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The optimal combination of substrate chemistry with physiological fluid shear stress

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ABSTRACT

Osteoblasts on implanted biomaterials sense both substrate chemistry and mechanical stimulus. The effects of substrate chemistry alone and mechanical stimulus alone on osteoblasts have been widely studied. This study investigates the optimal combination of substrate chemistry and 12 dyn/cm^2 physiological flow shear stress (FSS) by examining their influences on primary rat osteoblasts (ROBs), including the releases of ATP, nitric oxide (NO), and prostaglandin E_2 (PGE_2). Self-assembled monolayers (SAMs) on glass slides with –OH, $–CH_3$, and $–NH_2$ were employed to provide various substrate chemistries, whereas a parallel-plate fluid flow system produced the physiological FSS. Substrate chemistry alone exerted no observable effects on the releases of ATP, NO, and PGE_2. Nevertheless, when ROBs were exposed to both substrate chemistry and FSS, the ATP releases of NH_2 were upregulated about 12-fold compared to substrate chemistry alone, while the ATP releases of CH_3 and OH was similarly increased 7-fold at the peak. Similar trends were observed for the releases of NO and PGE_2. The expressions of ATP, NO, and PGE_2 followed the pattern of NH_2-FSS > Glass-FSS > CH_3-FSS ≈ OH-FSS. ROBs on NH_2 produced the optimal combination of substrate chemistry with the physiological FSS. The F-actin organization and focal adhesion (FA) formation of ROBs on various SAMs without FSS were examined. NH_2 produced the best results whereas CH_3 and OH produced the worst ones. Inhibition of FAs and/or disruption of F-actin significantly decreased the releases of FSS-induced PGE_2, NO, and/or ATP. Consequently, a mechanism was proposed that the best F-actin organization and FA formation of ROBs on NH_2 lead to the optimal combination of substrate chemistry with the 12 dyn/cm^2 physiological FSS. This mechanism gives guidance for the design of implanted biomaterials and bioreactors for bone tissue engineering.

1. Introduction

Scaffolds and seeded cells such as osteoblasts are two important components of bone tissue engineering. Scaffolds provide physical support and living microenvironment for seeded cells [1]. The properties of scaffolds, including scaffold chemistry [2,3], stiffness [4] and architecture [5], determine cellular responses, tissue growth, and even clinical success of bone tissue engineering. The design and selection of scaffold chemistry is the first step for scaffold applications. To provide guidance for designing and selecting scaffold chemistry, the effect of substrate chemistry on osteoblasts has been widely studied by using 2D [6–8] or 3D [2,9] systems and some important results were reported. For instance, Healy et al. [7] demonstrated enhanced attachment of osteosarcoma cells to NH_2 surfaces compared to CH_3 surfaces and they were able to guide spatial distribution of cells by controlling the distribution of NH_2 and CH_3. Similarly, Garcia et al. [8] observed different osteoblasts adhesion and mineralization on well-defined NH_2, COOH, CH_3 and OH surfaces. In 3D scaffolds, Reis et al. found that sulfonic and phosphonic groups grafted on the starch and polycaprolactone (SPCL) scaffolds significantly promoted osteoblast proliferation compared to virgin SPCL [9].

In addition to substrate chemistry of scaffolds, mechanical loading is indispensable for mechanosensitive osteoblasts to produce normal bone tissue and maintain normal bone functions [1,10]. Generally, the mechanical loading is applied to osteoblasts either through scaffolds stretch [11], compression [12] or fluid flow through scaffolds [13]. Especially, fluid shear stress (FSS) is regarded as the principal mechanical stimulus responsible for bone

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adaption and remodeling [14–16]. In bone tissue *in vivo*, the physiological FSS caused by interstitial fluid flow is 8–30 dyn/cm^2 on the basis of the model presented by Weinbaum et al. [17]. In this study, 12 dyn/cm^2 were employed to represent physiological FSS.

Accordingly, appropriate substrate chemistry and 12 dyn/cm^2 FSS are both required to realize bone tissue engineering. The osteoblast responses to substrate chemistry under 12 dyn/cm^2 FSS seem more significant and instructive for scaffold design than the osteoblast responses to substrate chemistry alone.

The aim of this study is to examine the osteoblast responses to various substrate chemistries under 12 dyn/cm^2 physiological FSS, including ATP, nitric oxide (NO), and prostaglandin E_2 (PGE_2) releases, and find an optimal combination of substrate chemistry with the physiological FSS. The productions of NO and PGE_2 are early responses of osteoblasts to FSS and play important roles in bone formation and remodeling [18–20]. Thus, NO and PGE_2 productions were selected as parameters to indicate the early responses of ROBs. Besides, ATP, as a key energy provider, is the precondition for other follow-up cellular responses including the productions of NO and PGE_2, cell proliferation and differentiation. Moreover, ATP release is one of the fastest responses of ROBs to FSS and can be employed to indicate the sensitiveness of ROBs to FSS [21]. Therefore, ATP release was considered as well. Self-assembled monolayers (SAMs) on glass slides were employed to provide a variety of surface chemistries, including –OH, $–CH_3$ and $–NH_2$, and a parallel-plate fluid flow system was applied to produce 12 dyn/cm^2 physiological FSS. The results demonstrated that ROBs on NH_2 surfaces had better responses than on OH and CH_3 surfaces, indicating that NH_2 is the optimal combination of substrate chemistry with 12 dyn/cm^2 FSS. The different cytoskeleton organization and focal adhesion formation on various substrate chemistries were responsible for this optimal combination.

2. Materials and methods

2.1. Preparation and characterization of SAMs on glass slides

Blank glass slides were cleaned by acetone and ethanol, and then rinsed with deionized water (DIW). All cleaned slides were stored in DIW before introduction of chemical groups. To introduce –OH groups, the cleaned slides were dipped into freshly prepared Piranha solution (concentrated H_2SO_4: 30% H_2O_2 = 7:3, v/v) at 80 °C for 1 h, and then were rinsed with excessive DIW and blown dry with nitrogen. The obtained slides were labeled as OH slides. To get NH_2 slides, the OH slides were dipped into 1% solution of (3-aminopropyl) triethoxysilane (Alfa Aesar, USA) in acetone, and then treated by refluxing 1 mL distilled water for 30 min. The obtained slides were rinsed with ethanol and DIW, and blown dry with nitrogen [22]. For CH_3 slides, the OH slides were dipped into 5% solution of chloro(dimethyl) octadecylsilane (Sigma–Aldrich, USA) in hexane for 1 h, and then rinsed with ethanol and DIW, blown dry with nitrogen [9]. Finally, all slides were placed in Petri dishes (Corning, USA), soaked with 75% alcohol overnight and rinsed with sterile phosphate-buffered saline (PBS).

Water contract angles of the slides were characterized by using a Model 200 video-based optical system (Future Scientific Ltd. Co., Taiwan, China). Six specimens were measured for each kind of SAMs, and three different points were selected for each specimen. All measurements were performed at room temperature by dropping 5 μL of ultra-pure water.

2.2. Osteoblasts culture

Primary rat osteoblasts (ROBs) cultures were described in our previous publication [18]. Briefly, cells were isolated from minced rat calvarial chips, followed by 2 h of 0.125% collagenase and 0.25% trypsin at 37 °C shaking. Cultures were initiated in DMEM (Gibico, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sijiqing, China), penicillin (100 U/mL), streptomycin (100 μg/mL) and 0.05% L-glutamine, and maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The medium was changed every two days. After confluence, the cells were subcultured and identified by von Kossa staining method according to previously reported procedure [23]. The forth, fifth, and sixth passage ROBs were seeded on slides for experiments at a density of 2×10^5 cells/slide.

2.3. Fluid shear stress

A parallel-plate flow chamber apparatus was employed to provide steady FSS [24]. The dimension of the flow chamber was 7.50 cm (length, L) × 2.50 cm (width, W) × 0.03 cm (height, H). FSS was produced by circulating 10 mL DMEM *via* a peristaltic pump (JieHeng, China). The produced FSS (τ, dyn/cm^2) was calculated according to equation $\tau = 6\eta Q/H^2W$, where η is the dynamic viscosity of the perfusate and Q is the flow rate. The employed FSS in this study was steady 12 dyn/cm^2. All components of the apparatus except the pump were maintained in a 37 °C incubator during the experiment, and the medium was continuously saturated with 5% CO_2/95% air. For FSS-loaded samples (denoted by X-FSS, X represents surface chemistry), slides with attached ROBs were mounted on the flow chamber and then exposed to FSS for a predetermined time. Other samples without FSS exposure (denoted by X, X represents surface chemistry) were kept in Petri dishes at 37 °C in a humidified atmosphere of 5% CO_2/95% air for the same predetermined time.

2.4. Determination of ATP, NO and PGE_2 releases

After seeded on SAMs for 48 h, ROBs (reaching ~80% confluence) were employed to examine the releases of ATP, NO and PGE_2. When ROBs on slides were exposed to FSS for a predetermined time (0, 1, 2, 3, 4, 5, 10, and 15 min for ATP determination, 0, 5, 10, 15, 30, 45, and 60 min for NO and PGE_2 determination), 2 mL of medium was withdrawn and replenished with an equal volume of fresh culture medium to maintain a constant circulating fluid volume. The same procedure was performed for samples without FSS exposure.

2.4.1. Release of ATP

The ATP release was detected by using an ATP Assay Kit (Beyotime, China) based on bioluminescence technology. Briefly, 500 μL of the withdrawn medium and 200 μL of luciferin–luciferase reagent were mixed for 5 s; thereafter the fluorescence intensity was measured by using Multifunctional Microplate Reader (Biotek Synergy HT, USA). The concentration of ATP was obtained from the standard curve plotted by using the supplied ATP standards and normalized to the total cellular protein using a BCA Protein Assay Kit (Biotech, China).

To ensure that the detected ATP was all extracellular ATP, the plasma membrane integrity was confirmed through surveying the lactate dehydrogenase (LDH) levels of the conditioned media by using a LDH Assay Kit (Jiangcheng, China).

2.4.2. Releases of NO

The concentration of NO was detected by using Nitric Oxide Assay Kit (Beyotime, China) on the basis of Griess Reagent. The withdrawn medium was allowed to react with Griess Reagent for