did not impair the proliferation of cells (mean of pixels under standard condition at day 1: 197 ± 23, under dynamic conditions at day 1: 9,825 ± 1,208; at the 7th day: 15,025 ± 1,704) (Fig. 3A). The same tendency was observed when DPSCs were seeded onto the surface of freeze-dried albumin coated allografts under dynamic and standard culture conditions as well (standard condition: mean of reduced Alamar Blue (%) at 3rd day: 14.5 ± 2.23; mean at 7th day: 33.7 ± 0.06; dynamic condition: mean of reduced Alamar Blue (%) at 1st day: 33.5 ± 2.23; mean at 7th day: 60.9 ± 1.09) (Fig. 3B).

The surface of other commonly used bone grafts such as lyophilized bovine bone (BioOss) or artificial hydroxyapatite did not support the cell attachment either (Fig. 4). However, in contrast to allografts, even albumin coating failed to improve seeding efficiency and proliferation, indicating that there is a specific interaction between human albumin and human bone that is needed for the effect (Figs. 4 and 5).

We observed significant differences in the macro-, micro-, and nanostructue of freeze-dried human bone allograft and hydroxyapatite or lyophilized bovine bone (BioOss) with scanning electron microscopy (Fig. 4). Hydroxyapatite exhibits the most compact structure with a low number of micro-pores. The texture of lyophilized bovine bone (BioOss) is rich in large inter-connecting channels. Contrarily, human allograft can be characterized by a segmented surface in which pores of various size brake the continuity of the surface. By coating the surface of scaffolds with freeze-dried albumin amorphous protein chips mask the apparent differences in their micro- and nanostructure (Fig. 4).

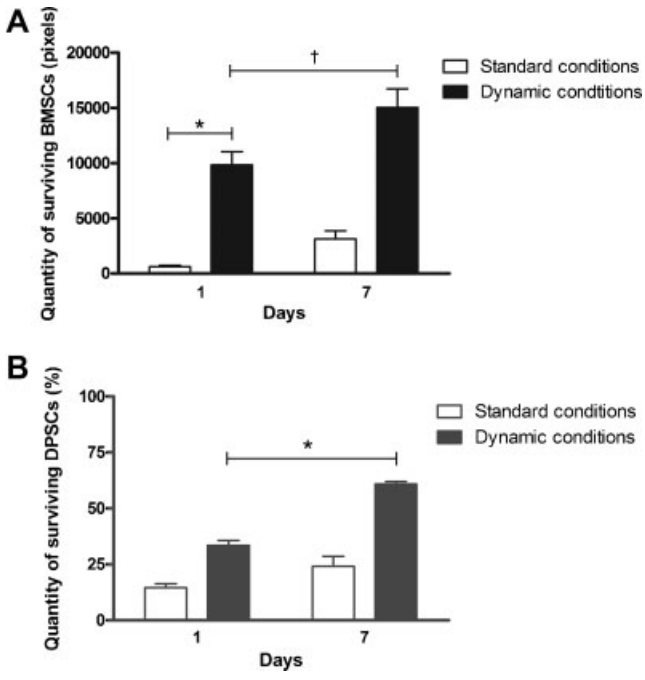

Figure 3. Attachment of BMSCs and DPSCs onto the surface of freeze-dried albumin coated allografts under dynamic conditions in a rotating bioreactor. The dynamic cell culture conditions markedly improved the initial attachment ($p(\dagger) < 0.05$) of both BMSCs (A) and DPSCs (B) which retained their capability of proliferating ($p^* < 0.05$).

Implantation of albumin coated bone grafts into a rat model of delayed bone healing resulted in better integration of the albumin-coated grafts than the native ones (Fig. 5). The implanted graft was located in the defect in each case, but only the albumin-coated grafts had significant ingrowth of new bone from the host resulting in union of the defect.

Soaking and repeated freeze-drying may alter the physical characteristics of the graft, therefore, we compared the hardness of uncoated and albumin coated bone allografts (Fig. 6.). The micro-hardness of freeze-died human bone allograft was not affected by albumin coating (55.1 N ± 7.7 vs. 53.9 N ± 7.9, respectively).

DISCUSSION

Our results showed that the biocompatibility of freeze-dried human bone allograft with MSCs can be improved by albumin coating. The freeze-dried albumin layer withstands the agitation under dynamic cell culture conditions and does not influence the mechanical strength of the human bone but significantly increases the proliferation rate of MSCs on the surface. After implantation in a delayed bone-healing model, albumin coating improved the ingrowth of new bone from the host. Interestingly, albumin only works on human bone surface but not on hydroxyapatite or bovine bone scaffolds.

There might be common reasons behind the low incorporation rate and the low biocompatibility with MSCs of freeze-dried human bone allograft. The desinfection of donor bone kills not just the potentially contagious microorganisms but destroys most of the biologically active proteins, such as cell-adhesive proteins.[13–15] Therefore, such a way prepared freeze-dried bone allograft consists of inorganic components of the bone in majority (mineralized bone) and just a few organic materials. Cell-adhesive proteins play a crucial role both in the attachment and proliferation of cells on the surface of scaffolds.[24] The lack of adhesive proteins may result in the low attachment rate of MSCs on the surface of freeze-dried human bone allograft. We suspect the same reason behind the stagnating number of MSCs on the surface of lyophilized bovine bone graft and hydroxyapatite which was demonstrated in the present study.

The simple coating of freeze-dried human bone allografts with cell-adhesive molecules , for example,

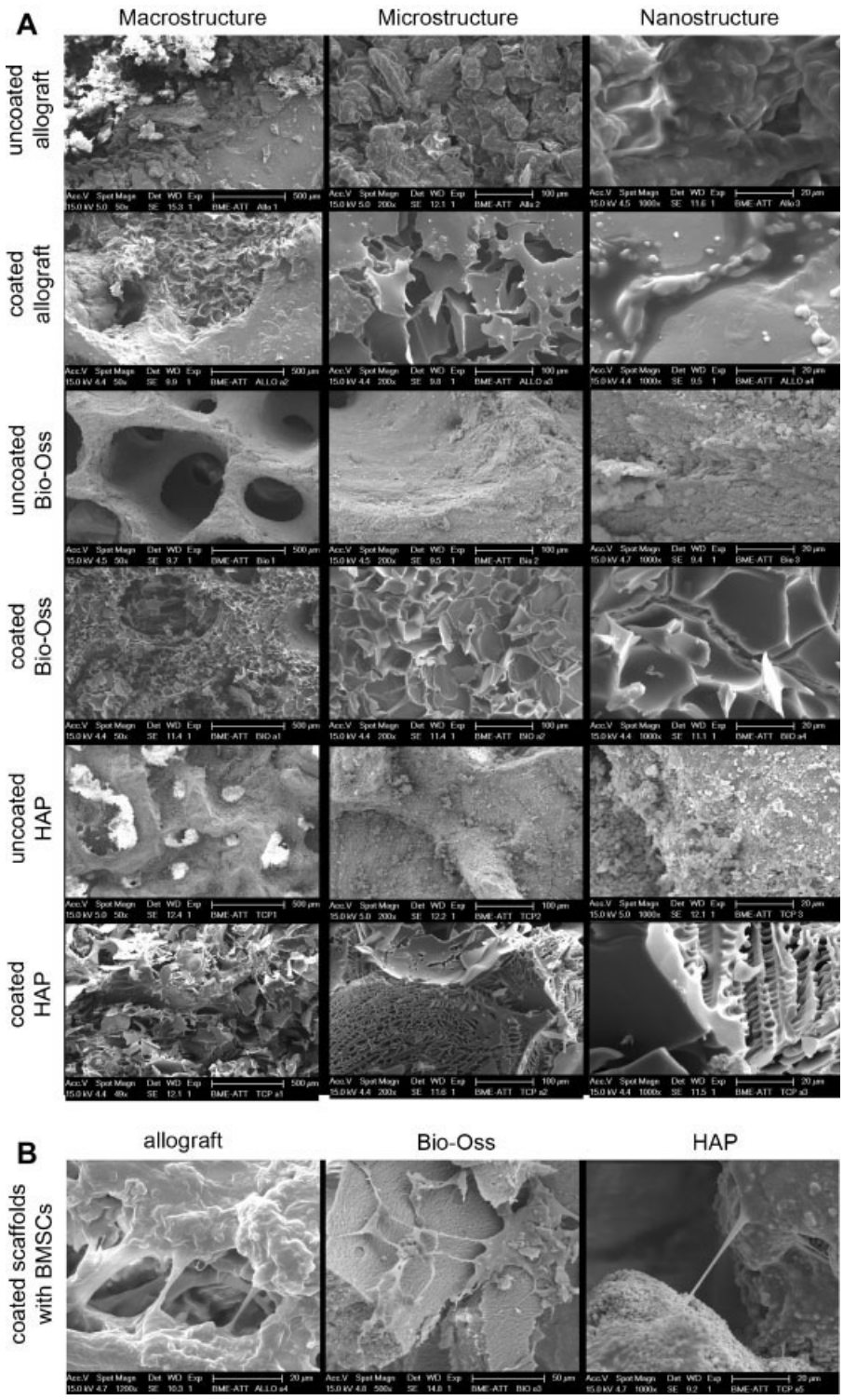

Figure 4. Macro-, micro-, and nanostructure of albumin coated and uncoated bone grafts. Scanning electron microscopy shows significant differences in the texture of mineralized allograft, hydroxyapatite (HAP), and lyophilized bovine bone graft (BioOss). Hydroxyapatite has the most compact structure with low porosity. On the other hand, high connectivity and thin wall-thickness typifies BioOss. The structure of human mineralized bone allograft is different from the other two. It is more compact than BioOss and its surface contains multiple micro-pores. By coating the surface of bone grafts with albumin amorphous protein chips mask the apparent differences in the micro- and nanostructure (A). (B) The attachment of BMSCs on the surface of albumin coated bone grafts. It can be observed that BMSCs do not cover the surface in a monolayer but rather span the pores from side to side.

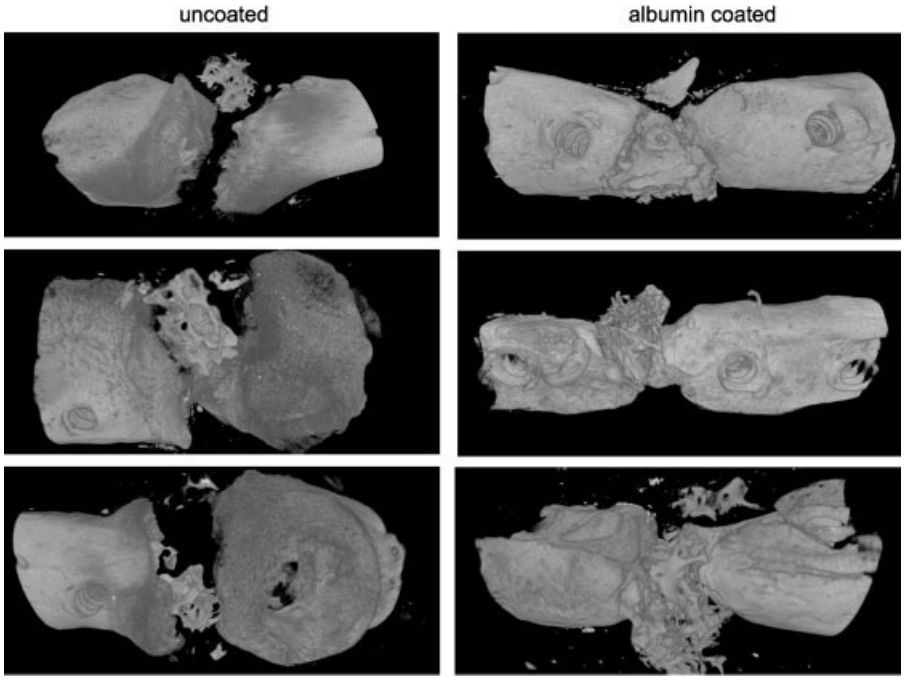

Figure 5. In vivo biocompatibility of a bone graft with or without albumin coating. Reconstructed 3-dimensional μCT images are shown of rat femora after 4 weeks of implantation of the graft. The left column shows that without albumin coating the bone graft does not integrate into the bone ends and there is no bony consolidation. In contrast, when the grafts were coated with albumin there is good ingrowth from the rat femur and a bony callous is formed.

collagen I and fibronectin was not appropriate to supply the long-term attachment and proliferation of MSCs. It is possible that the mineralized surface of freeze-dried human bone allograft does not contain adequate binding ligands which can permanently anchor collagen I and fibronectin. Under physiologic bone formation, collagen, and other structure proteins first build up the texture of the bone tissue followed by mineral deposition. Therefore it is not surprising that working in the opposite direction, that is, putting structure proteins on top of a mineralized scaffold does not yield optimal results.[25]

When the cells are seeded on the surface of freeze-dried albumin coated allograft the protein absorbs water and creates a miroenvironment for MSCs with high local albumin content. Since serum is a well-known supporting agent for cell proliferation, this microenvironment probably increases the viability of freshly deposited cells. These cells then easily regain their metabolic activity and start to deposit their own biofilm which further supports proliferation. The human bone structure and pore size play a crucial role in this mechanism. As it was observed in our electron microscopic images, MSCs are not forming a monolayer on the surface of bone but rather span the pores and establish minimal contact with the surface. This is in stark contrast to cell culture in traditional 2D monolayers on plastic surfaces and possibly explains why the proliferation-inducing properties of serum outweigh attachment factors. The larger pore size of lyophilized bovine bone graft or the smaller pores on the surface of synthetic hydroxyapatite do not provide optimal spatial arrangements for the MSCs. The lack of cell proliferation on other graft materials further supports this explanation. This mechanism provides an opportunity to colonize the surface of freeze-dried allografts in a simple rotating bioreactor system, which is frequently used in clinical tissue-engineering applications. In addition to bone marrow, proliferation of dental pulp-derived derived MSCs was also increased by albumin coating, making this technology suitable for dental applications.

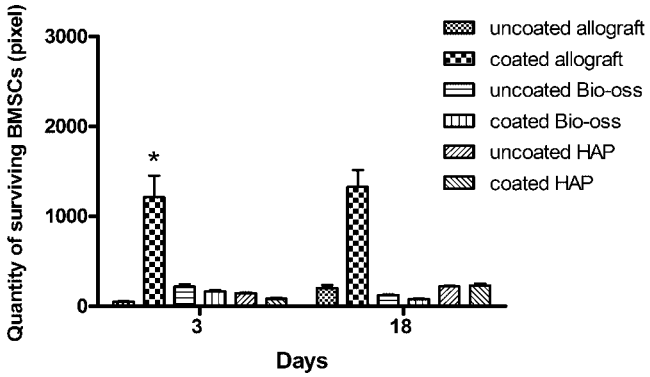

Figure 6. Attachment and proliferation of BMSCs on the surface of albumin coated and uncoated bone grafts. The coating of hydroxyapatite (HAP) and lyophlized bovine bone (BioOss) with freeze-dried human serum albumin does not improve the attachment and proliferation of BMSCs on the surface of grafts in contrast with mineralized bone allografts. $^{*}p < 0.05$.

The preliminary in vivo testing of albumin coated bone grafts showed an increased ingrowth on new bone compared to the uncoated ones. This observation highlights that early colonization of the graft with

bone marrow derived MSCs is a crucial step in the initiation of bone formation. Since serum albumin is a main constituent of growth media in standard stem cell culture, it can be hypothesized that the better union capacity was mainly due to the favorable conditions for MSCs in the graft achieved by albumin coating.

Our results indicate that allografts can be colonized in vitro with bone marrow or dental pulp derived MSCs in a rotating bioreactor system before transplantation. Since albumin coating does not alter the appearance and physical strengths of the allograft, this might be integrated into the manufacturing process of human cancellous bone grafts as a simple biocompatibility improving step.

ABBREVIATIONS

alpha-MEM	Minimal Essential Medium alpha
BMSC	Bone Marrow derived Stem Cell
DMEM	Dulbecco's Modified Eagle's Medium
DPSC	Dental Pulp derived Stem Cell
FCS	Fetal Calf Serum
MSC	Mesenchymal Stem Cell
PBS	Phosphate Buffered Saline

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