In vitro analysis of biodegradable polymer blend/hydroxyapatite composites for bone tissue engineering

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Abstract: Blends of biodegradable polymers, poly(caprolactone) and poly(D,L-lactic-*co*-glycolic acid), have been examined as scaffolds for applications in bone tissue engineering. Hydroxyapatite granules have been incorporated into the blends and porous discs were prepared. Mechanical properties and degradation rates *in vitro* of the composites were determined. The discs were seeded with rabbit bone marrow or cultured bone marrow stromal cells and incubated under physiological conditions. Polymer/ceramic scaffolds supported cell growth throughout the scaffold for 8 weeks. Scanning and transmission electron microscopy, and histological analyses were used to characterize the seeded composites. This study suggests the feasibility of using novel polymer/ceramic composites as scaffold in bone tissue engineering applications. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 47, 324–335, 1999.

Key words: biodegradable; hydroxyapatite; poly(caprolactone); PLGA; tissue engineering

INTRODUCTION

Bone substitutes are often required to replace damaged tissue due to disease, trauma, or surgery. Current bone substitutes do not exhibit the physiological or mechanical characteristics of true bone. While autogenic and allogenic grafts can be used successfully under certain conditions, these grafts suffer from problems associated with additional harvesting costs, donor site morbidity, and graft availability. Metal implants cannot perform as well as healthy bone or remodel with time. To help address the need for better bone substitutes, tissue engineers seek to create synthetic, three-dimensional scaffolds made from porous bioceramic and/or polymeric materials to induce the growth of normal bone tissue. One example of a bioceramic used as a bone substitute is hydroxyapatite (HA). Although HA is osteogenic, it is brittle and difficult to process into complex shapes.[1] Recently, copolymers of poly(lactic acid) and poly(glycolic acid), (PLGA), have been studied as scaffold materials for applications in bone tissue engineering.[2–4] Although the copolymers are biodegradable, they typically do not demonstrate the mechanical properties of trabecular bone. PLGA has been blended with HA in an attempt to improve mechanical properties as well as increase the osteoconductive nature of the composite.[5] Other polymer systems that are being investigated for bone substitutes include biodegradable polyarylates[6] and poly(propylene fumarates).[7]

Although many new polymer systems are being developed, it remains a challenge to seed relatively thick scaffolds with cells (>1 mm) and maintain cell viability for prolonged periods. Ishaug et al.[4] report mineralization of seeded osteoblasts up to 240 μm deep into 1.9-mm thick PLGA scaffolds. Others have reported cell penetration limitations of 100–300 μm into scaffolds *in vitro*.[8,9] We have prepared synthetic, polymer/ceramic discs (diameter and thickness, 12 mm and 1 mm, respectively), to be used as temporary supports for bone growth that can be fully seeded with cells. These porous scaffolds consist of blends of PLGA, poly(caprolactone) (PCL) and HA. The scaffolds were

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seeded with cultured rabbit bone marrow stromal cells or fresh bone marrow and examined *in vitro* for 8 weeks. Two-dimensional *in vitro* studies with cultured bone marrow stromal cells conducted in our laboratory demonstrated that PCL is a comparable substrate to PLGA for supporting cell growth.[10] The degradation rates of the composites can be optimized by adjusting the composition and molecular weights of the polymers such that the degradation rate is complementary to the new bone formation rate. The composite has the potential to obtain mechanical strengths that are comparable to trabecular bone. Furthermore, the porous material is easily cut, molded, and shaped.

The present article describes the preparation and *in vitro* analysis of polymer/bioceramic composite scaffolds with potential applications in bone tissue engineering.

MATERIALS AND METHODS

Materials

Poly(caprolactone) (*M*w 65 kDa)(Aldrich, Milwaukee, WI), poly(D,L-lactic acid-*co*-glycolic acid), [*M*w 40kDa-65kDa, (65:35)] (Aldrich), HA [$Ca_{10}(PO_4)_6(OH)_2$,] (Aldrich), and $CHCl_3$ (Fisher, Pittsburgh, PA) were all used as received. Phosphate-buffered saline (PBS) tablets were purchased from Sigma, St. Louis, MO. NaCl (Aldrich) was sieved into particles 150–250 μm in diameter using American Society for Testing and Materials (ASTM)-standard brass sieves (Fisher).

Molecular weight determination

Gel permeation chromatography was performed using a Styrogel column equipped with a Waters 510 programmable pump and a Waters 410 differential refractometer (Waters, Milford, MA). Molecular weights are relative to monodisperse polystyrene standards (Waters). The solvent used was tetrahydrofuran (THF).

Preparation of polymer scaffolds

Polymer scaffolds were prepared using a particulate-leaching technique as described by Mikos et al.[11] The polymers were dissolved in chloroform at room temperature (7–10% w/v). Sieved NaCl (150–250 μm particle size), and HA (~10 μm particle size), were suspended in the solution and sonicated for 60 s. After evaporation of the solvent, the scaffold was weighed and immersed in distilled water. After 24 h at room temperature, the scaffold was removed from the water and dried. The weight of the scaffold was recorded. Polymer scaffolds (3–6-mm thick) that had been prepared using the solvent-casting technique, before leaching the NaCl, were cut into discs 12 mm in diameter. The discs were pressed at a pressure of 6000–10,000 psi using a Carver (Wabash, IN) hydraulic press, model 100. The applied pressure controlled the thickness of the discs. The 1-mm thick discs were immersed in distilled water to dissolve the NaCl. Homopolymer discs as well as blends of 10/90 and 40/60 were prepared (10% PCL and 90% PLGA; 40% PCL and 60% PLGA, w/w, respectively). The incorporation of HA was studied using 0–50% of HA (w/w). Porosity was 80% (as controlled by the amount of NaCl incorporated).

In vitro degradation studies

Weight loss during storage at 37°C in phosphate-buffered saline (pH 7.4) was determined for the scaffolds. The porous scaffolds were 1-mm thick with a diameter of 7 mm. The buffer solution was changed every 2 weeks as adapted from studies described previously by others.[6,12] The scaffold was removed, rinsed with distilled water, and air dried at room temperature for 24 h for measurement of weight loss at 1, 2, 3, 4, 6, and 8 weeks. The results reported are an average of three measurements.

Mechanical testing

Samples were cut into strips of 12-mm length, 1-mm thickness, and 6-mm width. Tensile strength and Young's modulus were determined with an Instron, Model 5500-R, using Merlin Software at a crosshead speed of 2 mm/min. The load cell was an Instron (Canton, MA) static load cell (100 N). Results reported are an average of five measurements.

Cell isolation and culture

Bone marrow stromal cells were isolated from the femurs of New Zealand White rabbits. All animals were anesthetized with an intramuscular injection (0.59 mL/kg) of a solution of 91% ketamine hydrochloride (Ketaject, 100 mg/mL, Aveco, Fort Dodge, IA) and 9% xylazine (Xylaject, 20 mg/mL, Mobay Corp., Shawnee, KS). The rabbit was positioned in the supine position and the lower abdominal wall, inguinal region, and lateral surfaces of both thighs and legs were shaved, depilated, and prepared for aseptic surgery. A 4-cm-long skin incision was made on the anterior aspect of the patella, and the quadriceps femoris muscle was displaced laterally. A drill and cutting burr was used to create a small femoral and tibial defect and a Fogarty balloon catheter was used to harvest bone marrow from the medullary canal. The bone marrow plugs were harvested by inflating the balloon and withdrawing it from the canal.

The bone marrow plugs were then mixed with 4 mL of heparinized Iscove's Modified Dulbecco's Medium (IMDM) tissue culture medium (GIBCO Laboratories, Grand Island, NY) in a test tube. The marrow was disaggregated by passing it gently through an 18-gauge IV catheter and syringe to create a single cell suspension. The suspension was centrifuged (250 g, 10 min) and some of the supernatant was dis-

carded to concentrate the cell number. Two milliliters of venous blood was taken from the femoral vein through a small incision and autogenous serum was obtained by centrifugation. After adding autogenous serum (10% of total volume), the viability of the cells was >90% as checked by the tryptan blue dye exclusion method.

In vitro studies

Cells were maintained in 75-cm^2 flasks in complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO), 15% fetal calf serum (GIBCO), gentamin sulfate (25 g/mL) (Sigma), L-ascorbic acid (50 g/mL) (Sigma), sodium-glycerophosphate (10 mM) (Sigma), penicillin G (50 g/mL) (GIBCO), and dexamethasone (10 nM) (Sigma). Cells were passaged every 2–3 days. After 10–14 days, cells had grown to confluency. Discs of polymeric blends with HA were sterilized by soaking in two changes of ethanol for 30 min, then soaking in four changes of PBS solution for 2 h. The discs were immersed in 24 well plates (in individual 15-mm wells). One milliliter of the cell suspension was then pipetted onto the top of the polymer/ceramic disc directly giving a concentration of 40,000 cells/mL. One milliliter of media was added to each well. Discs were incubated at 37°C in a 5% CO_2 atmosphere. Media was changed every 2–3 days. Discs were removed after incubation with cells at 2, 4, and 8 weeks for evaluation by electron microscopy and histological staining.

Electron microscopy analysis and preparation

The specimens were post-fixed in 1% OsO_4 buffered with PBS for 1 h at room temperature. The sample was washed in three changes of distilled H_2O, and dehydrated in an ethanol series (50%, 70%, 80%, 90%, and 100%). At this point, 1/2 of the sample was processed for scanning electron microscopy (SEM) using a procedure described below. The sample for transmission electron microscopy (TEM) was infiltrated in a mixture of EPON-Araldite (EA) and 100% EtOH (1:1) overnight at room temperature. After 24 h, the infiltration solution was replaced with EA, and infiltrated for an additional 48 h. The samples were placed in flat molds, filled with resin, and the resin was polymerized at 60°C for 48 h. The EA resin blocks were cut with a DDK diamond knife, on a Reichert–Jung (NuBlock, Germany) Ultracut E ultramicrotome. Thin (100 nm) sections were picked up on 200-mesh copper grids and stained with uranyl acetate and lead citrate. The sections were viewed on a Hitachi H-7100 TEM at 50 keV (Hitachi, San José, CA). Digital images (TIFF image format) were collected with an AMT Advantage 10 image acquisition system (AMT, Rowley, MA).

Dehydrated samples for SEM were critically point dried (CPD) using a Polaron, Energy Beam Sciences (Agawam, MA) E3000 CPD apparatus. The samples were dried from CO_2 at 38°C and 1200 psi. Dried samples were mounted on aluminum specimen stubs, and coated with gold using a

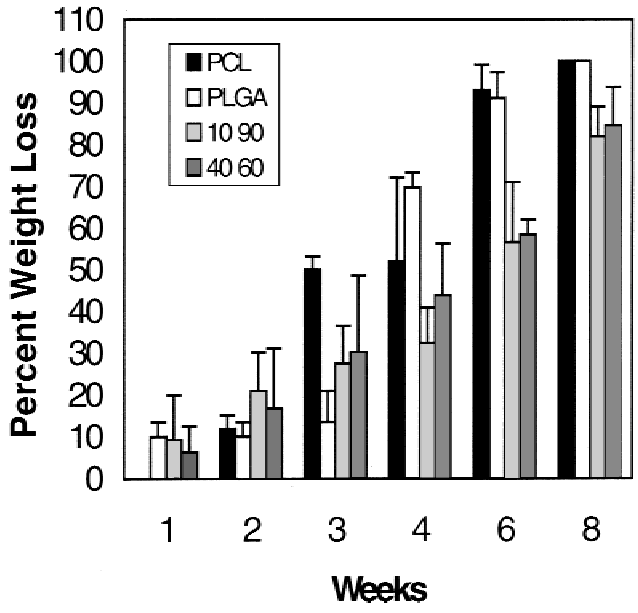

Figure 1. Weight loss of polymer scaffolds (without HA) after *in vitro* degradation (means ± SD, $n = 3$).

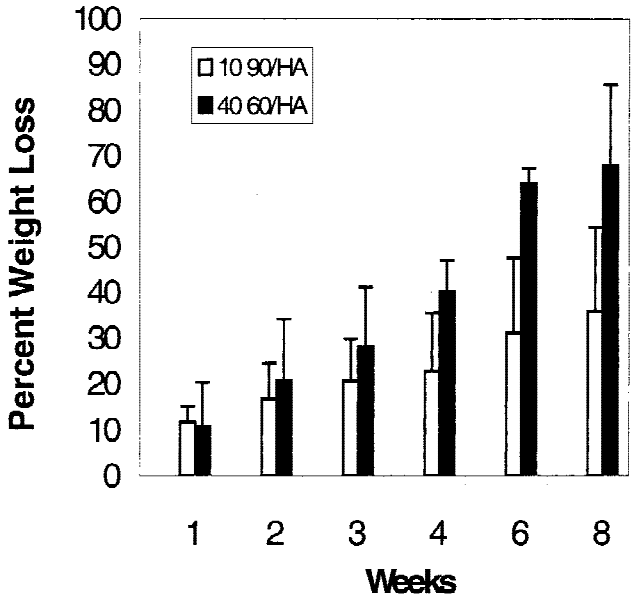

Figure 2. Weight loss of polymer blends incorporated with 10% HA (w/w) after *in vitro* degradation (means ± SD, $n = 3$). Two-tailed, unpaired t tests were conducted at each time point: 1 week, $p = 0.782$; 2 weeks, $p = 0.689$; 3 weeks, $p = 0.470$; 4 weeks, $p = 0.127$; 6 weeks, $p = 0.068$; 8 weeks, $p = 0.098$.

TABLE I
Mechanical Properties of the Porous Scaffolds (Means ± SD, $n = 5$)

	Tensile Strength (MPa)	Young's Modulus (MPa)
Trabecular bone	1.2	50–100
PCL	1.1 ± 0.1	11.8 ± 4.0
PLGA	0.45 ± 0.08	2.4 ± 0.7
10/90	0.40 ± 0.1	2.5 ± 0.7
10/90 + 10% HA	0.51 ± 0.08	12.5 ± 3.2

Pelco (Silver Springs, MD) SC-2 Sputter Coater. The samples were viewed using a Hitachi H-2460N SEM at 5 keV. Images were recorded digitally (TIFF image format) using a PC-based Quartz PCI image management system (Quartz Imaging Corp., Vancouver, Canada).

Histological preparation and analysis

Seeded discs were prepared for histology after 2, 4, and 8 weeks in culture. The samples were fixed in 2.5% glutaraldehyde. Standard dehydration in sequentially increasing alcohol solutions to 100% ethanol was performed, followed by immersion in xylene, next in paraffin-saturated xylene, and then in molten paraffin. Tissue blocks were sectioned at 5 μm and stained with Hematoxylin and Eosin (H & E) as well as Masson's Trichrome. Slides were prepared in triplicate then imaged into a PC using a video capture program (VidCap 2.0). The images were transferred into Adobe Photoshop 4.0 and then further transferred into NIH Image 1.61 for analysis. Images were used to quantify tissue ingrowth by histomorphometric techniques. The polymer/ceramic composite was distinguishable from tissue by its gray level intensity.

Statistical analysis

Histological analysis results reported are an average of 10 fields analyzed per stained slide, thus analyzing the entire cross section of the disc. Three slides for each specimen were analyzed. Unpaired, two-tailed *t* tests were performed at each time point (2, 4, and 8 weeks) to determine any significant changes in seeding with cultured bone marrow stromal cells versus fresh bone marrow. The results are expressed as means ± standard deviations.

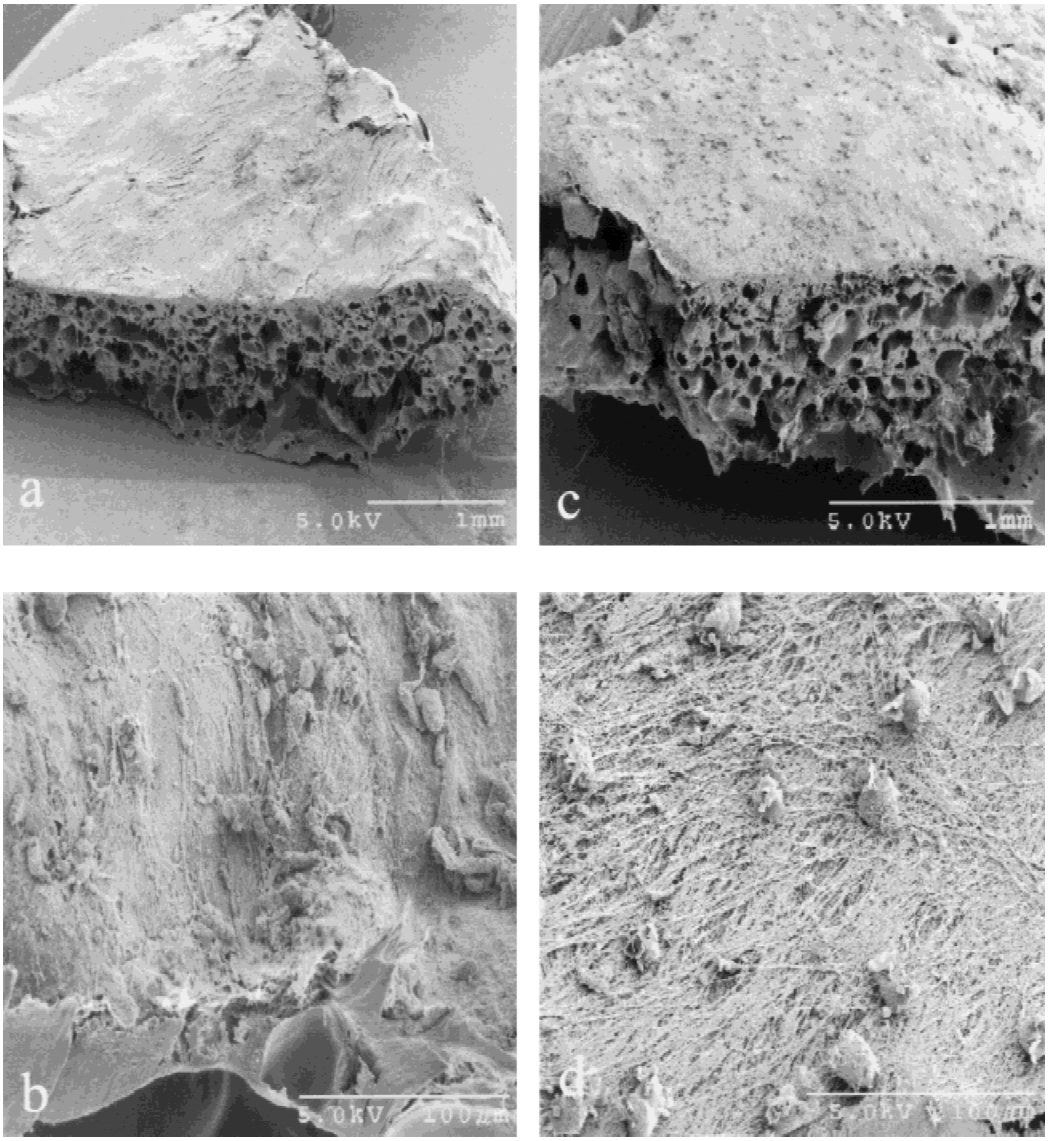

Figure 3. SEM micrographs of 10/90 blend + 10% HA seeded with: (a) cultured bone marrow stromal cells after 2 weeks of incubation (cross-section); (b) cultured bone marrow stromal cells after 2 weeks of incubation (surface); (c) fresh bone marrow after 4 weeks of incubation (cross-section); and (d) fresh bone marrow after 4 weeks of incubation (surface).

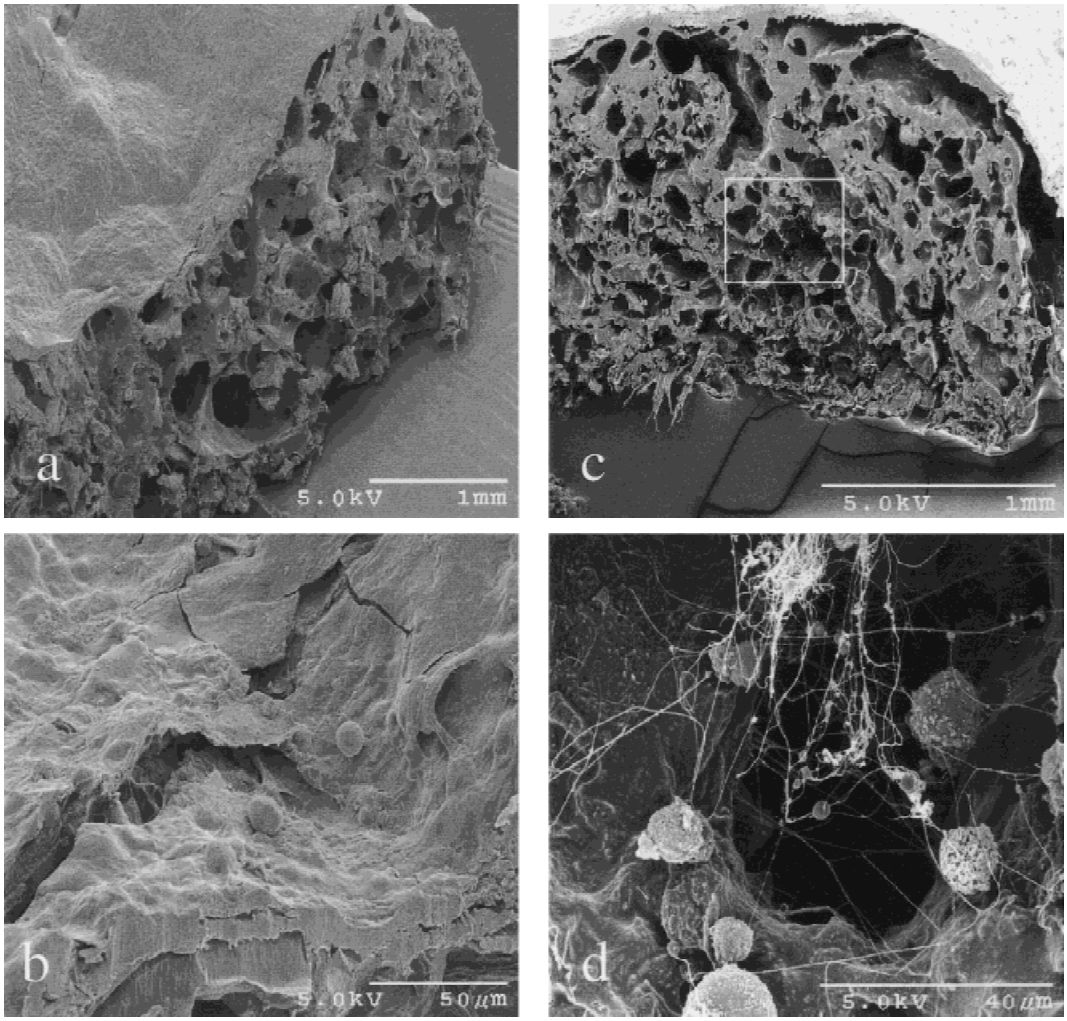

Figure 4. SEM micrographs of 10/90 blend + 10% HA at 8 weeks of incubation seeded with: (a) cultured bone marrow stromal cells (cross-section); (b) cultured bone marrow stromal cells (surface); (c) fresh bone marrow (cross-section); and (d) fresh bone marrow, higher magnification of 4(c) (cross-section).

In vitro degradation studies are an average of three samples measured. Unpaired, two-tailed *t* tests were performed on the polymer/ceramic composites at each time point. The results are expressed as means ± standard deviations.

Mechanical testing results are an average of five samples measured. Unpaired, two-tailed *t* tests were performed to compare the tensile strength and modulus differences for the 10/90 and 10/90 + HA samples. The results are expressed as means ± standard deviations.

RESULTS

Polymer/bioceramic scaffolds

Polymer/ceramic discs were prepared with controlled thickness and porosity. A solvent-casting technique described previously by Mikos et al.[11] was used. Sheets of the polymer composite were prepared and before particulate leaching, discs were cut from the sheets (thicknesses of 3–6 mm). Discs were pressed using a Carver hydraulic press to a desired thickness and diameter of 1 mm and 12 mm, respectively. The discs were immersed in water to dissolve the NaCl, and the resulting porous discs were examined. The discs were easily cut with scissors (both NaCl-containing discs and porous discs), and retained their shapes after cutting or molding. Molecular weights were determined by GPC for PLGA and PCL (PLGA: Mw = 4.4×10^4 g/mol, PDI = 1.6; PCL: Mw = 7.5×10^4 g/mol, PDI = 1.5).

Polymer degradation

The weight loss of the composites over an 8-week period was determined by gravimetric analysis. Figure 1 displays the weight loss of the homopolymers and blends during an 8-week period. The degradation rates of the blends containing 0, 10, 20, 30, and 50%

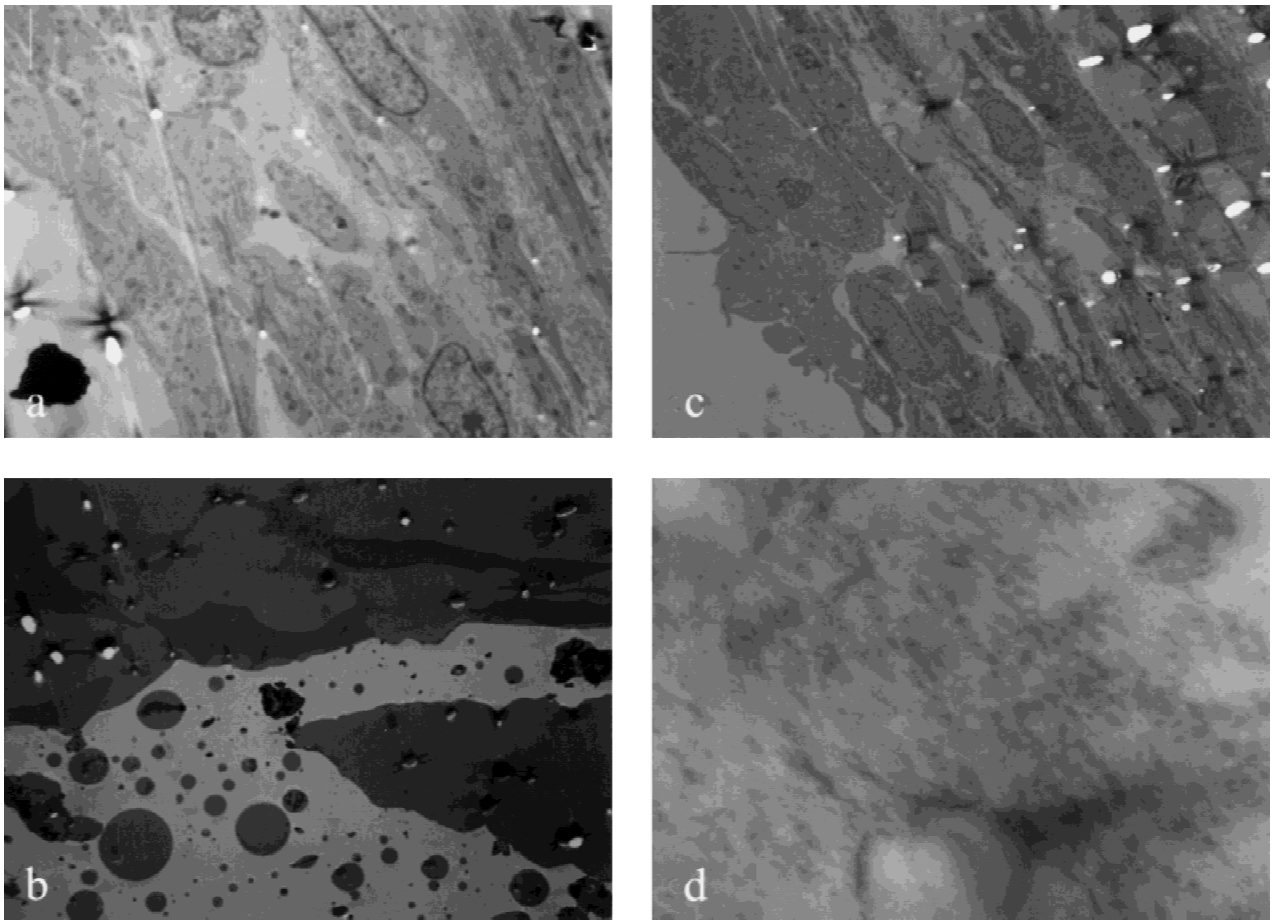

Figure 5. TEM micrographs of 10/90 blend + 10% HA after 2 weeks of incubation: (a) cultured bone marrow stromal cells in layers on the surface of the scaffold (original magnification ×3500), (b) fresh bone marrow laying down collagen (original magnification ×60,000); and after 4 weeks: (c) cultured bone marrow stromal cells producing organized collagen (original magnification ×15,000), (d) fresh bone marrow cells at surface with ribosomes and extracellular matrix (original magnification ×9000).

HA were also determined (data not shown). Blends containing 50% HA were visibly chalky. Figure 2 displays the weight loss of the blends incorporated with 10% HA. There was a trend toward significance as the degradation period increased, although there was never a statistically significant difference in degradation (1 week, $p = 0.782$; 2 weeks, $p = 0.689$; 3 weeks, $p = 0.470$; 4 weeks, $p = 0.127$; 6 weeks, $p = 0.068$; 8 weeks, $p = 0.098$). Further studies are being conducted with a larger number of samples. Based on the trend toward significance, the 10/90 blend with 10% HA was used for the *in vitro* experiments.

Mechanical properties

Strips were cut from sheets of the polymer composites (PCL, PLGA, 10/90, and 10/90 + 10% HA). An Instron was used to determine tensile strength and Young's modulus (Table I). The 10/90 + 10% HA composite demonstrated mechanical properties ~1/3 those of trabecular bone. There was improvement in tensile strength when HA was incorporated into the blend system (*t* test: $p = 0.018$); 10/90 + HA demonstrated a statistically significant increase in Young's modulus over 10/90 (*t* test: $p = 0.001$). Further experiments are being conducted to examine mechanical properties of the composites under compression. The results of the tests under compression would also constitute an appropriate assessment of the scaffold for load-bearing applications.

Electron microscopy

Seeded and unseeded discs were cultured for 8 weeks under physiological conditions. After 2, 4, and 8 weeks, the discs were removed and fixed in 2.5% glutaraldehyde. SEM and TEM were used to examine cellular activity on the surface as well as deep within the scaffolds.

SEM revealed cell growth on the surface of the seeded discs at each time point regardless of the cell source. Figure 3 displays SEM micrographs of discs at 2 and 4 weeks seeded with cultured bone marrow

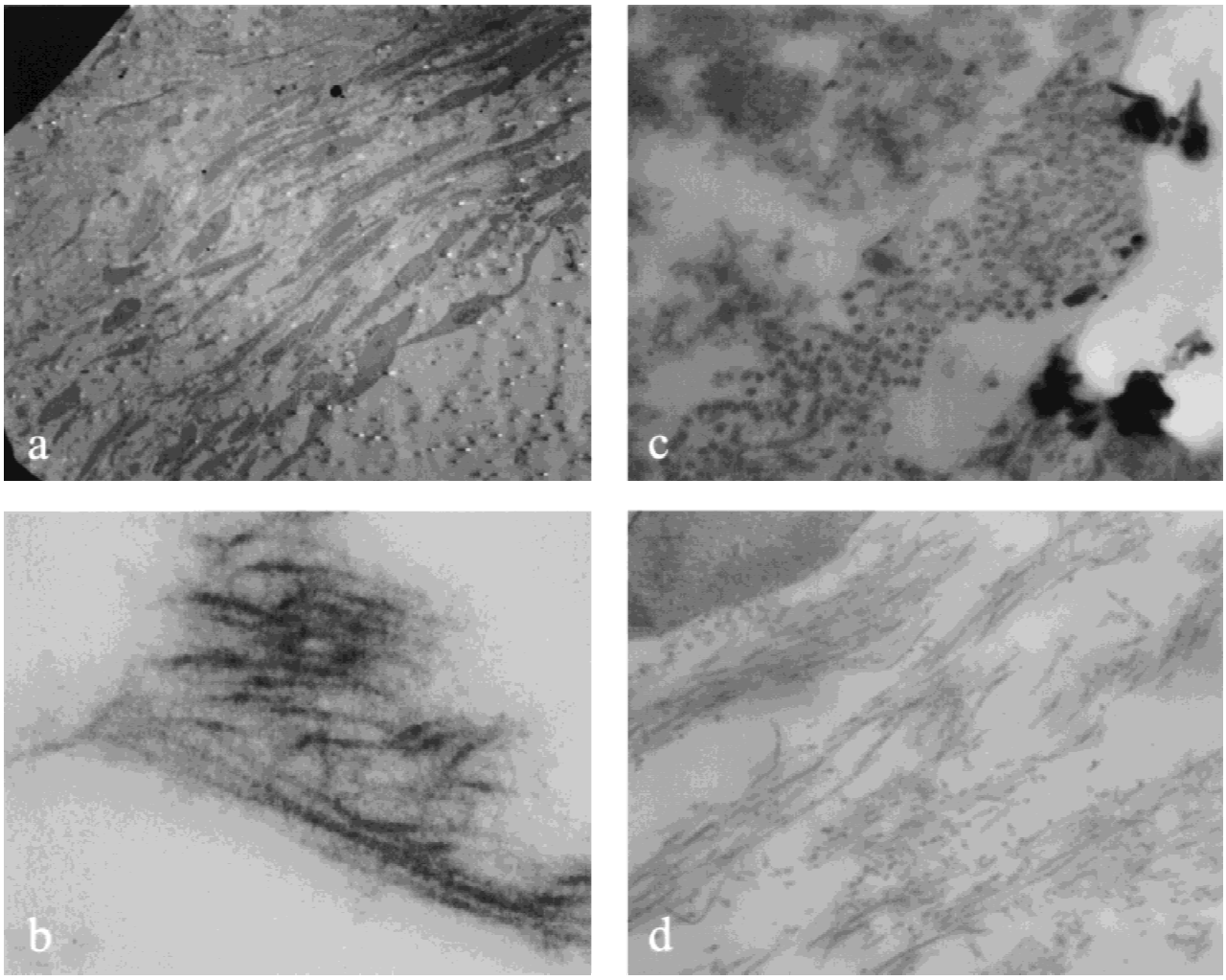

Figure 6. TEM micrographs of cultured bone marrow stromal cells on 10/90 blend + 10% HA after 8 weeks of incubation: (a) layers of cells on surface (original magnification ×1000); (b) banded collagen on surface (original magnification ×100,000); (c) cells producing collagen near polymer matrix 200 μm deep into scaffold (original magnification ×70,000); and (d) organized collagen at surface (original magnification ×10,000).

stromal cells [Fig. 3(a,b)] and fresh bone marrow [Fig. 3(c,d)]. Unseeded discs did not display any cellular activity (not shown). Figure 4 displays SEM micrographs of the samples after 8 weeks in culture. Both the cultured bone marrow stromal cell samples and fresh bone marrow samples sustain thick layers of cells on the surface as well as cellular activity within the scaffolds.

TEM was used to further characterize the cellular activity within the scaffolds. Figure 5 displays TEM micrographs of the scaffolds after 2 weeks in culture [Fig. 5(a) and (b), cultured and fresh bone marrow, respectively] and after 4 weeks in culture (Fig 5(c) and (d), cultured and fresh bone marrow, respectively). Figure 5 displays images that are abound with vital cells. Dying cells were rarely observed (seen as cells beginning to lose their shape and break down). The beginning of collagen formation is also seen. Figures 6 and 7 display TEM micrographs of the cultured bone marrow stromal cell samples (Fig. 6) and fresh bone marrow seeded samples (Fig. 7) after 8 weeks in culture. Thick, confluent layers of cells are seen on the surface. Analysis at 0.1 mm–0.5 mm deep into the scaffold-displayed cellular activity, including live cells and collagen formation, regardless of cell source.

Histology

Slides of vertical cross-sections of the discs were prepared and stained with Hematoxylin and Eosin (H & E) and Masson's Trichrome. Figure 8 displays representative slides of the 2-, 4-, and 8-week samples [cultured cells—Fig. 8(a–c)—versus fresh bone marrow—Fig. 8(d–f)] after H & E staining. Figure 9 displays fresh bone marrow and cultured bone marrow stromal cells on specimens after 8 weeks of incubation

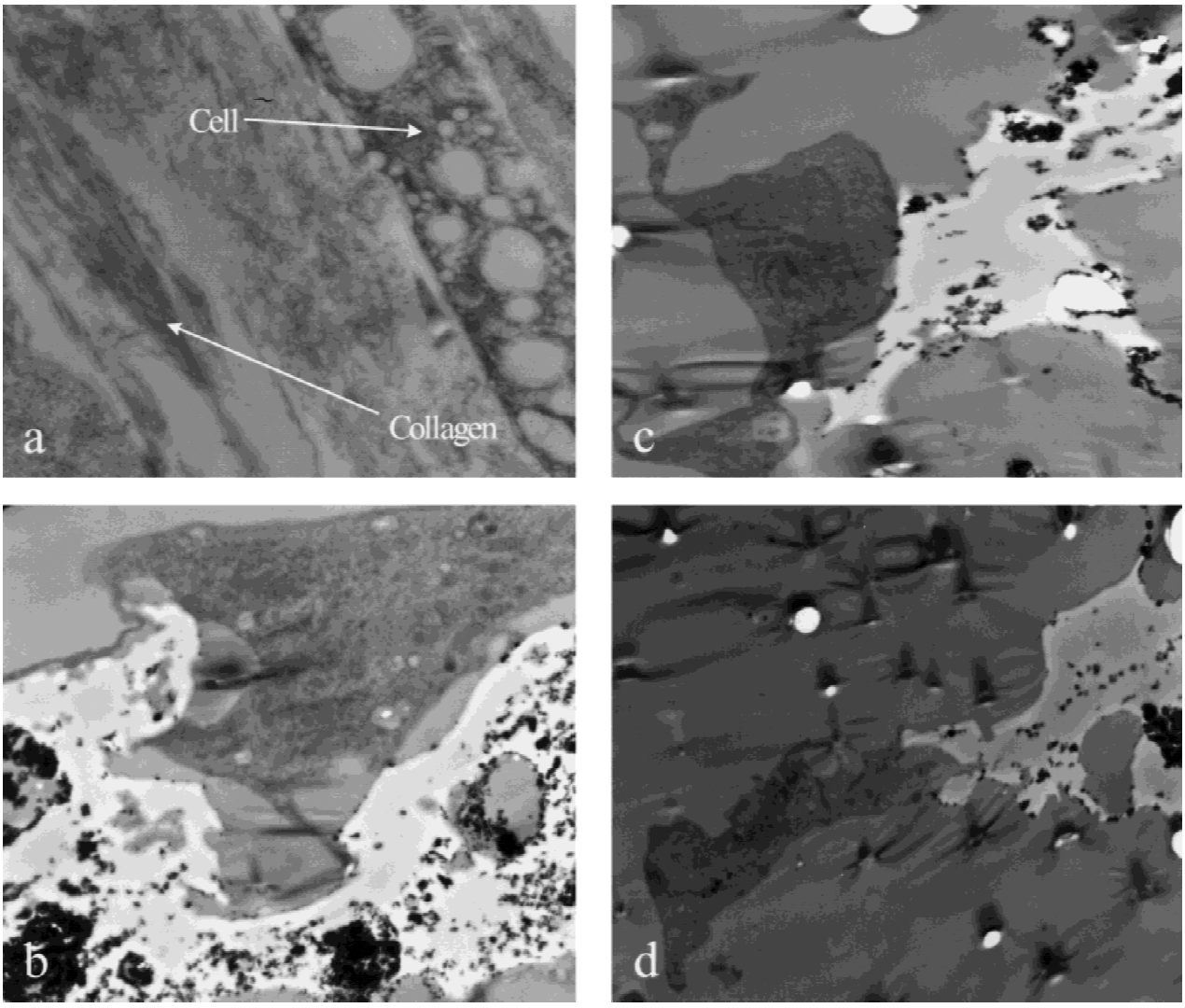

Figure 7. TEM micrographs of fresh bone marrow on 10/90 blend + 10% HA after 8 weeks of incubation: (a) cell, tissue and collagen forming on surface (original magnification ×17,000); (b) cell 0.2-mm deep into and on scaffold (original magnification ×6000); (c) cell on scaffold 0.3-mm deep (original magnification ×8000); and (d) cell 0.5-mm deep (original magnification ×5000).

stained with Masson's Trichrome. Cells are evident throughout the scaffold with many cells on the surface. The blue color seen in Figure 9 is a positive stain for collagen and is more abundant around the surface of the scaffold. Figure 10 indicates there is no significant difference in the degree of tissue ingrowth for samples seeded with fresh bone marrow compared with samples seeded with cultured bone marrow stromal cells (*t*-test results: $p = 0.823$ at 2 weeks, $p = 0.159$ at 4 weeks, and $p = 0.244$ at 8 weeks).

DISCUSSION

We have blended PCL with PLGA and HA as scaffolds for bone tissue engineering applications. We have incorporated PCL to potentially improve cell growth as well as modify degradation time and mechanical properties. PCL has been shown to be nontoxic to cells.[13] The mechanical properties (tensile strength and Young's modulus) are ~1/3 those of trabecular bone (Table I). There was a statistically significant increase in Young's modulus when HA was incorporated into the blend. The tensile strength values did not improve significantly with the addition of HA, and the 10/90 blend without HA shadows the mechanical properties of pure PLGA. These results indicate that the interfacial characteristics of the polymer and HA will be important in improving the mechanical properties, particularly the mechanical strengths. The blend system will allow us to modify the mechanical properties by adjusting molecular weights and composition. Studies to improve the interfacial bonding between the HA and the polymers are currently in progress.

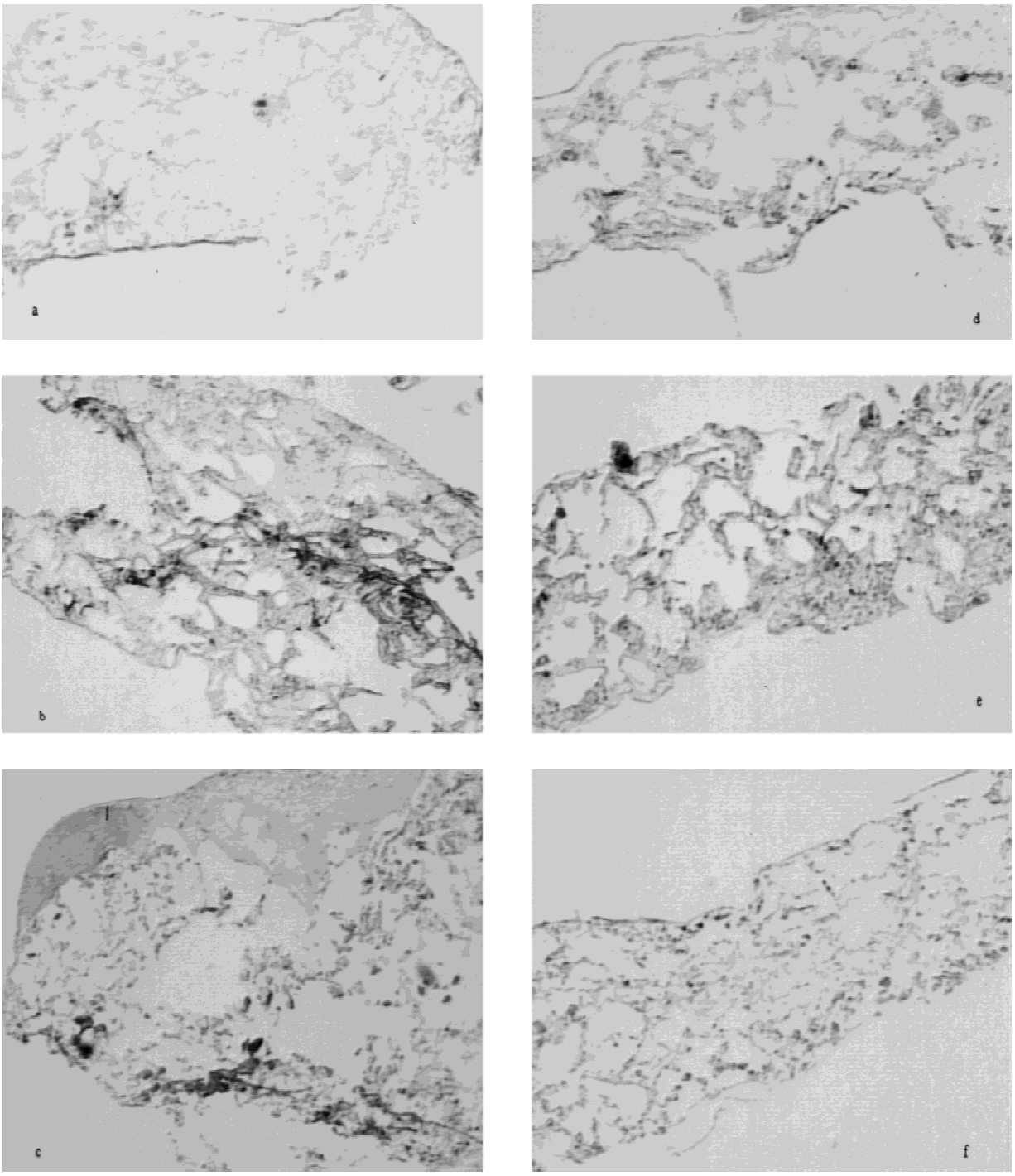

Figure 8. H & E stained cross-sections of cultured bone marrow stromal cells (a–c) and fresh bone marrow (d–f) seeded samples: (a) 2 week-, (b) 4 week-, and (c) 8-week cultured cells; and (d) 2-week, (e) 4-week, and (f) 8-week fresh bone marrow.

We have seeded the composites with fresh bone marrow as an indicator of the potential of the material to be used as a clinical bone graft. We also examined cultured bone marrow cells as a cell source in an attempt to isolate the osteoblast-like cells from the bone marrow in a preliminary study to determine the osteogenic potential of the material. Many research groups are focusing on polymer/ceramic composites for bone substitutes. Some are combining polymers and ceramics to improve mechanical strength, whereas others are adding ceramics to their polymeric materials to increase the osteoconductive nature of the scaffold. Attawia et al.[5] have prepared porous polymer/ceramic scaffolds from PLGA (50:50) and HA in equal amounts. Osteoblast cells from rat calvaria were cultured in the scaffolds *in vitro.* These results are

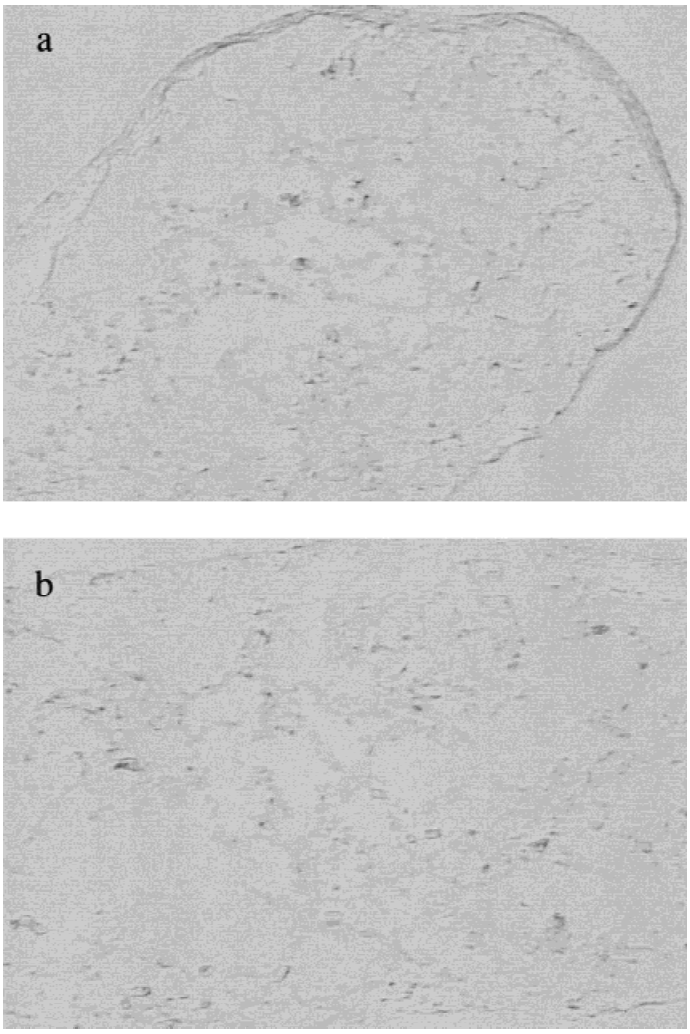

Figure 9. Masson's Trichrome stained cross-section of scaffold with (a) cultured bone marrow stromal cells and (b) fresh bone marrow after 8 weeks.

comparable to those of Ishaug-Riley et al.[2–4]; they seeded PLGA (75:25) with cultured osteoblast-like cells from rat calvaria both *in vitro* and *in vivo.* To increase the mechanical strengths of polymer/ceramic composites, Higashi et al.[14] combined PLA and HA in the form of pellets. The pellets were implanted into a rat femur for 8 weeks. The composite was found to be completely resorbed (PLA: molecular weight = 45 kDa). Higashi et al.[14] also found that the solubility of HA is increased because of the acidity of the degradation products of the polymer and concluded that the calcium and phosphate ions released by the dissolved HA were helpful in new bone formation. Shikinami et al.[15] prepared PLA/HA composites using a novel compression molding technique (PLA: molecular weight = 250 kDa) and obtained high mechanical strengths.

Different polymer systems currently being studied include PHB/PHV copolymers [poly(hydroxybutyrate-*co*-valerate)] as well as polyarylates and poly(propylene fumarates). Rivard et al.[16] prepared porous foams from PHB/PHV (91:9) and incubated the foams with osteoblasts from rat periosteum. After 5 weeks, however, cell proliferation was greatest on the collagen sponge used as a control. Ertel and Kohn[6] have rigorously examined polyarylates for use as bone substitutes, and Yaszemski et al.[7] have recently studied poly(propylene fumarate) as an *in situ* polymerized graft.

The combination of PCL, PLGA, and HA results in a unique, shapeable material as a potential scaffold for bone tissue engineering. Electron microscopy results indicate cell growth throughout the scaffold, and this is confirmed by histological analyses. Figures 3 and 4 display SEM micrographs of the seeded scaffolds after 2, 4, and 8 weeks *in vitro.* Both the cultured bone marrow stromal cell samples and fresh bone marrow samples sustain thick layers of cells on the surface as well as cellular activity within the scaffolds. TEM results demonstrate live cells throughout the scaffold producing collagen 500 μm deep into the scaffold as shown in Figures 5–7. Layers of cells are seen growing

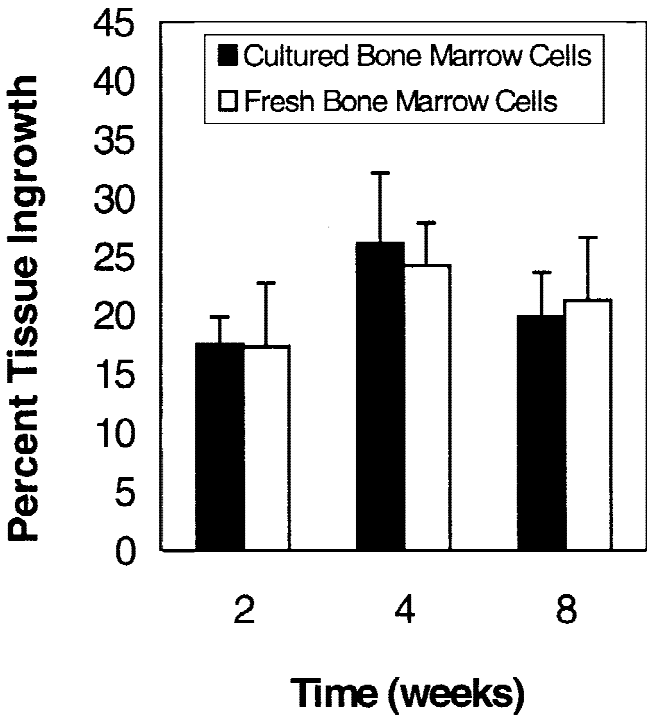

Figure 10. Histomorphometric analysis of percent tissue ingrowth of positive H & E stain after *in vitro* studies (means ± SD, $n = 30$).

on the surface of the scaffold in Figure 5(a). Figure 5(b,c) displays collagen formation on the surface of the scaffolds. Figure 5(d) also displays cellular activity on the surface after 4 weeks *in vitro.* After 8 weeks of incubation with cultured bone marrow stromal cells, collagen is seen on the surface as well as deep within the scaffold (Fig. 6). Figure 7 displays TEM micrographs of the fresh bone marrow seeded samples after 8 weeks *in vitro.* Cells are near the polymer blend (the HA granules have stained black and are seen dispersed in the polymer matrix) in the center of the scaffold. After 8 weeks of incubation, there is collagen production throughout the scaffolds regardless of cell source.

Histological analysis confirms cellular production and proliferation throughout the scaffolds. Figures 8 and 9 demonstrate that the amount of tissue ingrowth throughout the 10/90 blend + 10% HA scaffold is visibly similar with both the cultured cells and the fresh bone marrow. Thicker, confluent cell growth is observed on the surface of the scaffolds. TEM, SEM, and histological staining confirmed multilayers of cells grown to confluence on the surface of the discs after 8 weeks. The Masson's Trichrome stain demonstrates collagen production throughout the scaffolds (stained blue) with an increased amount of collagen evident on the surface. Histomorphometric analysis also indicates that seeding with fresh bone marrow is comparable to cultured bone marrow stromal cells (Fig. 10). After 8 weeks of incubation, the scaffolds contained approximately 20% tissue ingrowth. The polymer/ceramic composite was distinguished from cells and extracellular matrix (tissue) and the tissue ingrowth was quantified. Both electron microscopy and histological analyses results indicate that the simpler seeding of fresh bone marrow at the time of implantation is significantly comparable to the timely 2 week culture of the bone marrow to obtain primary bone marrow stromal cells ($p > 0.05$).

CONCLUSIONS

We have developed novel composites for use as bone substitutes. The polymer/bioceramic scaffolds are biodegradable and shapeable. After *in vitro* culture with fresh bone marrow and bone marrow stromal cells, electron microscopy and histological analyses revealed viable cells and the formation of collagen 500 μm deep into the scaffold. We are currently conducting *in vivo* experiments to explore the potential of this material to be used as synthetic, resorbable bone substitutes. Future studies involve improving the mechanical properties of the composite scaffold and binding growth factors to the composites to optimize tissue growth within the scaffolds.

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