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The responses of osteoblasts to fluid shear stress depend on substrate chemistries

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ABSTRACT

Natural bone tissue receives chemical and mechanical stimuli in physiological environment. The effects of material chemistry alone and mechanical stimuli alone on osteoblasts have been widely investigated. This study reports the synergistic influences of material chemistry and flow shear stress (FSS) on biological functions of osteoblasts. Self-assembled monolayers (SAMs) on glass slides with functional groups of OH, CH_3, and NH_2 were employed to provide various material chemistries, while FSS (12 dynes/cm^2) was produced by a parallel-plate fluid flow system. Material chemistry alone had no obvious effects on the expressions of ATP, nitric oxide (NO), and prostaglandin E_2 (PGE_2), whereas FSS stimuli alone increased the production of those items. When both material chemistry and FSS were loaded, cell proliferation and the expressions of ATP, NO and PGE_2 were highly dependent on the material chemistry. Examination of the focal adhesion (FA) formation and F-actin organization of osteoblasts before FSS exposure indicates that the FA formation and F-actin organization followed similar chemistry-dependence. The inhibition of FAs and/or disruption of F-actins eliminated the material dependence of FSS-induced ATP, PGE_2 and NO release. A possible mechanism is proposed: material chemistry controls the F-actin organization and FA formation of osteoblasts, which further modulates FSS-induced cellular responses.

Introduction

Natural bone tissue receives both chemical cues and mechanical stimuli in physiological environments. Accordingly, in bone tissue engineering application, osteoblasts anchoring to a scaffold require appropriate chemical and mechanical stimuli to produce functional 3D bone tissue constructs. Generally, the chemical stimuli is provided by material chemistry of scaffolds, whereas mechanical stimuli is applied *in vitro* through scaffolds stretch, fluid flow shear, or hydrostatic compression [1,2]. Although the effects of material chemistry alone or mechanical loading alone on osteoblasts have been extensively studied [1,2,3–7], their synergistic contribution has not been investigated so far. Thus, it is essentially important to investigate the synergistic effects of material chemistry and mechanical loading on osteoblast responses and understand the potential mechanism.

It is well known that material chemistry modulates cellular responses [3–5,8–13] and tissue formation [6,7] *in vitro* and *in vivo*. For instance, Healy [3] demonstrated enhanced osteosarcoma cells attachment to NH_2 surfaces compared to CH_3 surfaces and guided spatial distribution of cells by controlling the distribution of NH_2 and CH_3. Similarly, Garcia [9] reported divergent osteoblasts adhesion and mineralization on well-defined NH_2, COOH, CH_3, and OH surfaces. In 3D scaffolds, Reis [7] found that sulfonic and phosphonic groups grafted on the starch and polycaprolactone (SPCL)[1] blended scaffolds significantly promoted the proliferation of osteoblasts comparing with virgin SPCL scaffolds. Brodbeck et al. [6] employed a rat cage implant system with various surface chemistries to demonstrate increased *in vivo* apoptosis and reduce foreign body giant cell formation on hydrophilic and anionic implants compared to hydrophobic and cationic implants. In addition to material chemistry, appropriate mechanical loading is indispensable for the formation of normal bone tissue and maintenance of normal bone functions. For bone tissue *in vivo*, fluid shear stress (FSS) due to interstitial fluid flow through the canalicular spaces is regarded as the

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[1] *Abbreviations used:* SPCL, starch and polycaprolactone; Fss, fluid shear stress; ATP, adenosine triphosphate; NO, nitric oxide; PGE2, prostaglandin E2; SAMs, self-assembled monolayers; DIW, deionized water; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase.

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principal mechanical stimuli responsible for bone adaption and remodeling [14–16]. The exact mechanisms by which osteoblasts sense and transform FSS into biological signals that are responsible for bone adaption and remodeling remain elusive. However, integrins and integrin-medicated cytoskeleton rearrangement are regarded as critical factors in this mechanotransduction [17–21]. Moreover, there are evidences that the initial formation of FAs is also a key factor in this mechanotransduction [22–28]. For example, the number and morphology of FAs considerably influence the FSS-induced short-term and mid-term responses, including the releases of adenosine triphosphate (ATP), nitric oxide (NO), and prostaglandin E_2 (PGE_2), which further regulate cell proliferation [14–15,22]. In addition, it has been reported that FSS-induced NO release requires complete cytoskeleton [28].

Previous studies have shown that both material chemistry and FSS are important regulators for osteoblasts behavior; however, their synergistic contributions have not been sufficiently studied. The aim of this study was to investigate the synergistic role of material chemistry and FSS in osteoblast responses and understand the possible mechanism of these responses. We chose self-assembled monolayers (SAMs) terminated with –OH, $–CH_3$, and $–NH_2$ functional groups to provide chemical stimuli and a parallel-plate fluid flow system to produce 12 dynes/cm^2 FSS. The F-actin organization and FA formation of ROBs on various SAMs before FSS exposure were also investigated in order to understand the possible mechanism responsible for the synergistic responses.

Materials and methods

Preparation and characterization of SAMs on glass slides

Blank glass slides were initially cleaned using acetone and ethanol, and subsequently rinsed with deionized water (DIW). All cleaned slides were stored in DIW before the 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 subsequently rinsed with excessive DIW and blown dry with nitrogen. The obtained slides were labeled as OH slides. To prepare NH_2 slides, the OH slides were dipped into 1% solution of (3-aminopropyl) triethoxysilane (Alfa Aesar, USA) in acetone, and subsequently treated by refluxing 1 mL distilled water for 30 min. The obtained slides were rinsed with ethanol and DIW, and blown dry with nitrogen [29]. 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 subsequently rinsed with ethanol and DIW, and blown dry with nitrogen [30]. Finally, all the slides were placed in Petri dishes (Corning, USA), soaked in 75% alcohol overnight, and rinsed with sterile phosphate-buffered saline (PBS).

To indicate that the preparation of the SAMs is successful, 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. In addition, the wettability has a significant effect on cell adhesion. Moderate wettability surface can make osteoblast adhesion be better than hydrophobic and hydrophilic surface [31].

Osteoblasts culture

Primary rat osteoblasts (ROBs) cultures were described in our previous paper [32]. Briefly, the calvaria bone of 1–5 d old SD rats was removed and placed in Petri dish containing PBS buffer. The periosteum and surrounding connective tissue were removed by using tweezers. The calvaria bone was washed with PBS and cleaned with DMEM until the surface of bone is white and transparent, immersed into a small amount of fetal calf serum. The calvaria bone was cut into about $1 \times 1 \times 1$ mm pieces, coated uniformly in 25 ml culture flask. 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 2 days. After confluence, the cells were sub-cultured and identified by using the von Kossa staining method according to previously reported procedure [32]. The fourth to sixth passage of ROBs were used for experiments at a density of 2×10^5 cells/slide.

Fluid shear stress

A parallel-plate flow chamber apparatus was employed to provide FSS [33]. 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 using a peristaltic pump (JieHeng, China). The produced FSS (τ, dynes/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 12 dynes/cm^2. All the 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, where X represents surface chemistries), slides with attached ROBs were first mounted on the flow chamber and then exposed to FSS for a predetermined time. Other samples without FSS exposure (denoted by X, where X represents surface chemistries) were kept in Petri dishes at 37 °C in a humidified atmosphere of 5% CO_2/95% air for the same predetermined time.

Determination of ATP, NO, and PGE_2 releases

After seeded on SAMs for 48 h (reaching ~80% confluence), ROBs were employed for the determination of ATP, NO, and PGE_2 releases. After ROBs on slides were exposed to FSS for a predetermined time, 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.

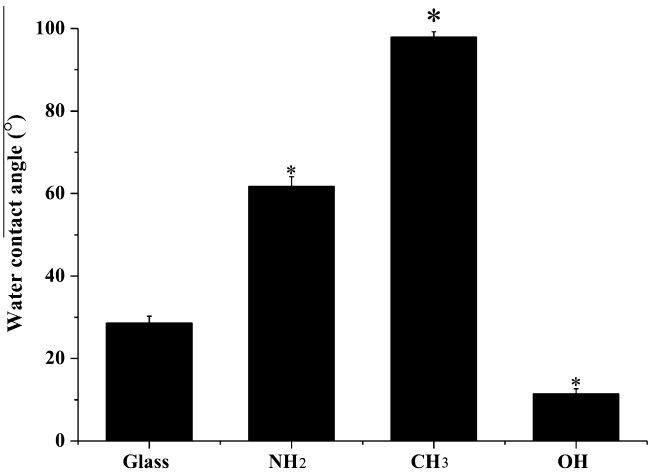

Fig. 1. Water contact angles of various SAMs with terminal NH_2, OH, and CH_3 compared to blank glass slides. The results were shown as mean ± SD. "*" represents that NH_2, OH, and CH_3 are significantly different from blank glass slides ($P < 0.05$).