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Suppression of apoptosis by enhanced protein adsorption on polymer/hydroxyapatite composite scaffolds

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Abstract

Bone tissue engineering is a promising alternative to bone grafting. Scaffolds play a critical role in tissue engineering. Composite scaffolds made of biodegradable polymers and bone mineral-like inorganic compounds have been reported to be advantageous over plain polymer scaffolds by our group and others. In this study, we compared cellular and molecular events during the early periods of osteoblastic cell culture on poly(l-lactic acid)/hydroxyapatite (PLLA/HAP) composite scaffolds with those on plain PLLA scaffolds, and showed that PLLA/HAP scaffolds improved cell survival over plain PLLA scaffolds. Most cells (MC3T3-E1) on PLLA/HAP scaffolds survived the early culture. In contrast, about 50% of the cells initially adhered to the plain PLLA scaffolds were detached within the first 12 h and showed characteristics of apoptotic cell death, which was confirmed by TUNEL staining and caspase-3 activation. To investigate the mechanisms, we examined the adsorption of serum protein and adhesion molecules to the scaffolds. The PLLA/HAP scaffold adsorbed more than 1.4 times of total serum protein and much greater amounts of serum fibronectin and vitronectin than pure PLLA scaffolds. Similarly, significantly larger amounts of individual adhesion proteins and peptides (fibronectin, vitronectin, RGD, and KRSR) were adsorbed on the PLLA/HAP scaffolds than on the PLLA scaffolds, which resulted in higher cell density on the PLLA/HAP scaffolds. Furthermore, $\beta 1$ and $\beta 3$ integrins and phosphorylation of Fak and Akt proteins in the cells on the PLLA/HAP scaffolds were significantly more abundant than those on PLLA scaffolds, which suggest that enhanced adsorption of serum adhesion proteins to PLLA/HAP scaffolds protect the cells from apoptosis possibly through the integrin-Fak-Akt pathway. These results demonstrate that biomimetic composite scaffolds are advantageous for bone tissue engineering.

1. INTRODUCTION

Bone is a specialized connective tissue with mineralized extracellular matrix (ECM). Bone grafting is routinely performed to restore bone loss due to trauma or disease. Although autogenous cancellous bone is considered to be the gold standard for bone grafts, there are

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several limitations, including inadequate availability, increased operative blood loss from the two-site surgery, and potential donor-site morbidity. Allogeneic grafts are also problematic due to risks in infection, allograft fracture, pathogen transmission, and immune rejection. It has, therefore, become necessary to find suitable alternatives, particularly when a large graft is required. Bone tissue engineering is a promising alternative to bone grafting. In the tissue engineering approach, the following components are generally required: cells that can differentiate into osteoblasts, an artificial extracellular matrix (i.e., scaffold), and regulating bioactive molecules that promote cell recruitment, growth, differentiation, and mineralized bone tissue formation [1–4]. These components have to be in concert with each other to achieve optimal tissue regeneration.

It is well known that hydroxyapatite (HAP) is the major component of natural bone mineral and has been found to possess good mechanical properties, biocompatibility, and osteoconductivity [5]. For these reasons, sintered HAP or other calcium phosphate apatites have been widely used for repair and replacement of damaged or traumatized bone tissues [6–8]. However, these materials have major disadvantages for tissue engineering applications: they are lack of desired degradability in a biological environment, are brittle, and their processing capacity into a pre-designed highly porous structure is limited. In contrast, polymers have a greater processing flexibility. Biodegradability can be imparted into polymers through molecular design [4]. For these reasons, polymeric materials have received considerable attention and are widely studied for bone repair and bone tissue engineering applications [4, 9,10]. However, biodegradable polymers often do not provide satisfactory osteoconductivity. Research results from our group and others consistently suggest that the strategy of using composite scaffolds of biodegradable polymers and bone mineral-like inorganic compounds is a viable approach for bone tissue engineering [11–13]. We developed highly porous composite scaffolds using biodegradable polymers and micro-and nano-sized HAP using a thermally induced phase separation (TIPS) technique [14,15]. These PLLA/HAP scaffolds showed significant improvement in mechanical properties over the plain polymer scaffolds while still maintaining a high porosity. The *in vitro* study confirmed that the scaffolds promoted cell seeding uniformity and osteoblastic differentiation [11].

Although the composite materials have been considered better choices as bone tissue engineering scaffolds, few reports discussed the underlying mechanisms. In this study, we compared cellular and molecular events during the early periods of osteoblastic cell culture on PLLA/HAP composite scaffolds with those on control PLLA scaffolds to understand the mechanisms of enhanced osteoblast seeding and differentiation on the PLLA/HAP scaffolds. We demonstrated that PLLA/HAP improved cell viability over pure PLLA polymer scaffolds. We evaluated the adsorption of total serum protein as well as adhesion proteins such as fibronectin and vitronectin in particular to these scaffolds. Furthermore, we examined integrins and integrin-mediated signaling such as phosphorylation of focal adhesion kinase (FAK) and Akt to understand the mechanisms of enhanced cell survival in relation to the adhesion proteins on the scaffolds.

2. MATERIALS AND METHODS

2.1. Preparation of the PLLA and PLLA/HAP scaffolds

The preparation has been reported in detail earlier [14]. Briefly, PLLA was dissolved in dioxane to make a 5% (w/v) solution. To prepare the composite scaffold, HAP powder (platelets ranging from 10 to 100 μm in size) was added into the prepared PLLA solution (HAP/PLLA = 1/1 in weight ratio). A solid-liquid phase separation technique and a subsequent solvent sublimation process were used to generate the highly porous PLLA and PLLA/HAP composite scaffolds. The PLLA/dioxane solution and PLLA/HAP/dioxane mixture were cooled to -18°C to induce solid-liquid phase separation. The phase-separated samples were dried under vacuum (0.5

mmHg) at between -5°C and -10°C for 7 days to remove the residual solvent. The PLLA and PLLA/HAP disks with a diameter of 8 mm and thickness of 1.5 mm were prepared.

The prepared PLLA and PLLA/HAP porous scaffolds had similar internal structures to those reported before [14]. The pores were interconnected throughout the scaffolds. The pore size ranged from tens to hundreds of micrometers. The PLLA scaffolds had a porosity of 92.7%, and the PLLA/HAP scaffolds had a porosity of 89.2% [14].

2.2. Cell culture

The MC3T3-E1 osteoblasts (clone 4) [16] were passaged in the ascorbic acid-free α -MEM (Quality Biological, Gaithersburg, MD) supplemented with 10% FBS (Gibco BRL, Grand Island, NY), 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin in a humidified incubator at 37°C with a CO_2/air ratio of 5:95. The PLLA and PLLA/HAP scaffolds were assembled on the bottoms of custom-made 12-well Teflon culture plates. The scaffold-containing culture plates were sterilized with ethylene oxide following the manufacturer's procedure (H.W. Anderson Products, Chapel Hill, NC). The sterilized PLLA and PLLA/HAP scaffolds were soaked in ethanol for 1 h and then exchanged with phosphate-buffered saline (PBS) three times (30 min each). The scaffolds were then washed with α -MEM containing 10% FBS two times (2 h each). One million cells were seeded on each scaffold. The cell-scaffold constructs were cultured in α -MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml ascorbic acid, and 5 mM β -glycerophosphate on an orbital shaker (75 rpm) [11] for up to 14 days.

2.3. DNA assay

The amounts of cells on scaffolds were determined using a DNA assay. The cell-seeded scaffolds were washed with PBS twice (5 min each), and homogenized using a Tissue Tearor for 1 min at 20000 rpm. The cell lysates were subject to procedures of DNA assay as previously described [11,17].

2.4. TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) assay

The MC3T3-E1 cell/scaffold constructs were fixed in 4% paraformaldehyde. Paraffin-embedded disk specimens were cut into 5- μm -thick cross sections. The TUNEL assay was performed following the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Roche, Germany). Numbers of TUNEL positive cells and total cells were counted in four areas ($200 \times 400 \mu\text{m}^2$) per section and three sections per group under microscope.

2.5. Protein adsorption to the PLLA and PLLA/HAP scaffolds

Protein adsorption studies were carried out as previously described with minor modifications [18]. The scaffolds were wetted with ethanol (1 h), and PBS (3 times, 30 min each), and followed by the incubation in 10% solution of FBS or human serum (Sigma, St. Louis, MO) in PBS. In a separate experiment, wetted scaffolds were incubated in 5 $\mu\text{g}/\text{ml}$ fibronectin (Sigma), vitronectin (Biosource, Carmarillo, CA), RGDS (Sigma), or KRSRGGG (a known osteoblast-adhesive peptide [19], synthesized by University of Michigan Protein and Carbohydrate Structure Facility, higher than 90% in purity) in PBS. After 4 h of incubation, the scaffolds were removed from the protein solution, tapped on the Whatman paper, and then transferred to a microtube. The scaffold was washed with 600 μL PBS under gentle agitation for 5 min. The washing solution was then discarded and 600 μL fresh PBS was replaced to wash again. A total of three washes were conducted to remove free and loosely adsorbed proteins (there was a negligible amount of proteins in the third washing solution). The remaining proteins (adsorbed) on the scaffold were recovered by incubation in 2 % sodium

dodecyl sulfate (SDS) solution for 1 h after homogenization. The procedures were repeated two more times and the three solutions were pooled in one tube. The total amount of protein was measured using MicroBCA assay (Pierce, Rockford, IL).

To examine the effect of adhesion proteins or peptides on the adhered cell number on the scaffolds, one million cells were seeded on each scaffold that was previously incubated in a protein or peptide solution, and cultured in the medium without FBS. After 24 h of culture, the DNA amounts within the scaffolds were measured.

2.6. Western blot analysis

The eluted serum protein samples (25 μ l out of 400 μ l in total) were subject to fractionation through 5% (for fibronectin) or 10 % (for vitronectin) SDS-PAGE. The fractionated proteins were transferred on to a PVDF membrane (Sigma). The blots were washed with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0), and blocked with Blotto (5% non-fat milk in TBST) at room temperature for 1 h. The blots were incubated in anti-human fibronectin and vitronectin antibodies at room temperature for 1 h. After washing with TBST, the blots were incubated in anti-goat IgG-horseradish peroxidase-conjugated antibodies (Sigma), and then in Chemiluminescence reagent (SuperSignal West Dura, Pierce). The relative densities of the protein bands were analyzed with QualityOne (Biorad).

For the detection of caspase-3, integrins β 1 and β 3, phospho-FAK, phospho-Akt and β -actin, the cell-seeded scaffolds were cultured. After culture, they were washed twice with ice-cold PBS, lysed in a buffer consisting of 10 mM Tris-Cl (pH7.5), 150 mM NaCl, 1 mM EDTA (pH8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, and complete protease inhibitor cocktail tablet (Roche). The crude extracts were sonicated briefly three times and incubated on ice for 15 min. After centrifugation, the supernatants were saved and used for analyses. Forty μ l of samples from the total of 400 μ l cell lysates was ran through 4–12 % gradient SDS-PAGE. Procedures similar to the above-described were used. Antibodies against fibronectin, vitronectin, integrins (β 1 & β 3) and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 and anti-phospho-Akt were from Cell Signaling Technology (Beverly, MA). Anti-phospho-FAK was from Biosource.

2.7. Statistical analysis

Student's T-test was performed, and the difference was regarded as significant if *p* value was lower than 0.05.

3. RESULTS

3.1. Cell numbers and apoptosis

The DNA contents of the cell-seeded scaffolds were measured to quantify the amounts of MC3T3-E1 cells on the PLLA and PLLA/HAP scaffolds. Cells that adhered on both types of scaffolds were statistically identical at 4 and 8 h after cell seeding (Fig. 1). The DNA contents were significantly different on the PLLA and PLLA/HAP scaffolds at 12 h of culture; the DNA content in the PLLA scaffolds was reduced by approximately 50% at this time point, whereas the DNA content in the PLLA/HAP scaffolds was virtually not altered. Therefore the resultant DNA content in the PLLA/HAP scaffolds was significantly greater than that in the PLLA scaffolds at 12 h and thereafter. The histological analysis of the constructs (data not shown) was consistent with the DNA results.

Next, the mode of cell death was examined considering that physicochemical properties of the scaffolds may affect the micro-environment surrounding the cells [20] and consequently lead to the onset of apoptosis. TUNEL assay revealed that more than 60% of the cells on the PLLA

scaffolds underwent apoptosis during the initial 12 h (Fig. 2A, B). In contrast, the apoptosis levels on PLLA/HAP scaffolds were significantly lower, at a level of ~20%. The apoptotic cells on PLLA scaffolds dropped to 35% after 24 h. Considering that apoptosis precedes cell detachment, the result was in line with the data on DNA contents. Apoptosis was also confirmed by activation of caspase-3, which is a common executor of apoptosis [21,22]. Consistent with TUNEL assay, cleavage of procaspase-3 (to activate caspase-3) occurred at profound levels in the cells on PLLA scaffolds at all the time points measured (Fig. 2C). Activation of caspase-3 in the cells on PLLA/HAP scaffolds was substantially lower than that on PLLA scaffolds at 4 h after cell seeding, and dramatically decreased thereafter to a non-detectable level. These results indicate that the PLLA/HAP composite scaffolds substantially rescued osteoblasts from apoptosis and had led to a higher density of osteoblasts on the scaffolds, which might in turn contribute to the subsequent osteoblastic differentiation.

3.2. Protein adsorption

Because the scaffolds were pre-treated with serum-containing medium in our culture system and would be exposed to blood in potential future clinical applications, it was examined whether serum proteins adsorbed to the scaffolds might contribute to the observed rescue of cells from apoptosis. Pre-treatment of the scaffolds with fetal bovine serum (FBS) resulted in increased DNA contents in both scaffolds, compared to pre-treatment with PBS (> 3 folds). A significant difference in DNA content between the PLLA and PLLA/HAP scaffolds was observed after the pre-treatment with a FBS-containing solution (Fig. 3A). The PLLA/HAP scaffolds adsorbed 40% more serum protein than the PLLA scaffolds, as measured using the MicroBCA assay (Fig. 3B). SDS-PAGE result corroborated with the greater amounts of adsorbed serum proteins to the PLLA/HAP scaffolds (Fig. 3C). Furthermore, the profile of adsorbed serum protein on the PLLA/HAP scaffolds was different from that on the PLLA scaffolds. Every protein band from the PLLA/HAP scaffolds had a higher intensity (1.1-to 9.6-fold) than that from the PLLA scaffolds (Table 1). Similar results were obtained for human serum protein adsorption using western blot analysis (data not shown). Fibronectin secreted by osteoblasts has been reported to support the survival of osteoblasts in the matrix [23], which prompted us to examine the adsorption of serum fibronectin as well as vitronectin. Western blot analyses revealed that serum fibronectin and vitronectin adsorbed to both types of scaffolds, and that the PLLA/HAP scaffolds adsorbed greater amounts of these proteins than the PLLA scaffolds: 74% and 157% higher, respectively (Fig. 3D).

The adsorptions of individual adhesion proteins and peptides to the PLLA and PLLA/HAP scaffolds were investigated to understand their effects on cell densities (numbers). These proteins and peptides were adsorbed 1.5 to 6.4-fold more on the PLLA/HAP scaffolds than on the PLLA scaffolds (Fig. 4A). The PLLA/HAP scaffolds adsorbed 2.0- and 2.1-fold more fibronectin and vitronectin than albumin ($p < 0.05$). The PLLA scaffolds, however, did not show statistically significant differences in adsorption among fibronectin, vitronectin, and albumin. To examine the effect of these adhesion proteins on cell density on the scaffolds, the PLLA and PLLA/HAP scaffolds were soaked in a single protein or peptide solution (5 $\mu\text{g/ml}$), and the cells were seeded on thus-treated scaffolds and cultured for 24 h in the absence of serum. Adsorption of fibronectin, vitronectin, RGDS, and KRSRGGG increased the cell survival at significantly higher levels on the PLLA/HAP scaffolds than on the PLLA scaffolds (Fig. 4B). These results suggest that the PLLA/HAP composite scaffolds provide osteoblasts with a more favorable micro-environment through enhanced adsorption of serum adhesion proteins supporting cell survival during the initial culture period.

3.4. Integrins and integrin-mediated signals

The above-presented results demonstrated that PLLA/HAP scaffolds adsorbed greater quantities of fibronectin and vitronectin (integrin-binding proteins) than plain PLLA scaffolds,

and substantially suppressed apoptotic cell death. We therefore examined the cell receptors of integrins in the cells on the scaffolds to understand the mechanisms. Fibronectin and vitronectin are known to bind integrin $\alpha 5 \beta 1$ and integrin $\alpha v \beta 3$ [21,22]. Upon binding of a ligand to an integrin, the signal relays to downstream via β subunits. Thus, the expression of integrins $\beta 1$ and $\beta 3$ was examined in the cells on the scaffolds. After 8 h of cell seeding, both β integrins were present at much higher levels in the cells on the PLLA/HAP scaffolds than on PLLA scaffolds (Fig. 5). Furthermore, the signals activated and phosphorylated Fak and Akt, which are known to rescue cells from apoptosis [24,25]. These results strongly suggest that the enhanced adsorption of serum fibronectin and vitronectin on the PLLA/HAP scaffolds protect the cells from apoptosis through the pathway of integrin-Fak-Akt.

4. DISCUSSION

Extracellular matrix (ECM) is produced and organized by cells. Cells can bind ECM components through a number of cell surface receptors. Recent studies indicate that ECM does much more to cells than simply tethering them to certain locations. Rather, it is now generally agreed that cell-ECM interactions are responsive to environmental conditions, and can profoundly affect many aspects of cell structure and function, including cytoskeletal organization, cell proliferation, and gene expression (reviewed in [20]). A scaffold, artificial ECM, can also affect the cell behavior, and should play an active regulating role as well as passive anchorage to promote tissue regeneration. The importance of the biodegradability and mechanical strength of a scaffold is widely recognized. However, cell-scaffold interactions have received less attention. Considering the specificity in the interactions between cells and ECM, it is critical to understand how chemical composition of a scaffold affects physicochemical properties such as binding characteristics of the surface, to regulate cell behavior.

Previously, we found that PLLA/HAP composite scaffolds advantageously support osteoblastic cell differentiation over plain PLLA scaffolds [11]. We also observed that the numbers of cells adhered on both scaffolds were not significantly different after 5 h of cell seeding, but there was significant difference after 1 week of culture. In this study, a more precise time frame was determined and the underlying mechanisms were investigated. It was demonstrated the first time that incorporation of bone-mineral mimicking HAP into a biodegradable polymer scaffold protects cells from apoptosis by enhancing adsorption of serum proteins. In addition to PLLA/HAP scaffolds, poly(lactic-co-glycolic acid)/HAP (PLGA/HAP) scaffolds were also found to protect osteoblasts against apoptosis (data not shown). Consistent with our findings, Shu et al. [26] recently reported that MC3T3-E1 cells cultured on sintered dense HAP disks were more differentiated than those on polystyrene plastic culture dishes as measured by osteogenic markers. However, they reported that the cell attachment and apoptosis were similar on these two different surfaces. Those results could have been complicated by the surface treatment of the polystyrene Petri dishes. Considering that the PLLA and PLGA are the most commonly used biodegradable polymers for biomedical applications, our results provide not only unrevealed new information but also important insights on scaffold design for biomaterials scientists and tissue engineers. The rescue of cells from apoptosis led to a higher cell density on the scaffolds. A higher cell density should increase cell-cell interactions (increased cell clustering). Because the establishment of connexin-mediated intercellular gap junctions has been shown to enhance osteoblast differentiation [27–29], the apoptosis-suppressing effect of HAP could have contributed to the enhanced osteoblastic differentiation on PLLA/HAP composite scaffolds in long-term cultures [11] via the increased number of gap junctions.

In bone tissue engineering, mesenchymal stem cells obtained from bone marrow, peripheral blood, and adipose tissue are frequently used. These mesenchymal stem cells are rare in number

and have limited propagation capacity. Extensive propagation decreases the differentiation capacity. The apoptosis-suppressing characteristic of the HAP-containing composite scaffolds would provide a great advantage for these adult stem cells in tissue engineering applications.

Cell surface receptors can specifically bind to their ligands on a natural extracellular matrix or a biomimetic scaffold. If signals from cell surface receptors are not adequate for cells to survive, they undergo apoptotic cell death [21,22]. It is well known that fibronectin and vitronectin can bind to integrins of cells including osteoblasts and increase cell attachment and/or cell spreading on biomaterials in vitro [30–32]. It has been reported that in comparison with solid titanium or steel implants, HAP implants adsorb more serum fibronectin and vitronectin, and bind more purified integrins [31,32]. These reports although based on solid implant materials, corroborate with our finding that the porous 3D PLLA/HAP composite scaffolds adsorbed greater amounts of serum protein than pure PLLA scaffolds. Furthermore, greater amounts of fibronectin and vitronectin were present on PLLA/HAP scaffolds, on which osteoblasts expressed much higher levels of $\beta 1$ and $\beta 3$ integrins. These integrins can bind to fibronectin and vitronectin with specific α subunits and also transduce signals into the cells. Activation of Fak and then Akt protein is known to play a role in matrix-induced cell survival signaling [24,25]. Phosphorylation of Fak in cells on PLLA/HAP scaffolds was at much higher level than that on PLLA scaffolds. Activation of Akt, an anti-apoptotic molecule, in its downstream pathway was detected only in the cells on PLLA/HAP scaffolds. The activation of caspase-3 (a common executor of apoptosis) by cleaving procaspase-3 was substantially decreased in cells on PLLA/HAP scaffolds. These data corroborate one another, strongly suggesting that the highly expressed integrins in the cells on PLLA/HAP scaffolds could have provided the survival signals upon ligation to the ligands. Therefore, it is very plausible that that enhanced adsorption of serum fibronectin and vitronectin rescued cells on PLLA/HAP scaffolds from apoptosis.

However, there could be other factors that contribute to the significant differences in cell response during the initial period of culture on the two types of scaffolds. First, even though adhesion protein-mediated cell survival is likely the cause, possible effects from growth factors cannot be excluded. Growth factors are known to enhance cell survival; deprivation of growth factors induces cell apoptosis [33,34]. Growth factors, either adsorbed to the PLLA/HAP scaffolds or de novo synthesized by cells upon the interactions between integrins and their ligands, may also increase cell survival. Second, in addition to the amount of proteins adsorbed to the scaffolds, the conformation of the adsorbed proteins on the PLLA and PLLA/HAP scaffolds could be different. As suggested by others [35], calcium in HAP may play a role in unfolding the adhesion molecules, which in turn expose the cell-adhesive epitopes recognized by specific cell surface integrins. Lastly, pore surface roughness of PLLA/HAP scaffolds also may contribute to the characteristics of protein adsorption, since the protein adsorption and cell response can be affected by topology as well as chemical composition of the incorporated bone-like mineral component [18,20].

5. CONCLUSION

In summary, the bone matrix-mimicking HAP-incorporated biodegradable polymer scaffolds (PLLA/HAP scaffolds) substantially rescued osteoblasts from apoptotic cell death during the initial period of culture. The PLLA/HAP scaffolds enhanced the adsorption and altered the profile of adsorbed serum proteins including higher amounts of fibronectin and vitronectin compared to the PLLA scaffolds. Pretreatment of the PLLA/HAP scaffolds with adhesion proteins or peptides increased the cell numbers on the scaffolds. Integrins $\beta 1$ and $\beta 3$ (signaling components of cell surface receptors for fibronectin and vitronectin) were highly expressed in the cells on PLLA/HAP scaffolds compared to those on PLLA scaffolds. Furthermore, phosphorylations of Fak and Akt were relayed in the cells on PLLA/HAP scaffolds, which

likely explain the observed suppression of caspase-3 activation. These results present evidence that the PLLA/HAP composite scaffolds provide a more favorable micro-environment for osteoblast survival than plain PLLA scaffolds through, at least in part, the enhanced adsorption of adhesion proteins and the suppression of apoptosis. These results provide valuable information for designing optimal scaffolds for tissue engineering.

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Figure 1. DNA contents in the scaffolds versus *in vitro* cultivation time. * Significantly different between PLLA/HAP and PLLA scaffolds ($p < 0.05$, $n = 4$).

Figure 2. Apoptotic cell death. (A) Fluorescence microscopic views of TUNEL (+) cells in green color, (B) Proportion of TUNEL (+) cells versus *in vitro* cultivation time, (C) Western blot analysis for caspase-3 in the cells cultured on the scaffolds. Lower panel was presented as loading

control (staining with Ponceau S). Forty μ l of cell lysates from a total of 400 μ l were separated in SDS-PAGE. * Significantly different between PLLA/HAP and PLLA scaffolds ($p < 0.05$, $n = 4$). Similar results were obtained from three independent experiments. Representative data are shown.

Figure 3.

(A) DNA contents within a construct; (B) amounts of adsorbed serum proteins to the scaffolds determined using MicroBCA assay; (C) profiles of protein adsorption to the scaffolds (Adsorbed proteins were fractionated in a 4–12% polyacrylamide gels followed by staining with coomassie blue); (D) Western blots for fibronectin and vitronectin adsorbed to the scaffolds from serum. Twenty five μ l from a total of 400 μ l of recovered serum proteins adsorbed to the scaffolds were separated in SDS-PAGE. * Significantly different between PLLA/HAP and PLLA scaffolds ($p < 0.05$, $n = 4$). Similar results were obtained from three independent experiments. Representative data are shown.

Figure 4.

(A) Amount of adsorbed proteins or peptides to the scaffolds, (B) DNA contents within the protein-pres soaked scaffolds. * indicates significant difference between PLLA/HAP and PLLA scaffolds; # indicates significant difference between a protein/peptide solution and PBS (p<0.05, n=4).

Figure 5.

Western blot analyses for integrins $\beta 1$ and $\beta 3$, Fak, Akt and caspase-3 proteins in the cells cultured in the scaffolds for 8 h (Forty μ l of cell lysates from a total of 400 μ l were separated in SDS-PAGE). Beta-actin serves as a loading control. Similar results were obtained from two independent experiments.

Table 1
Band intensities of human serum proteins adsorbed on PLLA and PLLA/HAP scaffolds

Approximate size of proteins (kDa)	PLLA	PLLA/HAP	Ratios(PLLA/HAP to PLLA)
180	0.063	0.124	2.0
150	0.042	0.067	1.6
86	0.045	0.160	3.6
81	0.067	0.315	4.7
68	2.057	2.322	1.1
60	0.033	0.192	5.8
47	0.008	0.058	7.1
45	0.007	0.065	9.6
26	0.200	0.667	3.3
<15	0.081	0.328	4.0