INFLUENCE OF DYNAMIC FLOW ON INTEGRIN EXPRESSION OF RAT CALVARIAL CELLS THREE-DIMENSIONALLY CULTURED ON PLAGA SCAFFOLDS IN ROTATING BIOREACTORS

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INTRODUCTION

Studies have suggested that the limited diffusion in static culture environments may constrain tissue ingrowth in tissue-engineered constructs [1]. To overcome the limitation associated with static culture, we have used the high aspect ratio vessel (HARV) rotating bioreactor to provide dynamic flow culture conditions for promoting tissue synthesis [2]. In previous studies, we have described the development of novel poly(lactide-co-glycolide) (PLAGA, 50:50) hollow microsphere based lighter than water (LTW) scaffolds that have density less than the media (1 g/cm^3) and we have also created "mixed" scaffolds by combining heavier than water (HTW, density > 1 g/cm^3) and LTW microspheres [2]. These scaffolds had trajectories that facilitated media perfusion in the rotating bioreactors, and differentiation and mineralization of osteoblastic cells seeded on these scaffolds were significantly enhanced. Osteoblastic cell adhesion is mainly mediated by integrins that are heterodimeric receptors composed of α and β subunits in specific combinations. The integrins allow the cell to respond to its extracellular environment as well as modulate cellular events that regulate remodeling. Studies have shown that different integrin subunits such as α2, α3, α4, α6, and β1 play important roles in the binding of osteoblasts to various surfaces [3]. The biomolecular events associated with successful osteoblastic cell growth on degradable materials under dynamic flow conditions are still unknown, and the measurement of integrin expression may provide further information on the ability of three-dimensional systems cultured in dynamic bioreactors to promote cellular attachment and growth. In this study, we further characterize the movement of mixed scaffolds of PLAGA (85:15) in rotating bioreactors, and seek to determine the effects of dynamic flow on integrin expression of rat calvarial osteoblastic cells cultured on the mixed scaffolds in rotating bioreactors *in vitro*.

MATERIALS AND METHODS

Scaffold preparation: The LTW and HTW biodegradable polymeric microspheres sized from 425-500 μm were fabricated using PLAGA copolymer in an 85:15 ratio as described previously [2]. Three-dimensional mixed scaffolds were fabricated into 4 mm x 2.5 mm cylindrical scaffolds by sintering the HTW and LTW microspheres in varying ratios at 80ºC for 3 hours [2].

Scaffold motion tracking: The movement of scaffolds was tracked using a real time microcapsule visualization unit [4]. The scaffold tracking system is comprised of a CCD camera that is rotated in synchrony with a rotating bioreactor, and scaffold motion was recorded digitally and analyzed using Image Pro (Phase 3 Imaging). Scaffolds were visualized in a bioreactor rotating at 36-rpm using the real time microcapsule visualization unit, and the instantaneous velocity values of the scaffolds were calculated by dividing the distance traveled by the scaffold by the time interval between frames.

Cell Culture: Rat calvarial osteoblast cells were isolated from 2-day old neonatal Sprague-Dawley rats by an enzymatic digestive method, and maintained to passage 3. Cells were seeded dynamically onto microcarrier scaffolds (HTW:LTW, 60:40) in the rotating bioreactor at a density of 2 x 10^4 cells/cm^2 for 24 hours, and the scaffolds were then separated into HARV vessels either maintained statically as controls or rotated at 36 rpm as rotating bioreactors. The cells were cultured in F-12 media supplement with 15% FBS, 1% P/S, 1% glucose, and 1% ß-glycerophosphate at 37°C and 5% CO_2. The media were changed every 3 days. At days 4, and 7, scaffolds were removed and characterized for integrin expression.

Enzyme-linked immunosorbent assay (ELISA): The expression of integrins was analyzed using ELISA. Briefly, the scaffolds with cells were lysed with 0.1% triton-100. The protein amount was estimated by lowry's method. Four micrograms of protein were added into each well of 96-well plate at room temperature for 1 hour. Goat anti-rat antibodies against integrin α2, α3, α4, α6, αM, β1 and β2 were added to the wells separately at 1:100, and incubated at 4°C overnight. After washing with PBS, alkaline phosphatase conjugated mouse anti-goat secondary antibody (1:100) was added into each well, and incubated at 4°C overnight. After washing with PBS, substrates p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3 indolyl-phosphate (BCIP) were added into each well, and incubated at room temperature for 2 hours. The absorbance was read at 620 nm using a plate reader.

Statistical Analysis: A two-tailed student t-test was used for comparing the results between the static and rotating culturing groups. A p value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The scaffold trajectories are shown in Fig. 1A-B. The aggregate densities of the LTW scaffolds and the mixed scaffolds (HTW:LTW, 80:20) were less than the surrounding medium, and the buoyant forces forced the scaffolds toward the center of the bioreactor vessel and kept the scaffolds from collisions with the bioreactor wall (Fig. 1A-B). By varying the ratio of LTW to HTW microspheres in the scaffolds, the velocity values of the scaffolds decreased from 103.1 ± 6.2 mm/s to 48.9 ± 7.5 mm/s (Fig. C). By assuming uniform flow past a single microcapsule on the surface of the scaffold, the velocity could be used to estimate the maximum fluid shear stress using the Stokes equation.

$$\sigma = \frac{-3\mu U}{2a}$$

Where σ is shear stress, μ is viscosity, U is flow velocity and a is the diameter of the microcapsule [2]. The estimated maximum shear stress decreased from 0.32 N/m^2 to 0.16 N/m^2 through changing ratio of HTW to LTW microspheres. Thus, the fluid shear stresses of the scaffolds can be adjusted by varying the ratio of HTW and LTW.

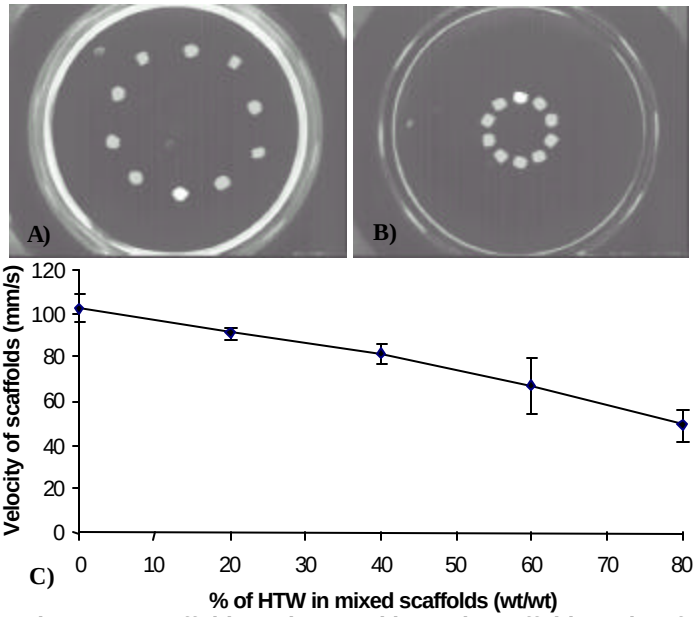

Figure 1. Scaffold motion tracking. A) scaffold made of LTW; B) scaffold with HTW to LTW ratio of 80:20; C) Velocity of scaffolds with various ratios of HTW to LTW).

Based on the ELISA assay, no significant difference was observed between the rotating and static conditions for the expression of integrins α2, α4, αM, β1 and β2 at day 4 (Fig. 2A). α3 and α6 integrin levels were significantly increased ($p < 0.05$) under rotating conditions compared to that in static cultures at day 4 (Fig. 2A). The expression of integrin α2, α3, α4, αM, β1 and β2 under rotating conditions was at the same levels as those in static conditions at day 7 (Fig. 2B). α6 integrin expression was significantly increased ($p < 0.05$) under rotating conditions compared to that in static cultures at day 7 (Fig. 2B). The results indicated that the expression of integrin α2, α4, αM, β1 and β2 in rat calvarial cells was not affected by dynamic flow at both day 4 and day 7, but dynamic flow up regulated the expression of integrin α3 and α6 at day 4, and up regulated the expression of integrin α6 at day7.

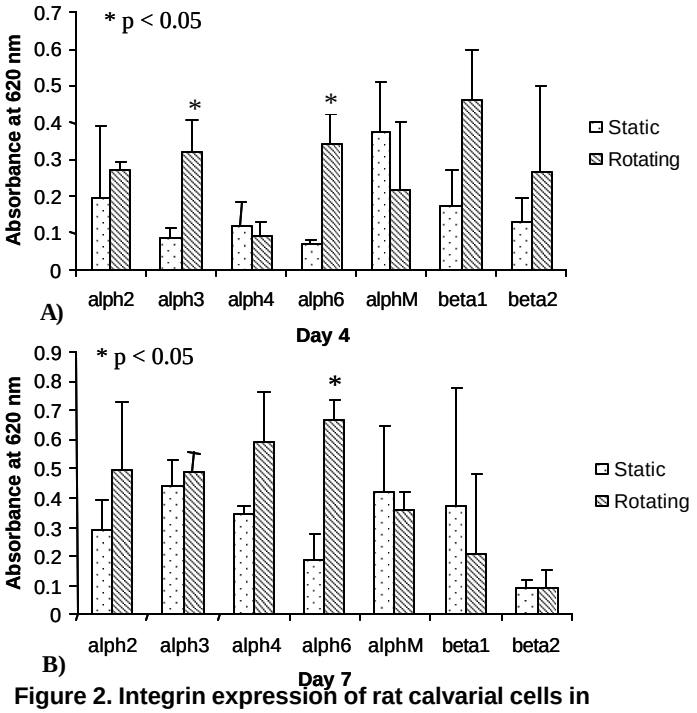

Figure 2. Integrin expression of rat calvarial cells in static and rotating bioreactors at A) day 4 and B) day 7.

CONCLUSIONS

These studies suggest that the motion trajectories and therefore the flow velocity around and through the scaffolds in rotating bioreactors can be manipulated by varying the ratio of HTW to LTW microspheres, and that the expression of some bone cell integrins may be enhanced under 3-D dynamic flow environment. These studies may provide a better understanding of cellular-polymer interaction under dynamic flow and aid in the design and optimization of bioreactor based tissue engineering of bone.

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