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Bone tissue engineering using a combination of polymer/Bioglass® composites with human adipose derived stem cells

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Abstract

Translational research in bone tissue engineering is essential for 'bench to bedside' patient benefit. However, the idea combination of stem cells and biomaterial scaffolds for bone repair/regeneration is still unclear. The aim of this study was to investigate the osteogenic capacity of a combination of poly(DL-lactic acid) (PDLLA) porous foams containing 5 and 40 wt% of Bioglass® particles with human adipose-derived stem cells (ADSCs) in vitro and in vivo. Live/dead fluorescent markers, confocal microscopy and scanning electron microscopy (SEM) showed that PDLLA/Bioglass® porous scaffolds supported ADSCs attachment, growth and osteogenic differentiation, which were confirmed by enhanced alkaline phosphatase activity (ALP). Higher Bioglass® content of the PDLLA foams increased more ALP activity compared to PDLLA only group. Extracellular matrix deposition after 8 weeks in in vitro culture was evident by Alcian blue/Sirius red staining. In vivo bone formation was assessed using scaffold/ADSCs constructs in diffusion chambers transplanted intraperitoneally into nude mice and recovered after 8 weeks. Histological and immunohistochemical assays indicated significant new bone formation in the 40 wt% and 5 wt% Bioglass® constructs compared to the PDLLA only group. This study indicated the combination of a well-developed biodegradable bioactive porous PDLLA/Bioglass® composite scaffold with a high potential stem cells source – human ADSCs could be a promising approach for bone regeneration in clinical setting.

Key words: bone tissue engineering; adipose tissue derived stem cells; PDLLA/Bioglass® composite; biodegradable polymers; in vivo

Abbreviations

ADSCs	adipose-derived stem cells
ALP	alkaline phosphatase activity
CMFDA	5-chloromethylfluorescein diacetate
COL-I	collagen I
EthD-1	ethidium homodimer-1
FCS	fetal calf serum
FDA	Food and Drug Administration
HA	hydroxyapatite
MSCs	mesenchymal stem cells
NBF	neutral buffered formalin
OCN	osteocalcin
PBS	phosphate-buffered saline
PDLLA	poly(DL-lactic acid)
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
SEM	scanning electron microscopy
TCP	tricalcium phosphate
TIPS	thermally induced phase separation
UV	ultraviolet
αMEM	α-modified minimal essential medium

Introduction

Critical bone defects and fracture non-union remain major challenges in current clinical practice (Hofmann et al. 2008; Zong et al. 2010). From a patient's perspective, the ultimate goal is replacement of their damaged/lost bone with autogenous material, harvested with minimal donor side morbidity. Traditionally, small defects are covered using autologous bone taken from iliac crest or ribs but large bony defects require autografts or allografts whose application is limited in terms of material availability and successful tissue in-growth (Eppley et al. 2005). Furthermore, allografts carry the risk of disease transmission and host immune response (Horner et al. 2010; Saha et al. 2011; Yang et al. 2006). In contrast, xenografts are in plentiful supply but they carry even greater risks of immune rejection and in situ degeneration as well as disease transmission. Tissue engineering offers a promising alternative to permanent implants in the repair of damaged tissue, through the use of autologous stem cells seeded into a suitable scaffold (Ahsan and Nerem 2005; Blaker et al. 2003; Buma et al. 2004; Marot et al. 2010).

Stem cell populations for producing engineered bone can be obtained from a range of different sources such as bone marrow and peripheral blood. Adipose tissue has recently been demonstrated as a viable source of stem cells that can differentiate along adipogenic, myogenic, chondrogenic, neuronal and osteogenic lineage pathways in vitro (De Ugarte et al. 2003; Gabbay et al. 2006; Kokai et al. 2005; Zuk et al. 2002; Zuk et al. 2001) and/or in vivo lee (Hicok et al. 2004; Lee et al. 2003; Lee et al. 2010; Parker and Katz 2006; Wang et al. 2010; Wosnitza et al. 2007). To date, bone marrow has been the traditional source of human multipotent mesenchymal stem cells (MSCs) for skeletal tissue engineering but

adipose tissue appears to offer an alternative, more readily available and highly accessible stem cell source (Mehrkens et al. 2012; Sterodimas et al. 2009).

Over the past few years, increasing attention has been paid to composite scaffolds designed to be both bioresorbable and bioactive for applications in tissue engineering. Such scaffolds have comprised of polymers together with bioactive materials (Boccaccini et al. 2003; Hench and Paschall 1973; Maquet et al. 2004). Ideally, composite scaffolds should be porous on the macroscale to allow for cell ingrowth and migration, and on the microscale to allow the efficient transport of nutrients/oxygen, as well as the removal of cellular waste products (Hutmacher 2000). For bone tissue engineering, composite scaffolds have included combinations of poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and other bioresorbable polymers combined with hydroxyapatite (HA), tricalcium phosphate (TCP) and bioactive glasses or glass-ceramics in various scaffold architectures (Cao and Kuboyama 2010; Liao et al. 2009; Ngiam et al. 2009; Saha et al. 2011; Yang et al. 2004; Yang et al. 2006). Synthetic bioresorbable polymers based on lactic acid and their copolymers have been used in clinical applications such as surgical sutures or drug delivery systems for many years and have US Food and Drug Administration (FDA) approval (Howard et al. 2002; Peter et al. 1998). However, lactic acid-based synthetic polymers are not inherently bioactive and will not bond directly to bone. They are also initially highly hydrophobic and lack the mechanical strength required to meet the demands of orthopaedic surgery (Cohen et al. 1993; Liu and Ma 2004). The combination of such polymers with a bioactive component therefore takes advantage of both the osteoconductive properties (bioactivity) of HA, bioactive glasses and their strengthening effect on polymer matrices. A

composite scaffold system currently being developed is based on PDLLA/Bioglass®-filled composite foams produced by thermally induced phase separation (TIPS) (Maquet et al. 2004). Bioactive glasses, such as 45S5 Bioglass®, have successfully been used in clinical applications for bone restoration and augmentation (Gheysen et al. 1983; Hattar et al. 2002; Hench 1988; Hench and Wilson 1986). 45S5 Bioglass® is a commercial bioactive glass containing 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5 (in weight percentage) (Hench and Polak 2002). When implanted or in contact with biological/physiological fluids, Bioglass® forms tenacious bonds with hard (and sometimes soft) tissue through the rapid formation of a thin layer of carbonated hydroxyapatite on the glass surface. This gives rise to an adherent interface that resists substantial mechanical forces. These properties of 45S5 Bioglass® have guided its application in tissue engineering, mainly in combination with biodegradable polymers, to produce porous bioactive composites with foam-like structure (Boccaccini et al. 2003). Indeed, numerous in vivo and in vitro studies have shown that 45S5 Bioglass® can stimulate bone regeneration (Bosetti et al. 2003; Gough et al. 2004; Livingston et al. 2002; Loty et al. 2001; Tsigkou et al. 2007; Xynos et al. 2000). As a result, Bioglass® is said to be both osteoproductive and osteoconductive. Incorporation of Bioglass particles in to a biodegradable polymer scaffold can be advantageous as it will impart bioactivity to the polymer matrix whilst reducing the autocatalytic degradation of polymers such as polylactide (Blaker et al. 2005; Stamboulis et al. 2002).

We have previously fabricated composite scaffolds through incorporation of different concentrations (0, 5, and 40 wt %) of 45S5 Bioglass® particles into PDLLA. These composites have been developed into 3D foam-like structures and characterized in terms of

their chemical and mechanical properties (Maquet et al. 2003; Yang et al. 2006). However, there has been no previous detailed in vitro and in vivo investigation evaluating the effect of the composites on human ADSCs osteogenic differentiation and extracellular matrix deposition and mineralization.

The aims of this study were to examine the adhesion, growth and differentiation of ADSCs on 3D PDLLA/Bioglass® composite foams in vitro and in vivo, and to evaluate their potential in bone tissue engineering.

Materials and methods

All in vivo experiments were performed with the ethical approval of University of Leeds Animal Experimentation Ethics Committee under the Home Office project license (40/3361).

Cell culture and characterization

Human adipose-derived stem cells were purchased from Invitrogen (Catalog nos. R7788-115), following isolation from human adipose tissue collected during liposuction procedures and cryopreservation from primary cultures. Before cryopreservation, the ADSCs were expanded for one passage in MesenPRO RS™ Medium. The ADSCs express a flow cytometry cell surface protein profile as follows: positive CD29, CD44, CD73, CD90, CD105, CD166; negative CD14, CD31, CD45, Lin1 (Invitrogen 2012). ADSCs were expanded in α-modified minimal essential medium (αMEM) （Lonza BioWhittaker, Belgium）supplemented with 5% fetal bovine serum (FBS) (BioSera, Sussex, UK) and 1%

penicillin–streptomycin (Sigma, P0781) (basal medium). ADSCs (1×10^4 cells) were seeded into 90 mm culture dishes (Costar, Cambridge, MA, USA) and cloned as previously reported (Seo et al. 2004). The cells were plated at a density of 5000 cells/cm^2 and expanded to the fifth passage. These cells were then used for all experiments performed in our study. Mesenchymal stem cell surface markers were confirmed for cultured ADSCs using fluorescence-activated cell sorting analysis for CD29 and CD44 (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. The induction of osteogenesis and adipogenesis were as previously reported (Lu et al. 2012).

PDLLA/Bioglass® foam scaffolds

Purasorb® PDLLA (obtained from Purac Biochem, Goerinchem, The Netherlands) with an inherent viscosity of 1.62 dl/g was used for the preparation of high porosity foams. Bioactivity was introduced using a melt-derived bioactive glass powder (Bioglass® grade 45S5, US Biomaterials Co., Alachua, FL, USA). The powder had a mean particle size < 5 µm. The composition of the bioactive glass used was (in wt %): 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5. Polymer/Bioglass® porous composites were prepared by freeze-drying as previously described (Maquet et al. 2003). Three scaffolds were tested in this study. These comprised of two PDLLA/Bioglass® composite scaffolds at two concentrations of Bioglass® (5 and 40 wt% by weight) together with a third scaffold containing PDLLA foam only (no Bioglass®).

Cell culture on PDLLA/Bioglass® composite scaffolds in vitro

PDLLA and PDLLA/Bioglass® composite scaffolds were sterilized for 20 min each side using ultraviolet (UV) (254 nM wavelength) irradiation and cut into about 2 × 2 × 5 mm^3 cuboid blocks. The scaffolds were pretreated by incubating in αMEM for 24 h. To determine cell adherence, ADSCs were incubated with 5 mM CellTracker™ green (5-chloromethylfluorescein diacetate -CMFDA: Molecular Probes, Leiden, The Netherlands) for 45 min. CMFDA is only incorporated into the cell cytoplasm of viable cells and remains present through at least four cell divisions. The medium was then replaced and the cells were incubated for a further hour. Following trypsinisation and resuspension in serum-free αMEM, a uniform number of cells (usually 5×10^5 cells) was added to each of the universals containing pre-prepared individual scaffolds (three samples per group of scaffolds). Fluorescence images were taken after 5 h and 24 h as required. The medium was then removed and the scaffolds were transferred into 12-well plates where they were maintained in αMEM supplemented with 5% FBS for up to 8 weeks. Cultures were fed every 3 days and maintained at 37℃ and 5% CO_2. At various time points up to 8 weeks, samples were harvested and processed biochemically and histologically.

Fluorescence microscopy and cell adhesion

Fluorescence images were taken of ADSCs seeded on the different PDLLA/Bioglass® composite scaffolds (three samples per group) after 5 h and 24 h post-seeding. Images were obtained using an inverted microscope (Motic AE31, USA), equipped with a fluorescence filter. Three random images were taken per sample, producing a total of nine images for

each group. Analysis was performed retrospectively using SCANIMAGE software, which detects variations in image colour intensity and calculates individual cell parameters based on the fluorescence signal per pixel.

Scanning electron microscopy (SEM)

ADSCs were cultured on different PDLLA/Bioglass® composite scaffolds for 3 weeks as described previously. Scaffolds with or without ADSCs were then fixed in 2.5% glutaraldehyde overnight and freeze-dried. Samples were coated with 5 μm thickness gold and examined by scanning electron microscopy (XL30, Philips).

Live/Dead cytotoxicity assay

Cytotoxicity of the two PDLLA/Bioglass® composite scaffolds at two concentrations of Bioglass® (5 and 40 wt % by weight) together with a third scaffold containing PDLLA foam only (no Bioglass®) were determined with the Live/Dead cytotoxicity assay. CellTracker™ green and ethidium homodimer-1 (EthD-1) (Molecular Probes) were added to 6 week cultures of ADSCs on composite scaffolds to determine cell viability (green fluorescence) and cell death (red fluorescence) using laser scanning confocal microscopy (Leica TCS SP2, Germany). The results represent the mean values of three individual samples for each type of scaffold.

Alcian blue/Sirius red staining

ADSCs were cultured on the three different scaffolds (three samples each group) for 8

weeks in vitro. Samples were fixed with 10% neutral buffered formalin (NBF) and processed to paraffin wax. 5 μm sections were prepared and stained using Weigert's haematoxylin solutions before staining with 0.5% Alcian blue. After treatment with 1% molybdophosphoric acid, sections were stained using 0.1% Sirius red (Saha et al. 2013; Yang et al. 2006).

Alkaline phosphatase specific activity

ADSCs were cultured on the three different scaffolds (four samples each group) in 12-well tissue culture plates (5×10^5 cell/scaffold) in basal medium for 4 weeks. Cell-scaffold constructs were then washed with 1× phosphate-buffered saline (PBS) and stored at -80℃ until required. For each assay, the constructs were cut into small pieces (about 1 × 1 × 1 mm^3) and 0.5 mL 0.1% (v/v) Triton X-100 added before freeze/thawing twice. Total cell lysis in Triton X-100 was confirmed by light microscopy. Alkaline phosphatase activity was measured spectrophotometrically using p-nitrophenylphosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5 M, pH 10.3, at 37℃) and reading the absorbance using a mircoplate reader (Dynex MRX II, America). DNA content was measured using the PicoGreen® fluorescence reagent according to the manufacturer's instructions (Molecular Probes) (Green et al. 2004). Sample fluorescence was measured using a micro-plate reader (Thermo Scientific Fluoroskan Ascent, America) (excitation ~480 nm, emission ~520 nm) (Yang et al. 2006). Alkaline phosphatase specific activity was then expressed as nanomoles of p-nitrophenol/min/ng DNA (Saha et al. 2010).

In vivo studies

Male MF-1 nu/nu immunodeficient mice were used for all in vivo studies, and were purchased from Harlan (Loughborough, UK). ADSCs were trypsinised and seeded, in αMEM, onto sterile scaffolds (n=4 for each scaffold: 0%, 5% and 40% Bioglass® in PDLLA) for 24 h. Cell-scaffold constructs were then placed into diffusion chambers (Diffusion Chamber Kit, Millipore). Intraperitoneal transplantation of the diffusion chambers (Saha et al. 2013) was carried out under anaesthesia using a mixture (1:1, vol/vol) of Hypnorm (1:4 in sterile water) and Hypnorval (1:1 in sterile water) administered as intraperitoneal injections at 7-10 mL/kg body weight. After 8 weeks, the animals were sacrificed and the diffusion chambers were retrieved and fixed in 10% NBF for histological analysis.

Histology and immunohistochemistry

After fixation, cell-scaffold constructs were processed to paraffin wax embedding and 5 μm sections were prepared using a microtome (Leica 2035, Germany). For histology, sections were stained with Alcian blue/Sirius red as described previously and examined microscopically with or without polarized light. For immunohistochemical studies, sections were rinsed with 1 x PBS and were blocked in 20% normal goat serum for 5 min at room temperature. The primary antibodies used were as follows: Anti-collagen I (COL-Ⅰ) monoclonal antibody (1:100, Abcam ab6308) and/or Anti-osteocalcin (OCN) polyclonal antibody (1:200, Abcam ab35078) were incubated overnight at 4℃ (COL-I) or for 1 hour (OCN) at room temperature. Primary antibodies were detected using the Dako REAL

EnVision second antibody detection system (Dako, Carpinteria, CA, USA). All sections were counterstained with Harris's Haematoxylin. 1 x PBS instead of primary antibodies was used as negative control.

Statistical analysis of data

Values are expressed as mean ± SD. Experiments were performed at least three times and results of representative experiments are presented except where otherwise indicated. Statistical analysis was performed by analysis of variance and an independent samples t-test with SPSS v16.0 software. P values of less than 0.05 were judged to be statistically significant.

Results

Characterisation of ADSCs

Human ADSCs were identified based on 1) their ability to form adherent clonogenic cell clusters of fibroblast-like cells (Fig. 1a); 2) the ability of these cells to undergo osteogenesis following induction was indicated by the presence of calcified extracellular matrix deposition in ADSCs within 28 days of induction, as confirmed by Alizarin Red staining (Fig. 1b); 3) adipogenesis was determined by the presence of intracellular lipid vacuoles as indicated by Oil-Red-O staining within 4 weeks of adipogenic induction (Fig. 1c); 4) fluorescence-activated cell sorting analysis of ex vivo–expanded ADSCs demonstrated that 100.0% of the cells stained positive for mesenchymal stem cell surface markers CD29 and 99.9% for CD44 (Fig. 1d, eE).

PDLLA/Bioglass® composite scaffolds support cell attachment and growth

ADSCs were observed to attach rapidly to each of the scaffold surfaces after 5h of cell seeding (Fig. 2a-c). ADSCs adhered and spread well on the scaffolds after 24 h, as observed by the change from a rounded shape to a flattened and spread morphology (Fig. 2d–f). Qualitative analysis of both numbers of cells attached and spreading on each scaffold type revealed no significant visual difference between each group.

SEM analysis of PDLLA/Bioglass® scaffolds ± ADSCs

The effect of Bioglass® content on the structure of the PDLLA/Bioglass® composite scaffolds was investigated by varying the Bioglass® content in a PDLLA matrix and the resulting scaffolds were viewed using scanning electron microscopy (Fig. 3a-c). All of the scaffolds exhibited a high porosity irrespective of Bioglass® concentration. Following seeding with ADSCs, adhesion and cell growth could be observed on all of the scaffolds after 21 days in culture. The images provided evidence of both cells and extracellular matrix on the surfaces of all of the scaffolds (Fig. 3d-f) with extensive matrix deposition apparent.

ALP activity of ADSCs on PDLLA/Bioglass® composite scaffolds in vitro

After 4 weeks of culture in vitro, alkaline phosphatase specific activity of ADSCs in the 40 wt % Bioglass® constructs was significantly higher compared to that of both the 5 wt% Bioglass® group and the PDLLA only (control) group ($p < 0.01$). In addition, ALSP for

ADSCs in the 5 wt% Bioglass® constructs was also significantly increased compared to the control group ($p < 0.05$) (Fig. 4).

Cell viability of ADSCs cultured on PDLLA/Bioglass® composite scaffolds

After 6 weeks of culture in vitro, extensive cellular ingrowth and majority of the cells were viable in all of the cell-scaffold constructs (Fig. 5a-c). Furthermore, there were only a few dead cells in all of the groups. However, no qualitative differences were apparent between the constructs with 0, 5, and 40 wt% Bioglass®.

Collagen synthesis of ADSCs on PDLLA/Bioglass® composite scaffolds in vitro

After 8 weeks culture in vitro, Alcian blue/Sirius red staining revealed an extensive collagenous matrix in the 5 wt% and 40 wt% Bioglass® constructs, that was especially pronounced in the 40 wt% Bioglass scaffold constructs. In addition, what appeared to be collagenous matrix (based on Sirius red staining) was particularly noticeable at the edges of the 5 wt% and 40 wt% Bioglass® constructs compared with the PDLLA only control group. Ingrowth of ADSCs was also apparently greater in the 5 wt% and 40 wt% Bioglass® constructs compared with the PDLLA only control group (Fig. 5d-f), though this was not quantified.

Histological examinations of cell-scaffold constructs transplanted in vivo

After 8 weeks in vivo implantation, Alcian blue/Sirius red staining revealed extensive collagenous matrix staining throughout all of the constructs examined (Fig. 6a-c). However,

this was much greater, especially at the edges of the construct, in those constructs incorporating 5 wt% and 40 wt% Bioglass® compared with the PDLLA control group. Ingrowth of ADSCs in to the 5 wt% and 40 wt% Bioglass® scaffolds was also greater than that seen for the PDLLA only control group. Birefringence revealed the presence of highly organized collagen fibers in the 5 wt% and 40 wt% Bioglass® constructs but there was little evidence for this in the PDLLA control group (Fig. 6d-f). These results were confirmed using immunohistochemistry (Fig. 6g–i), where type I collagen staining was greatest in the 40 wt% Bioglass® constructs. Furthermore, the expression of the bone-associated protein osteocalcin (OCN) was apparently very high in the 5 wt% followed by 40 wt% Bioglass® composites in comparison with the PDLLA controls (Fig. 6j-l).

Discussion

Composite scaffolds comprising of PDLLA with Bioglass® particle additions are of current interest for bone tissue engineering (Blaker et al. 2003; Blaker et al. 2005; Maquet et al. 2003; Maquet et al. 2004; Roether et al. 2002; Yang et al. 2006). Adipose tissue is an abundant source of mesenchymal stem cells, which have shown promise in the field of regenerative medicine (Hicok et al. 2004; Lalande et al. 2011; Parker and Katz 2006). These cells can be readily harvested in large numbers with low donor-site morbidity. During the past decade, numerous studies have provided preclinical data on the safety and efficacy of adipose-derived stem cells, supporting the use of these cells in future clinical applications. In this study we investigated ADSC cell adhesion, growth, and osteogenic differentiation in composites made of PDLLA foams and Bioglass® particle inclusions in

vitro compared with PDLLA alone. The combination of ADSCs and PDLLA/Bioglass® foam was also characterized in an immunocompromised animal model. This is the first study addressing specifically the effect of 45S5 Bioglass® additions to a polymer composite on adipose derived stem cell behavior and their capacity for bone formation.

A number of studies have showed that human adipose-derived stem cells are an attractive source of cells for bone tissue engineering due to their ease of harvest, the low morbidity associated with liposuction, and their rapid expansion in vitro (Schaffler and Buchler 2007; Tobitan et al. 2011). ADSCs used in this study were purchased commercially and showed typical mesenchymal stem cell characteristics, confirmed using multiple differentiation and flow cytometry analysis. The results were consistent with previous publications (Lu et al. 2012; Zuk et al. 2002).

Cell adhesion to 3D porous scaffolds is important because it directly influences cell settlement on the scaffold and subsequent cell spreading, migration, proliferation, growth, and differentiation leading to effective scaffold colonization (Anselme 2000; Verrier et al. 2004). In the present study, adhesion and spreading of human ADSCs on each of the materials was observed by fluorescent, confocal, and scanning electron microscopies. The results showed that human ADSCs can attach and spread on each of the materials used (Fig. 2a–f). In addition, SEM examination also indicated that ADSCs anchored tightly on the surface of all three different scaffolds and produced large amounts of extracellular matrix after 3 weeks culture in vitro (Fig. 3d-f). This indicated that the three scaffold types had all successfully facilitated cell attachment and spreading without the need for modification of surface chemistry, which was similar to our previous work on human bone marrow stromal

cells (Yang et al. 2006). The Live/Dead assays indicated that none of the test materials were toxic to the cells, and all of the scaffolds were suitable for long term culture with ADSCs in vitro. There were no significant difference for the cell attachment and spreading on the different scaffold groups, which indicated that the additional Bioglass® didn't effect the cell attachment. However, the 40 wt% Bioglass® constructs appeared to significantly enhance human ADSCs alkaline phosphatase activity compared with the 5 wt% Bioglass® and PDLLA only constructs after 4 weeks, suggesting stimulated cell osteogenic differentiation towards the osteogenic lineage as ALP activity has been extensively used as one of the indicators for stem cell osteogenic differentiation (Birmingham et al. 2012; Mauney et al. 2004). After 6 weeks in vitro culture, Alcian blue/Sirius red staining revealed that extensive collagenous matrix was produced along with the increase of Bioglass® concentration.

The diffusion chamber system (Horner et al. 2008) used here for the in vivo studies offers definite advantages over conventional transplantation techniques in which host cells from various tissues would affect the development of the graft. In vivo analysis of PDLLA/Bioglass® composites seeded with ADSCs revealed extensive collagen matrix formation in the 40 wt% and 5 wt% Bioglass® constructs, indicative of new bone matrix formation. This was also confirmed by birefringence and type I collagen and OCN immunohistochemistry. Type Ⅰ collagen is the most abundant bone matrix protein, constituting up to 90% of the organic matrix (Young 2003). Its expression and deposition play an important role in mineralization and, consequently, bone formation (Tsigkou et al. 2007). OCN is a bone specific extracellular matrix protein and its expression and synthesis reflect a mature osteoblast phenotype, suggesting that the composite scaffolds had

supported ADSC osteogenesis. These provided direct evidence of that incorporation of 45S5 Bioglass® into the PDLLA matrix significantly enhanced ADSC osteogenic differentiation and bone-associated extracellular matrix deposition.

To date, there is no standard method to assess the different combination of stem cells and biomaterial scaffolds for the efficacy of tissue regeneration. Neuss et al (2008) have assessed 7 different stem cells and 19 different polymers for systematic screening assays to analyse parameters such as morphology, vitality, cytotoxicity, apoptosis and proliferation (Neuss et al. 2008). However, it was hard to conclude which combination could be better for which specific tissue regeneration. In this study, we selected a well-developed biodegradable bioactive porous PDLLA/Bioglass® composite scaffold and a high potential stem cells source – human ADSCs to test their capacity for bone tissue engineering both in vitro and in vivo, which provide evidence to guide for clinical translation of these technology. Furthermore, a number of studies showed that ADSCs are immunoprivileged cells that may be therefore available for cell replacement therapies in HLA-incompatible hosts, before and/or after osteogenic differentiation in vitro (McIntosh et al. 2009; Niemeyer et al. 2007; Puissant et al. 2005), which indicate the potential of using allogenic ADSC and PDLLA/Bioglass® composite to heal human bone defect in clinical setting, although more work needs to be done in this area to develop useful, safe and powerful applications of ASCs in the biomedical field (Rada et al. 2009).

Conclusions

This study has shown that PDLLA/Bioglass® composite scaffolds can provide appropriate surface chemistry, structure and microenvironment to support ADSCs growth and osteogenic differentiation for bone matrix formation, which indicated the potential of using the combination of a biodegradable bioactive porous PDLLA/Bioglass® composite scaffold with human ADSCs for bone regeneration in clinical bone augmentation.

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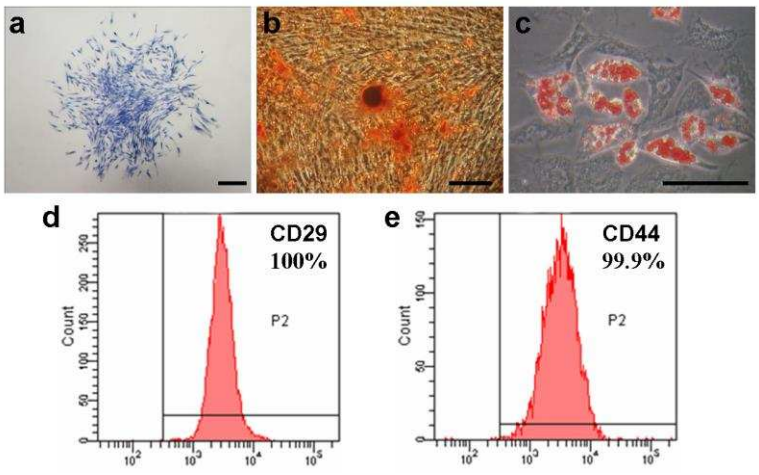

Figure 1. Characterization of human ADSCs. A) ADSCs were capable of forming a single-colony cluster when plated at a low cell density. B) Mineralized nodules formation by ADSCs after 4 weeks culture in osteogenic inductive conditions, which were confirmed by Alizarin red staining. (C) ADSCs formed Oil red O–positive lipid clusters after 4 weeks of adipogenic induction. D&E) Flow cytometric analysis of ex vivo–expanded ADSCs revealed expression of CD29 (100%) and CD44 (99.9%). Scale bars: 50 μm.

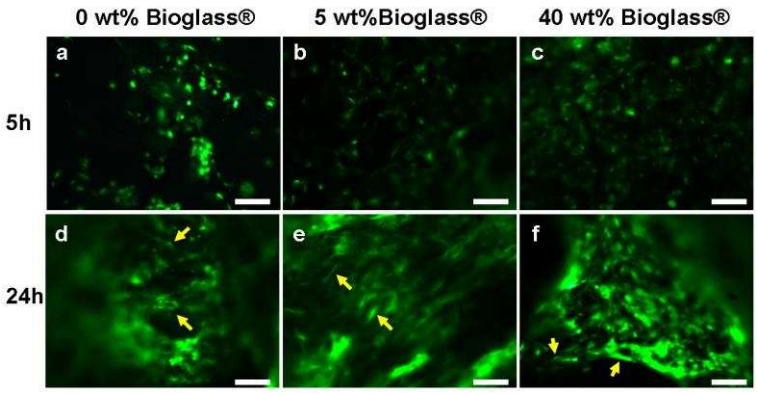

Figure 2. ADSCs attachment and spreading on 3D PDLLA/Bioglass® scaffolds. Representative images of viable human adipose derived stem cells (labeled with CMFDA) attachment (A-C: 5 hrs after seeding) and spreading (D-F: 24 hrs after seeding) to PDLLA/Bioglass® scaffolds. ADSCs adhered and spread well (yellow arrows) on the scaffolds after 24 h compare to that at 5 hrs. Scale bars: 50 μm.

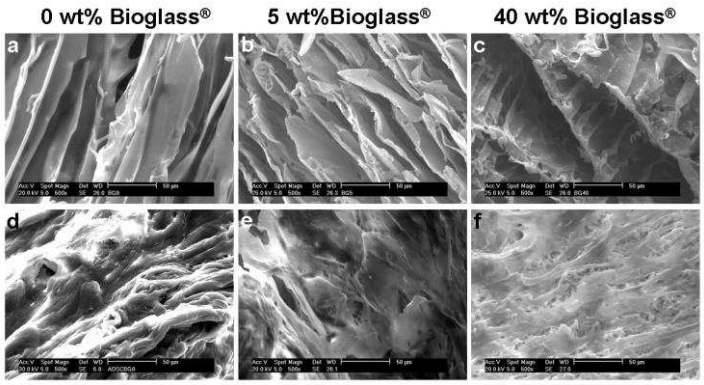

Figure 3. SEM images of different PDLLA/Bioglass® composite foams with/without ADSCs. SEM micrographs showing the microstructure of PDLLA composite foam (A & D), 5 wt% PDLLA/Bioglass® composite foam (B & E) and 40 wt% PDLLA/Bioglass® composite foam (C & F). A-C) scaffold alone. D-F) scaffolds with cells after 3 weeks of culture. Sale bars: 50 μm.

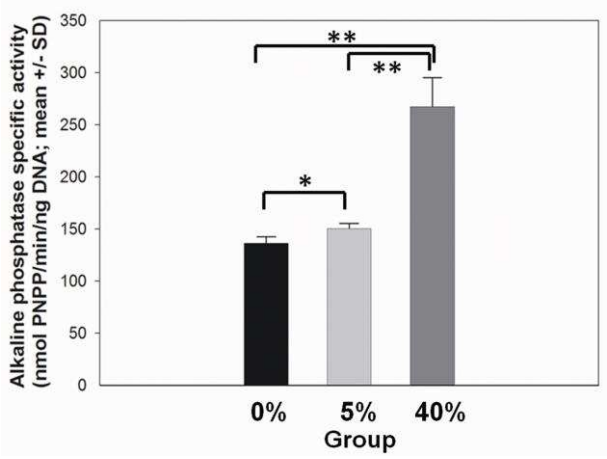

Figure 4. Alkaline phosphatase specific activity of ADSCs on scaffolds with different Bioglass® contents. Data obtained after 4 weeks culture of ADSCs on different scaffolds in basal media. *$p < 0.05$, **$p < 0.01$.

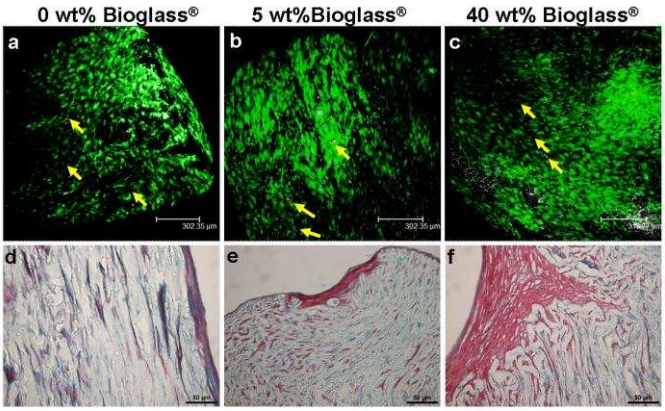

Figure 5. Cell viability and histological appearance of ADSCs on different 3D scaffolds. Confocal microscopy images (a-c) shows the majority of ADSCs maintained viable (green colour) with only a few necrotic cells (red colour: yellow arrows) on different scaffolds after 6 weeks in vitro culture. Alcian blue/Sirius red staining (d-f) shows collagen matrix (red colour) and tissue formation by the ADSCs on/within PDLLA/Bioglass® composites after 6 weeks in vitro culture. a & d） ADSCs cultured on PDLLA composite foam; b & e）ADSCs cultured on 5 wt % PDLLA/Bioglass® composite foam; c & f）ADSCs cultured on 40 wt %PDLLA/Bioglass® composite foam).

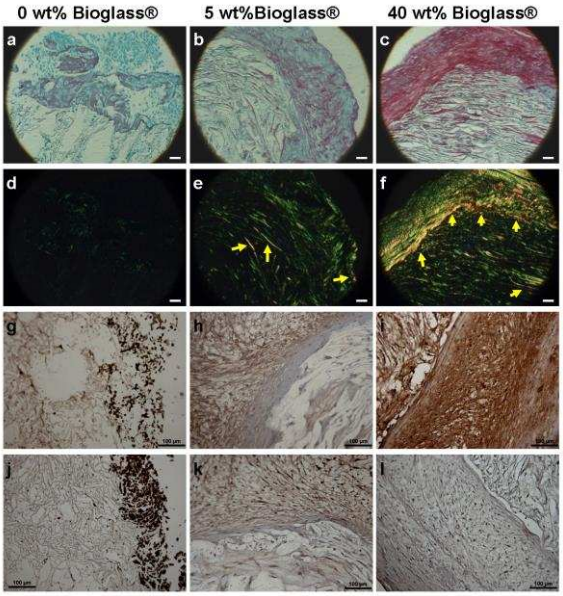

Figure 6. Histological and immunohistochemical examinations of human ADSCs-PDLLA/Bioglass® constructs after 8 weeks in vivo implantation. a-c) Alcian blue/Sirius red staining shows collagen matrix (red colour) and tissue formation by ADSCs on/within the scaffold in vivo; d-f) Birefringence images shows high organized type I collagen fibers (yellow arrows) within the extra cellular matrix; g-i) Type I collagen immunohistochemical staining (brown colour), and j-l) OCN immunohistochemical staining (brown colour). Scale bars: 100 μm.