OSTEOBLAST FUNCTIONS ON NANOPHASE TITANIA IN POLY-LACTIC-CO-GLYCOLIC ACID (PLGA) COMPOSITES

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ABSTRACT

Much work is needed in the design of more effective bone tissue engineering materials to induce the growth of normal bone tissue. Nanotechnology offers exciting alternatives to traditional bone implants since bone itself is a nanostructured material composed of nanofibered hydroxyapatite well-dispersed in a mostly collagen matrix. For this purpose, poly-lactic-co-glycolic acid (PLGA) was dissolved in chloroform and nanometer grain size titania was dispersed by various sonication powers from 0 W to 332.5 W. Previous results demonstrated that the dispersion of titania in PLGA was enhanced by increasing the intensity of sonication and that greater osteoblast adhesion was correlated with improved nanophase titania dispersion in PLGA. However, adhesion of osteoblasts to material surfaces, alone, is not adequate to determine long-term functions of cells on implant materials. For this reason, osteoblast subsequent functions on nanophase titania/PLGA composites were investigated *in vitro* in this study. Moreover, for the first time, results correlated better osteoblast long-term functions, such as alkaline phosphatase activity and calcium-containing mineral deposition, with improved nanophase titania dispersion in PLGA. Thus, the present study demonstrated that PLGA composites with well-dispersed nanophase titania can improve osteoblast functions necessary for increased orthopedic implant efficacy.

INTRODUCTION

Poly-lactic-co-glycolic acid (PLGA) was chosen as the model polymer in the current study since it is biodegradable and widely utilized in tissue engineering applications. Nanophase titania was utilized as the model ceramic due to its excellent biocompatibility. Since nanophase ceramics tend to significantly agglomerate when added to polymers, different sonication output powers were investigated in this study to enhance ceramic dispersion. The results from a previous study by this research group demonstrated that the adhesion of osteoblasts on PLGA/titania composites was enhanced at higher sonication powers.[1] Since osteoblast adhesion results alone, are not enough for evaluating cell responses to the composites, osteoblast long-term functions (total protein synthesis, collagen synthesis, alkaline phosphatase synthesis and calcium deposition) on the nanophase titania/PLGA composites were studied.

MATERIALS AND METHODS

Substrate preparation

Polymer/ceramic composites: PLGA pellets (50/50 wt.% polylactic/glycolic acid; Polysciences, Warrington, PA) were dissolved in 8 mL of chloroform at 50 °C in a water bath for 40 minutes. Nanophase titania powder (Nanophase Technologies, Romeoville, IL) was then added to the PLGA solution to give a 70/30 polymer/ceramic weight ratio. The purity of the titiania powder was 99.5+%, the particle size was 32 nm according to BET measurements and the crystal phase was 80% anatase/20% rutile. The composite suspensions were sonicated using a W-380 sonicator (Heat System – Ultrasonics, Inc.) with output power settings of 118.75 W to 332.5 W. After sonication, the suspension was cast into a Teflon petri dish, evaporated in air at room temperature for 24 hours and dried in an air vacuum chamber (50.8 kPa) at room temperature for 48 hours. Finally, the composite films (0.3 mm in thickness) were cut into 1×1 cm squares for cell experiments. All substrates are listed in Table 1.

Polymer films: PLGA pellets were dissolved in 8 mL chloroform, cast into a Teflon petri dish, evaporated in air at room temperature for 24 hours and vacuum dried for 48 hours at room temperature.

Then, the PLGA films (0.2 mm in thickness) were cut into 1×1 cm squares and used as polymer control substrates.

Ceramic compacts: Green titania (TiO_2) circular disks were prepared by dry pressing nanophase titania powders in a tool-steel die (10 mm in diameter) via a uniaxial pressing cycle from 0.6 to 3 GPa over a 10 min period into 0.8 mm in thickness. Then some disks were sintered by heating in air at the rate of 10 °C/min from room temperature to a final temperature of 600 °C, holding at 600 °C for 2 hours and cooling down at the same rate as the heating rate. Both green and sintered titania disks were used as ceramic control substrates.

Reference materials: Glass coverslips were etched in 1 N NaOH and prepared for experiments according to standard protocols. Glass was used as a reference material. All samples were degreased in acetone and ethanol according to established laboratory procedures.

Table 1. PLGA, TiO_2 and PLGA/TiO_2 composites substrates

Substrate	Parameters
PLGA	Pure PLGA, control
TCG	Green pure titania compacts, control
TCS	Sintered pure titania compacts, control
PTC25	PLGA/titania composites sonicated at 118.75 W for 10 min
PTC35	PLGA/titania composites sonicated at 166.25 W for 10 min
PTC45	PLGA/titania composites sonicated at 213.75 W for 10 min
PTC70	PLGA/titania composites sonicated at 332.5 W for 10 min

Cell culture and experiments

Human osteoblasts (bone-forming cells; CRL-11372 American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (P/S; Hyclone) under standard cell culture conditions, that is, a sterile, 37 °C, humidified, 5% CO_2/95% air environment.

Osteoblasts were seeded at a density of 100,000 cells/cm^2 onto the substrates of interest and were cultured in complete DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 50 μg/ml L-ascorbic acid (Sigma) and 10 mM β–glycerophosphate (Sigma) under standard cell culture conditions for 7, 14, 21 days. At the end of the prescribed time periods, osteoblasts were lysed using distilled water and three freeze-thaw cycles. Total protein content in the cell lysates was determined using a commercial Coomassie Plus™ - The better Bradford assay kit (Pierce Biotechnology, Inc.) and following manufacturer's instructions. In addition, collagen synthesis was determined. Briefly, aliquots of the distilled water supernatant were dried onto a microplate, mixed with 0.1% Sirius Red stain (Sigma) and incubated for one hour at room temperature. The microplate was washed five times with 0.01 M HCl for 10 seconds per wash. The collagen bound stain was then washed with 0.1 M NaOH for 5 minutes. The eluted stain was then mixed several times into a multichannel pipette and placed into a second microplate. Absorbance was then read at 540 nm in a spectrophotometer. Total collagen content was normalized by total protein synthesis and expressed mg/mg protein. An alkaline phosphatase assay kit, a commercial kit from Upstate Cell Signaling Solutions, was also used to assay alkaline phosphatase activity in the cell lysates prepared as described above. To determine calcium deposition by osteoblasts, the substrates of interest to the present study were treated with 0.6 N HCl at 37 °C overnight. After the prescribed time period, the amount of calcium present in the acidic supernatant was quantified using a commercially available kit (Sigma) and following the manufacturer's instructions. All numerical data were analyzed using standard analysis of variance (ANOVA) techniques; statistical significance were considered at $p<0.05$.

RESULTS

Total protein was significantly ($p < 0.05$) greater on the PTC45 composites and TCG after 7, 14, and 21 days than PTC25, PTC35, PTC70 composites and PLGA controls after corresponding days of culture. Total protein was significantly greater after 14 days than after 7 days of culture on the PTC25, PTC45, PTC70, TCG substrates and glass references; total protein was also significantly greater after 21 days than after 7 and 14 days of culture on the PTC25, PTC45, PTC70, TCG substrates and glass references.

Total collagen was significantly ($p < 0.05$) greater on the PTC45 composites and TCG after 7, 14, and 21 days than PTC25, PTC35 composites and PLGA controls after corresponding days of culture. Total collagen was significantly greater after 14 days than after 7 days of culture on the PTC25, PTC35, and PTC70 composites; PLGA, TCG, TCS controls and the glass reference.

After 7 days of culture, there was not significantly different amounts of alkaline phosphatase activity in osteoblasts among the PTC25, PTC35, PTC45, PTC 70, and PLGA substrates; while alkaline phosphatase activity was significantly higher on the TCG and TCS substrates than all the composites. Alkaline phosphatase activity was significantly ($p < 0.05$) greater on the PTC35 composites, TCG and TCS after 14 and 21 days of culture, than PTC45 and PTC70 composites and PLGA controls after corresponding days of culture.

After 7 days of culture, there were no detectable amounts of calcium deposited on all the composites, PLGA substrates and the glass reference; while there were significantly higher amounts of calcium on the TCG and TCS substrates than all the other substrates. After 14 days of culture, there were no detectable amounts of calcium deposited on the PTC45 and PTC70 composites; while there were significantly higher amounts of calcium on the TCG and TCS substrates than all the other substrates. After 21 days of culture, there were no detectable amounts of calcium deposited on the PTC70 composites and PLGA controls; while there were significantly higher amounts of calcium on the PTC35 composites than all the other composites substrates. Significantly higher amounts of calcium on TCG and TCS substrates than all the other substrates were also observed.

DISCUSSION

Results of a previous study conducted by this research group demonstrated that the dispersion of nanophase titania in PLGA was significantly enhanced by increasing the intensity of sonication. That is, higher ultrasonic energy broke large titania agglomerations into smaller titania particles, which were more easily dispersed in PLGA suspensions. Moreover, previous results correlated greater osteoblast adhesion with increased nanophase titania dispersion in PLGA. That is, when sonication power increased, ceramic agglomeration decreased which promoted titania dispersion and subsequently enhanced osteoblast adhesion. Initial events during cell-biomaterials interactions, such as cell adhesion affect long-term functions, such as proliferation, synthesis of proteins and calcium mineral deposition. In the present study, enhanced synthesis of alkaline phosphatase and deposition of calcium-containing mineral was observed on the titania/PLGA composites with increased nanophase titania dispersion in PLGA.

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REFERENCES

[1] H. Liu, E.B. Slamovich and T.J. Webster, "Improved Dispersion of Nanophase Titania in PLGA Enhances Osteoblast Adhesion", *American Ceramic Society Annual Meeting Proceedings*, in press, (2004)