REGULAR ARTICLE

Bone tissue engineering by 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 ideal combination of stem cells and biomaterial scaffolds for bone repair/regeneration is still unclear. The aim of this study is to investigate the osteogenic capacity of a combination of poly(DL-lactic acid) (PDLLA) porous foams containing 5 wt% 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 showed that PDLLA/Bioglass porous scaffolds supported ADSC attachment, growth and osteogenic differentiation, as confirmed by enhanced alkaline phosphatase (ALP) activity. Higher Bioglass content of the PDLLA foams increased ALP activity compared with the PDLLA only group. Extracellular matrix deposition after 8 weeks in the in vitro cultures was evident by Alcian blue/Sirius red staining. In vivo bone formation was assessed by using scaffold/ADSC constructs in diffusion chambers transplanted intraperitoneally into nude mice and recovered after 8 weeks. Histological and immunohistochemical assays indicated significant new bone

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formation in the 40 wt% and 5 wt% Bioglass constructs compared with the PDLLA only group. Thus, the combination of a well-developed biodegradable bioactive porous PDLLA/ Bioglass composite scaffold with a high-potential stem cell source (human ADSCs) could be a promising approach for bone regeneration in a clinical setting.

Keywords Bone tissue engineering · Adipose-derived stem cells · PDLLA/Bioglass® composite · Biodegradable polymers · In vivo

Abbreviations

ADSCs	Adipose-derived stem cells
ALP	Alkaline phosphatase
CMFDA	5-Chloromethylfluorescein diacetate
COL-I	Collagen I
FBS	Fetal bovine serum
MSCs	Mesenchymal stem cells
NBF	Neutral buffered formalin
OCN	Osteocalcin
PBS	Phosphate-buffered saline
PDLLA	Poly(DL-lactic acid)
SEM	Scanning electron microscopy
αMEM	Alpha-modified minimal essential medium
3D	Three-dimensional

Introduction

Critical bone defects and fracture nonunion 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 by using autologous bone taken from the 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, in situ degeneration and disease transmission. Tissue engineering with autologous stem cells seeded into a suitable scaffold offers a promising alternative to permanent implants in the repair of damaged tissue (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 wide range of 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. 2001, 2002) and/or in vivo (Hicok et al. 2004; Lee et al. 2003, 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. 2010).

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 are composed 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 cell ingrowth and migration and on the microscale to permit the efficient transport of nutrients/oxygen and the removal of cellular waste products (Hutmacher 2000). For bone tissue engineering, composite scaffolds have included combinations of poly(lactic acid), poly(glycolic acid) and other bioresorbable polymers combined with hydroxyapatite, tricalcium phosphate 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, 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 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 the osteoconductive properties (bioactivity) of hydroxyapatite, bioactive glasses and their strengthening effect on polymer matrices. A composite scaffold system currently being developed is based on poly(DL-lactic acid) (PDLLA)/Bioglass-filled composite foams produced by thermally induced phase separation (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 a 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 into a biodegradable polymer scaffold can be advantageous, as it will impart bioactivity to the polymer matrix, while reducing the autocatalytic degradation of polymers such as polylactide (Blaker et al. 2005; Stamboulis et al. 2002).

We have previously fabricated composite scaffolds through the incorporation of various concentrations (0, 5 and 40 wt%) of 45S5 Bioglass particles into PDLLA. These composites have been developed into three-dimensional (3D) foam-like structures and characterized in terms of their chemical and mechanical properties (Maquet et al. 2003; Yang et al. 2006). However, no previous detailed in vitro and in vivo investigation has evaluated the effect of the composites on the osteogenic differentiation and extracellular matrix deposition and mineralization of human adipose-derived stem cells (ADSCs). The aims of this study are 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 the University of Leeds Animal Experimentation Ethics Committee under a Home Office project licence (40/3361).

Cell culture and characterization

Human ADSCs were purchased from Invitrogen (catalog no. 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 flow cytometry cell surface protein profile expressed by the ADSCs is follows: positive CD29, CD44, CD73, CD90, CD105, CD166; negative CD14, CD31, CD45, Lin1 (Invitrogen 2012). ADSCs were expanded in alpha-modified minimal essential medium (αMEM; Lonza BioWhittaker, Belgium) supplemented with 5 % fetal bovine serum (FBS; BioSera, Sussex, UK) and 1 % penicillin–streptomycin (Sigma, P0781), termed basal medium. ADSCs (1×10^4 cells) were seeded into 90-mm culture dishes (Costar, Cambridge, Mass., 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. MSC surface markers were confirmed for cultured ADSCs by

using fluorescence-activated cell sorting analysis for CD29 and CD44 (eBioscience, San Diego, Calif., USA) according to the manufacturer's protocol. The induction of osteogenesis and adipogenesis was 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 by using a melt-derived bioactive glass powder (Bioglass grade 45S5, US Biomaterials, Alachua, Fla., USA). The powder had a mean particle size of <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 comprise 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 by using ultraviolet (254 nM wavelength) irradiation and cut into about $2 \times 2 \times 5$ mm^3 cuboid blocks. The scaffolds were pretreated by incubation in αMEM for 24 h. To determine cell adherence, ADSCs were incubated with 5 mM CellTracker^{TM} 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 trypsinization and resuspension in serum-free αMEM, a uniform number of cells (usually 5×10^5 cells) was added to each of the vials containing 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 °C and under 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 various PDLLA/Bioglass composite scaffolds (three samples per group) after 5 h and 24 h post-seeding. Images were obtained by 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 by 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

ADSCs were cultured on the various 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 gold at a thickness of 5 nm and examined by scanning electron microscopy (SEM; 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 (Molecular Probes) were added to 6-week cultures of ADSCs on composite scaffolds to determine cell viability (green fluorescence) and cell death (red fluorescence) by 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. Sections (5 μm thick) were prepared and stained by using Weigert's haematoxylin solutions before being stained with 0.5 % Alcian blue. After treatment with 1 % molybdophosphoric acid, sections were stained with 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 °C until required. For each assay, the constructs were cut into small pieces (about $1 \times 1 \times 1$ mm^3) and 0.5 ml of 0.1 % (v/v) Triton X-100 was added before freeze/thawing twice. Total cell lysis in Triton X-100 was confirmed by light microscopy. Alkaline phosphatase (ALP) activity was measured spectrophotometrically by using p-nitrophenylphosphate as a substrate in 2-amino-2-methyl-1-

propanol alkaline buffer solution (1.5 M, pH 10.3, at 37 °C) and reading the absorbance in a mircoplate reader (Dynex MRX II, USA). DNA content was measured by using the PicoGreen fluorescence reagent according to the manufacturer's instructions (Molecular Probes; Green et al. 2004). Sample fluorescence was measured in a micro-plate reader (Thermo Scientific Fluoroskan Ascent, USA; excitation ~480 nm, emission ~520 nm; Yang et al. 2006). ALP-specific activity was then expressed as nanomoles of p-nitrophenol/min per nanogram DNA (Saha et al. 2010).

In vivo studies

Male MF-1 nu/nu immunodeficient mice were used for all in vivo studies; they were purchased from Harlan (Loughborough, UK). ADSCs were trypsinized 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 induced by 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 killed and the diffusion chambers were retrieved and fixed in 10 % NBF for histological analysis.

Histology and immunohistochemistry

After fixation, cell-scaffold constructs were processed for embedding in paraffin wax and sections (5 μm thick) were prepared by 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× 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). Sections were incubated overnight with the primary antibodies at 4 °C (COL-I) or for 1 h (OCN) at room temperature. Primary antibodies were detected by using the Dako REAL EnVision second antibody detection system (Dako, Carpinteria, Calif., USA). All sections were counterstained with Harris's haematoxylin. PBS (1×), instead of primary antibodies, was used as a negative control.

Statistical analysis of data

Values are expressed as means ± 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 a t-test for independent samples with SPSS v16.0 software. P-values of less than 0.05 were judged to be statistically significant.
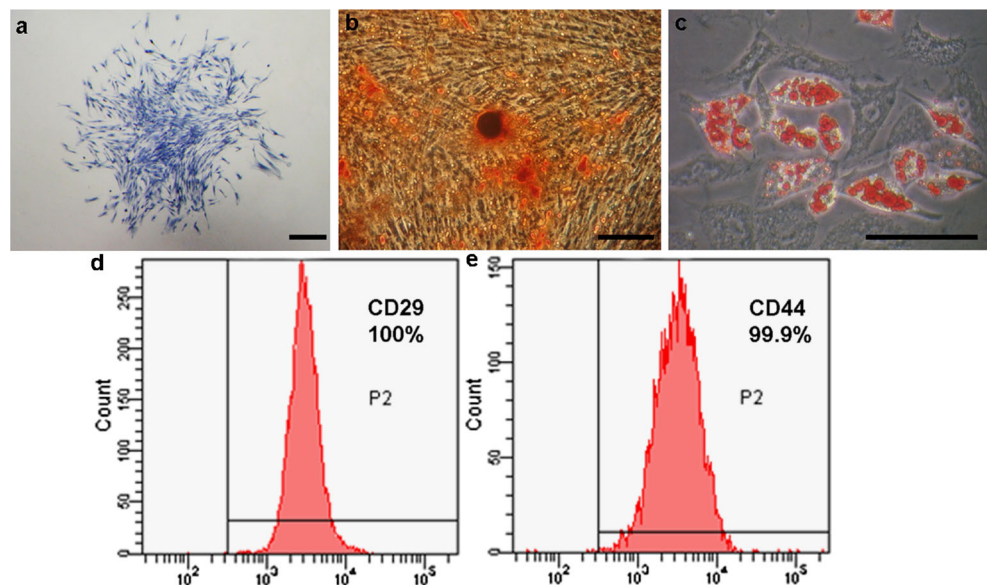
Results

Characterization of ADSCs

Human ADSCs were identified based on the following: (1) their ability to form adherent clonogenic cell clusters of fibroblast-like cells was examined (Fig. 1a); (2) their ability 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) their ability to undergo 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 positively for MSC surface marker CD29 and 99.9 % for MSC cell surface marker CD44 (Fig. 1d, e).

PDLLA/Bioglass composite scaffolds support cell attachment and growth

ADSCs were observed to attach rapidly to each of the scaffold surfaces after 5 h of cell seeding (Fig. 2a-c). ADSCs adhered and spread well on the scaffolds after 24 h, as seen by their 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.

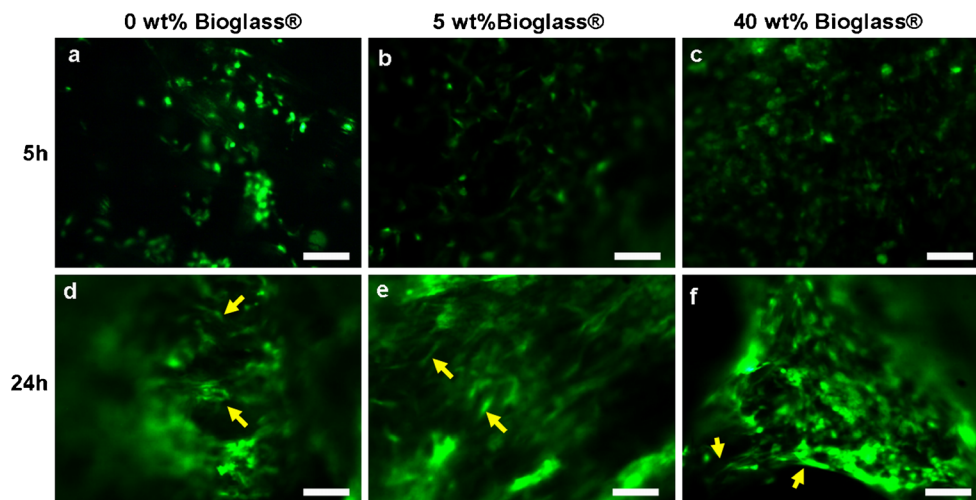### Analysis of PDLLA/Bioglass scaffolds with or without ADSCs by SEM

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. The resulting scaffolds were viewed by using SEM (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 being apparent.

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

After 4 weeks of culture in vitro, ALP-specific activity of ADSCs in the 40 wt% Bioglass constructs was significantly higher compared with that of both the 5 wt% Bioglass group and the PDLLA only (control) group ($P<0.01$). In addition, ALP-specific activity for ADSCs in the 5 wt% Bioglass constructs was also significantly increased compared with that of 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 had taken place and most of the cells were viable in all of the cell-scaffold constructs (Fig. 5a–c). Furthermore, only a few dead cells were seen in any of the groups. However, no qualitative differences were apparent between the constructs with 0, 5 and 40 wt% Bioglass.

Fig. 1 Characterization of human adipose-derived stem cells (ADSCs). **a** ADSCs were capable of forming a single-colony cluster when plated at a low cell density. **b** Mineralized nodule formation by ADSCs after 4 weeks of culture under osteogenic inductive conditions; confirmed by Alizarin red staining. **c** ADSCs formed Oil-red-O–positive lipid clusters after 4 weeks of adipogenic induction. *Bars* 50 μm. **d**, **e** Flow cytometric analysis of *ex-vivo*–expanded ADSCs revealed expression of CD29 (100 %) and CD44 (99.9 %)

Fig. 2 ADSC attachment and spreading on three-dimensional (3D) PDLLA/Bioglass scaffolds. Representative images of viable human ADSC (labelled with 5-chloromethylfluorescein diacetate) attachment (**a–c**: 5 h after seeding) and spreading (**d–f**: 24 h after seeding) on PDLLA/Bioglass scaffolds. ADSCs adhered and spread well (*yellow arrows*) on the scaffolds after 24 h compared with those at 5 h. One scaffold contained PDLLA foam only (*0 wt% Bioglass*®). The other two were composed of PDLLA/Bioglass composite at two different concentrations (by weight) of Bioglass (*5 wt% Bioglass*®, *40 wt% Bioglass*®). *Bars* 50 μm

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

After 8 weeks of culture in vitro, Alcian blue/Sirius red staining revealed an extensive collagenous matrix in the 5 wt% and 40 wt% Bioglass constructs; this was especially pronounced in the 40 wt% Bioglass scaffold constructs. In addition, material that 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), although 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 scaffolds incorporating 5 wt% and 40 wt% Bioglass compared with the PDLLA control group. Ingrowth of ADSCs into 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 fibres in the 5 wt% and 40 wt% Bioglass constructs but little evidence for this was seen in the PDLLA control group (Fig. 6d–f). These results were confirmed by using

Fig. 3 Images of various PDLLA/Bioglass composite foams with/without ADSCs by scanning electron microscopy. Scanning electron 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. *Bars* 50 μm
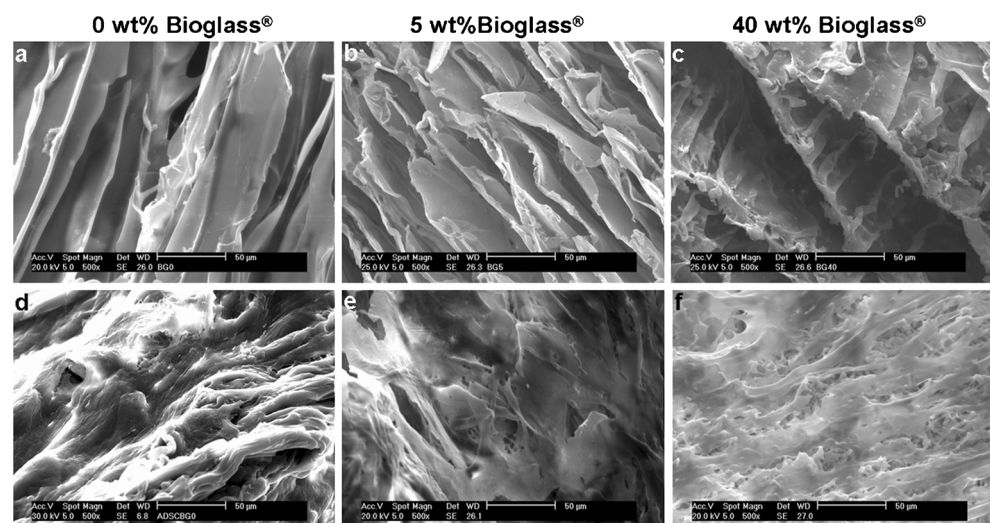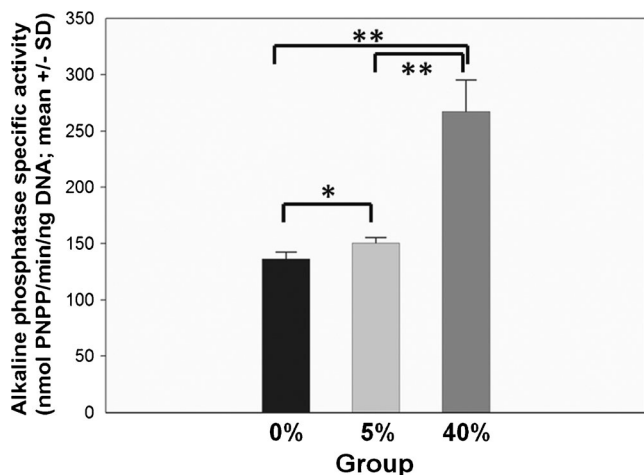
Fig. 4 Alkaline-phosphatase-specific activity of ADSCs on scaffolds with different Bioglass contents (*0%, 5%, 40%*). Data obtained after 4 weeks of culture of ADSCs on the various scaffolds in basal media. *$P < 0.05$, **$P < 0.01$

immunohistochemistry (Fig. 6g–i), which demonstrated that type I collagen staining was greatest in the 40 wt% Bioglass constructs. Furthermore, the expression of the bone-associated protein OCN was apparently extremely high in the 5 wt% followed by 40 wt% Bioglass composites in comparison with the PDLLA controls (Fig. 6j–l).

Discussion

Composite scaffolds composed of PDLLA with Bioglass particle additions are of current interest for bone tissue engineering (Blaker et al. 2003, 2005; Maquet et al. 2003, 2004;

Roether et al. 2002; Yang et al. 2006). Adipose tissue is an abundant source of MSCs, 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 ADSC, supporting their use in future clinical applications. In this study, we investigated ADSC 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 has also been characterized in an immunocompromised animal model. This is the first study addressing specifically the effect of 45S5 Bioglass additions to a polymer composite on the behaviour of ADSCs and their capacity for bone formation.

A number of studies have showed that human ADSCs are an attractive source of cells for bone tissue engineering because of their ease of harvest, the low morbidity associated with liposuction and their rapid expansion in vitro (Schaffler and Buchler 2007; Tobitan et al. 2011). The ADSCs used in this study were purchased commercially and showed typical MSC characteristics, as confirmed by 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, the adhesion and spreading of human ADSCs on each of the

Fig. 5 Cell viability and histological appearance of ADSCs on various 3D scaffolds. Confocal microscopy images (**a**–**c**) reveal that most ADSCs were viable (*green*) with only a few necrotic cells (*red*, *yellow arrows*) on the various scaffolds after 6 weeks in vitro culture. Alcian blue/Sirius red staining (**d**–**f**) shows collagen matrix (*red*) 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
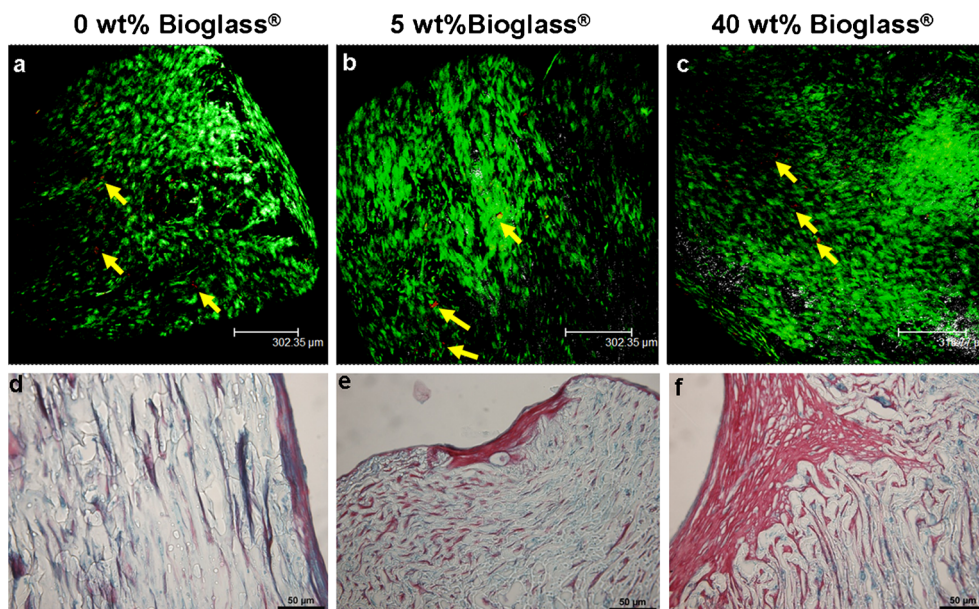
Fig. 6 Histological and immunohistochemical examinations of human ADSCs on PDLLA/Bioglass scaffolds after 8 weeks in vivo implantation. **a–c** Alcian blue/Sirius red staining shows collagen matrix (*red*) and tissue formation by ADSCs on/within the scaffold in vivo. **d–f** Birefringence images show high organized type I collagen fibres (*yellow arrows*) within the extracellular matrix. **g–i** Type I collagen immunohistochemical staining (*brown*). **j–l** Osteocalcin immunohistochemical staining (*brown*). *Bars* 100 μm
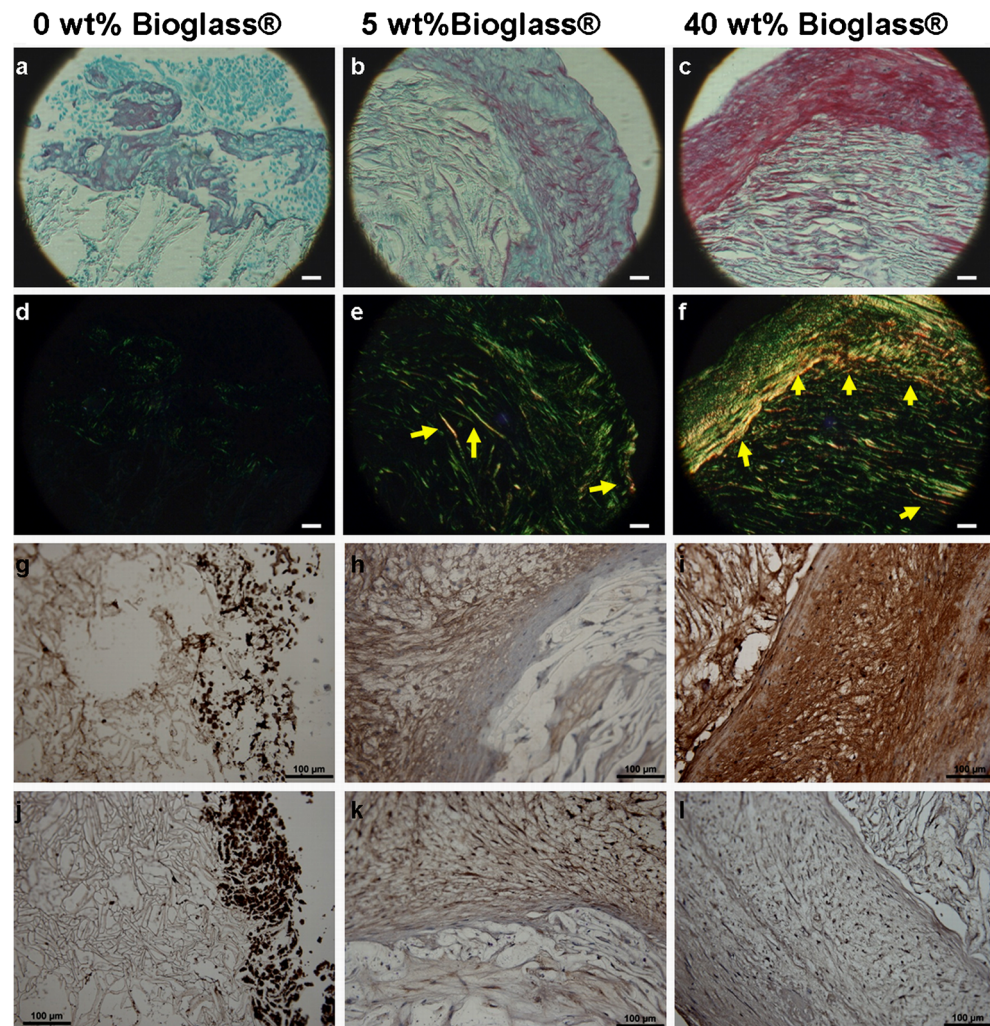
materials has been observed by fluorescent and confocal microscopy and SEM. The results reveal that human ADSCs can attach and spread on each of the materials used (Fig. 2a–f). In addition, examination by SEM has also indicated that ADSCs anchor tightly onto the surface of all three types of scaffolds and produce large amounts of extracellular matrix after 3 weeks of culture in vitro (Fig. 3d–f). This indicates that all three scaffold types successfully facilitate cell attachment and spreading without the need for the modification of surface chemistry, as we have previously demonstrated for human bone marrow stromal cells (Yang et al. 2006). The live/dead assays indicate that none of the test materials is toxic to the cells and that all of the scaffolds are suitable for long-term culture with ADSCs in vitro. No significant difference was seen for the cell attachment and spreading on the various scaffolds indicating that the added Bioglass does not effect cell attachment. However, the 40 wt% Bioglass constructs appear to significantly enhance human ADSC ALP 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

is 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 in the presence of an increase in 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 the host cells from various tissues would affect the development of the graft. In vivo analysis of PDLLA/Bioglass composites seeded with ADSCs has revealed extensive collagen matrix formation in the 40 wt% and 5 wt% Bioglass constructs, indicative of new bone matrix formation. This has also been confirmed by birefringence and immunohistochemistry for type I collagen and OCN. 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 support ADSC osteogenesis. These data provide direct evidence that the incorporation of 45S5 Bioglass into the PDLLA matrix significantly enhances ADSC osteogenic differentiation and bone-associated extracellular matrix deposition.

To date, no standard method is available to assess the various combinations of stem cells and biomaterial scaffolds for the efficacy of tissue regeneration. Neuss et al. (2008) assessed seven different stem cells and 19 different polymers for systematic screening assays in order to analyse parameters such as morphology, vitality, cytotoxicity, apoptosis and proliferation; however, a conclusion as to which combination was better for which specific tissue regeneration was difficult to make. In this study, we selected a well-developed biodegradable bioactive porous PDLLA/Bioglass composite scaffold and a high-potential stem cell source (human ADSCs) to test their capacity for bone tissue engineering, both in vitro and in vivo, in order to provide evidence to guide the clinical translation of this technology. Furthermore, a number of studies have shown that ADSCs are immunoprivileged cells that may therefore be 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), indicating the potential of using allogenic ADSCs and PDLLA/Bioglass composites to heal human bone defects in a clinical setting. Nevertheless, more work needs to be performed in this area to develop useful, safe and powerful applications of ADSCs in the biomedical field (Rada et al. 2009).

In conclusion, our study has shown that PDLLA/Bioglass composite scaffolds can provide an appropriate surface chemistry, structure and microenvironment to support ADSC growth and osteogenic differentiation for bone matrix formation. These results indicate the potential of using a combination of biodegradable bioactive porous PDLLA/Bioglass composite scaffolds with human ADSCs for bone regeneration in clinical bone augmentation.

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