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Impact of flow shear stress on morphology of osteoblast-like IDG-SW3 cells

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Abstract

This study constructed an in situ cell culture, real-time observation system based originally on a micro-fluidic channel, and reported the morphological changes of late osteoblast-like IDG-SW3 cells in response to flow shear stress (FSS). The effects of high (1.2 Pa) and low (0.3 Pa) magnitudes of unidirectional FSS and three concentrations of extracellular Type I collagen (0.1, 0.5, and 1 mg/mL) coating on cell morphology were investigated. IDGSW3 cells were cultured in polydimethylsiloxane microfluidic channels. Cell images were recorded real-time under microscope at intervals of 1 min. Cell morphology was characterized by five parameters: cellular area, cell elongation index, cellular alignment, cellular process length, and number of cellular process per cell. Immunofluorescence assay was used to detect stress fiber distribution and vinculin expression. The results showed that 1.2 Pa, but not 0.3 Pa of FSS induced a significant morphological change in late osteoblast-like IDG-SW3 cells, which may be caused by the alteration of cellular adhesion with matrix in response to FSS. Moreover, the amount of collagen matrix, alignment of fiber stress and expression of vinculin were closely correlated with the morphological changes of IDG-SW3 cells. This study suggests that osteoblasts are very responsive to the magnitudes of FSS, and extracellular collagen matrix and focal adhesion are directly involved in the morphological changes adaptive to FSS.

Keywords

Microfluidic chip; Flow shear stress; Osteoblast; Cell morphology

Introduction

Bone is one of the most adaptable tissues in the human body. The external mechanical load leads to modeling and remodeling of the structure and property of bone [1]. Bone cells

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

exhibit different cell morphology in response to changes of mechanical conditions [2]. For example, osteocytes in force-bearing fibulae bone showed a significant morphological changes different from non-force bearing caldaria bones in human body [3]. Some previous studies have demonstrated the alteration of osteoblasts, including morphology, expression and release of molecules, and cytoskeleton arrangement [4–9], after exposure to flow shear stress (FSS) [10, 11], which is recognized as a major form of the mechanical stimuli on bone cells under normal physiological condition [12, 13].

However, the results varied due to different frequencies, magnitudes, and models (oscillatory or unidirectional) of fluid flow. Also, the real-time change of cells during the fluid stress was not well investigated.

In recent years, a microfluidic chip has been used, which generates a local microenvironment closely mimicking cells exposure to fluid flow [14]. Moreover, polydimethylsiloxane (PDMS) channels make it possible to observe dynamic changes of cell morphology in situ [15, 16]. Some previous studies have shown the responses of calcium signals and the changes of morphology and cytoskeleton in osteoblasts using microfluidic devices [6, 17].

Based on this, we wanted to study the real-time morphological effects of FSS on osteoblasts in microfluidic channels. In this study, IDG-SW3 cells generated from long bones was used, which closely mimic late osteoblasts and early osteocyte cells that are programmed to be differentiated to mature osteocytes under appropriate condition [18]. The cells were cultured at 33 °C with the characteristics of osteoblasts. We subjected the cells to high (1.2 Pa) and low (0.3 Pa) levels of unidirectional FSS on different amounts of extracellular Type I collagen coated matrix. Furthermore, stress fiber distribution and vinculin expression were studied due to their known roles in maintenance of cell morphology and adhesion of cells with matrix.

Materials and methods

Materials

The cell culture reagents used in this study were alpha modified essential medium (α -MEM, Gibco), fetal bovine serum (FBS, Hyclone), penicillin and streptomycin (P/S, Sina-American), and trypsin (Sina-American). Interferon- γ (IFN- γ) and bovine serum albumin (BSA) were purchased from Invitrogen. Antibodies to vinculin (Sigma), secondary antibody goat anti-mouse IgG-FITC (Cwbio), and rhodamine phalloidin (Invitrogen) were used for cellular immunofluorescence staining. Materials required for device fabrication included SU-8 photoresist (MicroChem Corp.), AZ-50XT (AZ Electronic Materials), photoresist developer (MicroChem Corp.), trimethylchlorosilane (Alfa Aesar), and polydimethylsiloxane (GE Silicones). Type I collagen from rat tails (BD) was used for microfluidic channel coating.

Design and fabrication of the microfluidic chip

The microfluidic devices used had dimensions of 150 μ m channel height and 125 or 500 μ m width. According to the formula: $\tau = 6\mu Q/H^2 W$ (τ , FSS; μ , viscosity of medium; Q , volume

flow rate; H , height of channel; W , width of channel), FSS was set at 1.2 Pa ($W = 125 \mu\text{m}$) or 0.3 Pa ($W = 500 \mu\text{m}$) (Fig. 1a, b).

The microfluidic channel molding was produced by a photolithographic process to create the microchannels (Fig. 1c). A 150- μm -thick SU-8 was coated onto a silicon wafer, baked, and flood exposed to form a templet. Then soft lithography was used to fabricate the chip. A 10:1 mixture of PDMS prepolymer was thoroughly mixed, poured onto the templet, degassed under vacuum for air bubble removal, and kept at 80 °C for 20 min. Then the PDMS layer was removed from the templet and further kept at 80 °C for 40 min to ensure the stiffness. The channel inlet ports (small for linking to the fluid control device) and outlet ports (large for changing the medium easily) were punched through. Another 15:1 mixture of PDMS prepolymer was mixed, spin-coated onto 35 mm dish, and kept at 80 °C for 10 min to ensure the bonding of PDMS layer to the dish. The PDMS chips inside the 35 mm dish were kept at 80 °C for another 48 h for solidification. The microfluidic chips were sterilized under UV overnight before used.

Surface coating

Prior to cell culture experiments, the microfluidic channel surfaces were coated with 0.1, 0.5 or 1 mg/mL rat tail collagen type I. After rinsing three times with PBS, the channels were flushed with collagen solutions, and the microfluidic devices were incubated at 33 °C for 2 h. After that, α -MEM medium was flushed into the channels to remove the collagen solutions, and then the microfluidic devices were incubated at 33 °C for another 1 h.

Cell culture

IDG-SW3 late osteoblasts, a gift from Dr. Lynda Bonewald (Indiana University), were cultured on collagen-coated plates in α -MEM medium supplemented with 10% FBS, 50 U/mL of IFN- γ , 1% glutamine, and 1% penicillin/streptomycin at 33 °C and 5% CO₂. IDG-SW3 cells shows a continuous proliferation and immortalization at 33 °C in the presence of IFN- γ , to express a temperature-sensitive mutant of the SV40 large tumor antigen [18]. IDG-SW3 cell suspensions with a concentration of 1×10^6 cells/mL were loaded into microfluidic channels through the inlets using micropipettes. Cells were incubated at 33 °C with 5% CO₂ for 1 h, and then the medium in channels was removed and replaced by fresh supplemented medium. In the next 24 h the medium was replaced every 2 h to ensure the adhesion and growth of cells. The cells were then subjected to next FSS experiment.

System for in situ time-lapse on-chip cell observation

An in situ cell culture, real-time observation system was constructed based originally on a microfluidic channel, living cell incubation system (TOKAI HIT), a phase microscope, a micro-injection pump and a medium collector (Fig. 1c). The inlet and outlet of the channels were connected to polyethylene tubing coupled with a pump and a medium collector, respectively. Dynamic behaviors of the cells under FSS were visualized and the data were collected at 1 min intervals.

F-actin and vinculin staining

Immediately after 2 h of FSS stimulation, F-actin and vinculin staining were performed. The cells were fixed with 4% preheated paraformaldehyde (PFA) for 20 min at room temperature. After washed and permeabilized, cells were incubated with rhodamine phalloidin (1:40, Invitrogen) and anti-vinculin (1:40, Sigma) antibody overnight at 4 °C, respectively. For vinculin staining, FITC labelled secondary antibody (1:100, CWBIO) was added into the channels for 1 h at room temperature and followed by washing with TBS three times. Then the channels were flushed with anti-quenching glycerinum and observed under a fluorescence microscope.

Quantification of cell morphological changes

Each group at least had three repetitions and about 40–50 cells in each repetition ($n = 40$ –50). Cell morphology was defined by next five parameters: (1) cellular area, (2) cell elongation index (length/width), (3) cellular alignment (the angle between long axis of cells and the direction of fluid flow), (4) length of cellular process, and (5) number of cellular process per cell.

Statistical analysis

Statistical analysis was performed using GraphPad Prism5 statistics software (GraphPad). All data were expressed as mean $\pm$ SEM. Student *T* test was used for statistical analysis. Asterisks indicate the degree of significant differences compared with the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

Effects of FSS on IDG-SW3 morphology

Mouse late osteoblast-like IDG-SW3 cells were cultured on microfluidic chip and subjected to FSS for 2 h. The effects of magnitudes of unidirectional FSS (1.2 or 0.3 Pa) and the amount of extracellular type I collagen (0.1, 0.5 or 1 mg/mL on morphological changes of cells in microfluidic channels were investigated. The images of IDG-SW3 cells were taken every 1 min for 2 h, and then the five parameters mentioned were statistically analyzed to represent the cell morphology. Figure 2b shows the length (*L*) and width (*W*) of cells by red lines and the angle of cells and the direction of fluid flow by white lines, with the arrow direction as the direction of FSS. Figure 2a shows that cells under 1.2 Pa unidirectional FSS stimulation underwent a process of contraction and followed by a cell re-spreading, while the control cells under static condition maintained cell shapes during the same time period. Most of the cells reduced volume to the minimal after proximately 30 min of FSS treatment, and then re-spread to normal cell size after 2 h (Fig. 2c). Figure 2d shows that the mean cell minimal area during 2 h of FSS decreased up to approximately 30% of their original area. After 2 h of FSS treatment, the cell elongation index (length/width) decreased about 23%, which indicated that the circularity of the cells became larger (Fig. 2e). The angle of cells and the direction of fluid flow decreased from 42.6° to 32.6°, which showed a realignment of cells trend to the direction of fluid flow (Fig. 2f). Furthermore, in the screenshots of the video and magnified detail shown in Fig. 3a, FSS treated cells showed more and shorter

processes compared to static control cells. Statistical analysis showed that the length of cellular process decreased by about 34% (Fig. 3b), and number of cellular process per cell increased by 48% (Fig. 3c).

We also observed that 2 h of 1.2 Pa FSS treatment caused a rearrangement of F-actin. The stress fibers showed a homogeneous distribution in the entire cell, while in static cultured cells stress fibers distributed more in the cytoplasm near the cell periphery, arranged rarely in the nucleus region (Fig. 4a, c). Interestingly, the expression level of vinculin dramatically increased and more concentrated on the tips of cellular processes (Fig. 4b, c).

Furthermore, we detected the morphology of IDG-SW3 cells in response to low magnitude of FSS (0.3 Pa). After 2 h of treatment, no obvious difference was found for cellular area, cellular alignment and elongation index compared with that of static control cells (Fig. 5a–c). However, significant changes were detected in area and elongation index of cells treated with 1.2 Pa FSS compared with that of 0.3 Pa FSS treatment (Fig. 5a, c).

Effects of collagen matrix on IDG-SW3 morphology

Similar to those cultured on 0.5 mg/mL, IDG-SW3 cells cultured on 0.1 (Fig. 6d–f) and 1 mg/mL (Fig. 6a–c) collagen-coated plates underwent the process of contraction and re-spreading with an increase of cell circularity in IDG-SW3 cells during 1.2 Pa FSS stimulation. The time duration of shrinkage to minimal size was about 20 min for cells on 0.1 and 1 mg/mL collagen-coated plates (Fig. 6f, c), which was shorter than 30 min with 0.5 mg/mL collagen coating. From 0.1 and 0.5 to 1 mg/mL collagen coating, the percentage of contracted cells decreased from 94 and 75 to 56% (Fig. 7a), the contraction start time was postponed from 2.4 and 3.0 to 3.7 min (Fig. 7b), and the elongation index increased from 1.4 and 1.9 to 2 (Fig. 7c). After 2 h of FSS, the cells with 0.5 mg/mL collagen in the matrix were nearly re-spread out, while cells with 0.1 and 1 mg/mL collagen coating had not yet returned to the area before FSS, and the process of re-spreading was delayed (Fig. 7d).

Discussion

It is well known that bone cells remodel in response to mechanical loading [7, 8]. However, the real-time morphological response of osteoblasts to external FSS stimulation was not well investigated, and also the underlying cellular mechanism needs further study. In this study, IDG-SW3 cells underwent a process of contraction and re-spreading in response to 1.2 Pa FSS within a period of 2 h. FSS mechanical stimulation alters the interaction between cells and external environment, which leads to the reorganization of cytoskeleton. As an important mechanosensor, cytoskeleton links to focal adhesion and external cellular matrix and transfers extracellular mechanical stress to intracellular chemical response (outside-in) [8, 9, 19]. In the study, we observed a more homogeneous distribution of F-actin filament in the entire cell. Jackson et al. have also shown a more widely distribution of cytoskeleton cross-linking proteins throughout the MC3T3–E1 osteo-blasts after 2 h of FSS stimulation [20]. These results show a response of cytoskeleton to mechanical loading. Also, our results showed that 1.2 Pa of FSS dramatically increased the expression of vinculin, an important protein in focal adhesion [21], and redistributed to the tips of cellular processes, which may indicate an enhancement of cells adhering to the matrix in order to resist the mechanical

stress exerted by fluid flow. The increase of cell process number also indicated the efforts that cells maximized their adhesive capability to the matrix. This phenomenon was also observed in osteocytes subjected to 24 h of FSS [8].

In the study, IDG-SW3 cells realigned towards the direction of fluid flow, which was found in osteoblasts responding to mechanical tension [22]. However, Malone et al. have shown that no observable relationship was found between the direction of fluid flow and the orientation of actin fibers after a steady flow treatment, although actin fibers were orientated parallel to the long axis of the cell [9]. Ponik et al. have also shown that 24 h of FSS caused a long axis alignment of HUVEC, not MC3T3–E1 osteoblasts [8]. And they have found that MC3T3–E1 cells elongated when subjected to FSS compared to static controls. These are not consistent with the results in this study. We thought that the model and magnitude of mechanical stimulation and the cell type make a difference in the morphology response of osteoblasts. The morphological changes may be the prelude to changes in gene and protein expression, ultimately leading to the functional change of osteoblasts in response to mechanical stimulation.

Extracellular collagen through integrin and focal adhesion interacts with intracellular actin filaments, which plays an important role in mechanical response [23]. In this study, cells cultured on 0.1, 0.5, and 1 mg/mL collagen-coated plates all underwent the process of contraction and re-spreading, but 1 mg/mL collagen in matrix attenuated the process by delaying the contraction start time and decreasing contraction cell number. It is likely that higher amount of collagen in the matrix increases cell adhesion to matrix, thus inducing a less response to FSS. While the lower collagen concentration decreased the connection between cells and matrix, the morphological response is greater. Also, different collagen coating provide different surface stiffness. Previous studies have shown that different cell types have adapted responses to different ranges of stiffness to match their function, therefore the cellular response to matrix stiffness may be very different in different cell type [24]. Cells show different morphological spread on different surface stiffness produced by collagen matrix [25]. In the study, contraction re-spread process may also depend on the difference of surface stiffness induced by different collagen matrix.

Therefore, our study illustrates a successive mechanotransduction involving extracellular matrix (collagen)-focal adhesion (vinculin)-cytoskeleton (actin filament), these important cellular components work together to transfer mechanical signals to modulate cell function.

In the study, 0.3 Pa FSS did not cause an obvious morphological change in IDG-SW3 cells. Aryaei et al. have also shown that low shear stress was not sufficient to induce a cellular response in osteoblasts [10]. Physiologically, in the human body the magnitude of fluid flow shear stress is thought to be about 0.8–3 Pa [26]. Therefore, it is likely that there is a threshold of FSS that causes the changes of cell morphology, and 0.3 Pa flow stimuli may be below that threshold. However the limitations of this study is that only two magnitudes of FSS was investigated for only 120 min. In future, more experiments should be performed using different magnitudes of FSS for a longer time, which might be helpful to find the threshold.

Conclusions

Taken together, in this study, we have constructed a micro-fluidics system to observe and quantify in situ, the dynamic process of IDG-SW3 cell responses to FSS. Compared to static conditions, high intensity FSS (1.2 Pa), but not low FSS (0.3 Pa) treatment caused significant morphological changes in IDG-SW3 cells, including a process of cell contraction and re-spreads, an increase of cell circularity, and realignment of cells along the direction of fluid flow. These morphology changes may be associated with cell–matrix interaction, matrix surface stiffness and cytoskeleton organization.

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Fig. 1. Schematic (a) and photo (b) of the microfluidic chip and composition of the device. The channel height was $150\mu\text{m}$ and width was 125 or $500\mu\text{m}$, respectively. c The three layers of the device from top to bottom (the fluidic layer, PDMS layer, and 35 mm dish). The channel inlet and outlet ports were punched through to link the fluid control device (a microinjection pump and a syringe) and a medium collector, respectively. The whole device was plated in a living cell incubation system to keep temperature and observed under a phase microscope

Fig. 2.

IDG-SW3 cells underwent a contraction and re-spread process during 1.2 Pa FSS treatment.

a The morphology alteration of a representative cell during FSS and static treatment. **b** The method of measuring the length (L) and width (W) of cells by red lines and the angle of the cellular long axis and the direction of fluid flow by white lines, with the arrow direction as the direction of FSS (all downward in the figure). The cell area decreased and then increased during 2 h of FSS treatment (**c**, **d**), **c** Cells volume decreased to the minimum after approximately 30 min of FSS treatment. The cells contracted to about 30% of their original area. The ratio of cellular length and width (elongation index) decreased about 23% (**e**), and the cells showed a trend of realignment along the direction of fluid flow, the angle of the cell long axis and fluid flow direction decreased from 42.6° to 32.6° (**f**). $**P < 0.01$; $***P < 0.001$

Fig. 3. IDG-SW3 cells showed more **(b)** and shorter **(c)** processes after 1.2 Pa FSS treatment compared to static control. **a** Cells under microscope (left lane) and magnified details (right lane) of static cultured (above) and FSS treated (below) cells. The white arrowhead in **a** points out the cell processes. *** $P < 0.001$

Fig. 4. The treatment of 1.2 Pa FSS changed the distribution of F-actin (**a**) and expression of vinculin (**b**). Bar = 50 μm. **c** Showed the magnified details of **a** and **b**. The yellow arrows pointed out the distribution of F-actin and vinculin

Fig. 5. After 2 h of 0.3 Pa FSS treatment, cellular area (a), cellular alignment (b), and elongation index (c) of IDG-SW3 cells were not obviously changed, while 1.2 Pa fluid stimuli changed the elongation index compared with static control. * $P < 0.05$, ** $P < 0.01$

Fig. 6.

Both of 1 mg/mL (a–c) and 0.1 mg/mL collagen (d–f) coated matrix caused contraction and re-spreading during 2 h of 1.2 Pa FSS treatment, which was similar to that of 0.5 mg/mL collagen. On both 0.1 and 1 mg/mL collagen-coated matrixes, cells volume decreased to the minimum after approximately 20 min (c, f) and contracted to about 40% of their original area (a, d). Also, the ratio of cellular length and width (elongation index) decreased (b, e).

* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$

Fig. 7.

From 0.1 and 0.5 to 1 mg/mL collagen coating, the percentage of contracted cells decreased from 94 and 75 to 56% (**a**), the contraction start time was postponed from 2.4 and 3.0 to 3.7 min (**b**), and the elongation index increased from 1.4 and 1.9 to 2 (**c**). After 2 h of 1.2 Pa FSS, the cells with 0.5 mg/mL collagen in the matrix were nearly re-spread out, while cells with 0.1 and 1 mg/mL collagen coating had not yet returned to their original area, and the process of re-spreading was delayed (**d**). * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$