Effect of Fluid Shear Stress on Apoptosis and Osteogenesis of Mesenchymal Stem Cells

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Article

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Abstract

Mechanical cues, including fluid shear stress (FSS) of interstitial flow within bone cavities, induce osteogenic differentiation of mesenchymal stem cells (MSCs). However, whether FSS leads to the apoptosis of MSCs and its relation with osteogenic differentiation are still unclear. In this study, the effect of FSS on apoptosis and osteogenesis of MSCs is investigated. FSS is applied intermittently to MSCs through a cone-plate flow chamber system for 3 d. And results show that FSS inhibits the apoptosis of MSCs. Compared with static culture group, FSS promotes osteogenesis, as shown by the expression of three osteogenic differentiation markers, namely, alkaline phosphatase (ALP), osteocalcin (OCN), and collagen I (COL I). Double staining of individual cells shows that the relationship of apoptosis and osteogenic differentiation of MSCs is mutually exclusive. YAP may mediate the FSS-inhibiting apoptosis of MSCs. The results will further elucidate the mechanism of mechanical stimulation-induced bone remodeling.

Introduction

Mesenchymal stem cells (MSCs) are a self-renewing stem cells derived from the mesoderm and ectoderm during early embryonic development. MSCs are widely distributed in bone marrow and other tissues in animals. Under specific conditions in vitro, MSCs can differentiate into many cells, such as adipocytes, chondrocytes, osteocytes, myocytes, neurocytes, and endothelial cells. Mechanical loading, especially fluid shear stress (FSS) in bone marrow and periosteum, can affect MSCs’ growth, apoptosis, and differentiation.

Interstitial fluid flowing through lacunar or canalicular spaces in bones generates FSS on MSCs, which may further translate into biochemical signals to regulate the biological behavior of cells. FSS improves the osteogenic differentiation of MSCs. The osteogenic differentiation of adipose tissue-derived mesenchymal stem cells is also observed in vitro in response to pulsating fluid flow. Low-frequency (0.015, 0.044, or 0.074 Hz) pulsatile flow increases the expression of osteogenic differentiation factors, including collagen I (Col I), osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP), and bone morphogenetic proteins (BMP)-2, -4, and -7. Similarly, steady flow with a very low FSS of $10^{-3}$ to $10^{-5}$ Pa for 3 days significantly increases the osteogenic differentiation of MSCs, as shown by the expressions of alkaline phosphatase (ALP) and OPN. The expressions of osteogenic genes, such as ALP, Runx2, COL Iα, and OCN, show that intermittent flow has greater effects on the osteogenic differentiation of hMSCs than continuous flow through the regulation of ERK1/2 and FAK activity.

Cell apoptosis can be significantly influenced by mechanical cues. For example, FSS can regulate the fate of stem cells. The proliferation of MSCs exposed to 2 Pa FSS increases by 26.1% compared with static control. FSS inhibits TNF-α-induced apoptosis of MC3T3-E1 cells by activating extracellular kinase 5, in addition, the adhesion morphology of MSCs also influence their survival through their involvement with the cytoskeleton. In our previous study, the MSCs adhered to a small area of 314 μm².
tend to undergo apoptosis, whereas those adhered to a large area of more than 1256 µm² tend to survive. The disruption of cytoskeleton by cytochalasin B is found to be related to the apoptosis of MSCs. And the mechanical forces on MSCs are transduced into the nucleus to influence gene expression. However, whether a correlation exists between osteogenesis and apoptosis under FSS exposure is still unknown and needs to be studied.

In this study, we used a custom-made cone-plate flow chamber to apply constant wall FSS on MSCs, and to investigate how FSS at a physiological level regulates the apoptosis and osteogenic differentiation of MSCs.

**Results**

**A cone-plate flow chamber provides controllable FSS distribution**

The numerical simulation results indicated that uniform FSS can be obtained in the annular region (Figs. 1D and 1F). At the center of the circle plate, the value of FSS was nearly zero. The fluctuation of FSS distribution was increased in the outer region of the circular plate. As shown in Figs. 1E and 1G, the wall FSS on the bottom increased along with the distance to the center (in the range of 0-5 mm) and displayed a relatively stable value of 1 or 2 Pa.

The actual spreading area and circularity were measured based on the light-field images of single cell. Statistical analysis showed that no significant difference existed between the nuclear areas of MSCs upon exposure to FSS (1 and 2 Pa) compared with the control group. In addition, the differences of spreading area and circularity of cells were not statistically significant between static mode and FSS stimulation. The experimental results showed that FSS did not affect the spreading area, shape, or nuclear area of MSCs.

**MSCs apoptosis is inhibited by FSS**

After FSS exposure for 3 days, the apoptosis level of MSCs was detected by TUNEL assay. Typical photographs of each group are shown in Fig. 2A. The statistical results showed that compared with the control group, the average level of apoptosis decreased significantly in the FSS loading group (Fig. 2B). Compared with 2 Pa-FSS group, the average apoptosis level of 1 Pa-FSS group decreased.

**Osteogenesis of MSCs is enhanced by FSS**

To investigate the effect of FSS on osteogenic differentiation in MSCs, three types of osteogenic differentiation marker, namely, ALP, OCN and COL I, were detected after FSS exposure for 3 days. The total fluorescence intensity in a single cell was represented as the osteogenesis level of MSCs. Typical fluorescence photos of ALP, OCN and COL I are shown in Figs. 3A, 4A, and 5A. Statistical analysis showed that compared with static culture, MSCs expressed a significantly higher level of the protein of osteogenic marker ALP under FSS exposure (Fig. 3B). A similar trend was also observed for OCN (Fig. 4B) and COL I...
(Fig. 5B). Compared with the 1 Pa-FSS group, the expression levels of OCN and COL I were significantly higher in the 2 Pa-FSS group, whereas no significant difference was found in the expression of ALP between the two groups. The average fluorescence intensity of markers in each group was used to calculate the ratio of osteogenic differentiation. Differentiation ratios of the three markers in the loading group were significantly higher than those in the control group, and those in the 2 Pa-FSS group were significantly higher than those in the 1 Pa-FSS group (Figs. 3C, 4C, and 5C). These results demonstrated that FSS induced osteogenic differentiation in MSCs.

**Apoptosis and osteogenesis of MSCs are mutually exclusive**

Both TUNEL and ALP immunofluorescence were stained in a single cell to investigate the relationship between MSCs apoptosis and osteogenesis under the FSS exposure. Cells with high apoptotic levels had low osteogenic differentiation (Fig. 6A). The mean values of the relative fluorescence intensity of ALP and TUNEL in each group are shown in Fig. 6B. Compared with the control group, the FSS loading groups had higher osteogenesis level and lower apoptosis level. The osteogenesis level was higher and the apoptosis level was lower in the 1 Pa-FSS group than in the 2 Pa-FSS group.

**YAP nuclear translocation is regulated by FSS**

The nuclear transfer of YAP occurred when the mean YAP intensity in the nucleus was greater than that in the cytoplasm. Typical fluorescence photographs are shown in Fig. 7A. The percentage of cells with YAP nuclear localization showed that FSS reduced the YAP nuclear localization. Compared with the control group, the percentage of the cells with nuclear localization was significantly reduced in the FSS loading group, whereas that in 1 Pa-FSS group was lower than in the 2 Pa-FSS group (Fig. 7B).

**Discussion**

FSS was applied intermittently to MSCs through a cone-plate flow chamber system. After 3 days of FSS exposure, the spreading state, apoptosis, osteogenic differentiation, and nuclear transfer of YAP of MSCs were detected and compared with those under static culture condition. FSS does not affect the spreading area, shape, or nuclear area but upregulates the osteogenesis level, downregulates the apoptosis level, and reduces the YAP nuclear localization of MSCs.

A previous study demonstrated that the physiological FSS on osteocytes is between 0.8 and 3 Pa. In the present study, the FSS of physiological levels at 1 Pa and 2 Pa decrease the apoptosis of MSCs, and the 1 Pa-FSS group has the lowest apoptosis level. This finding is consistent with the previous result that the exposure of hMSCs to 2 Pa FSS induces 126.1% proliferation compared with static controls. Another study demonstrated that FSS inhibits TNF-α-induced apoptosis of MC3T3-E1 by activating extracellular kinase. Qi et al. found that increasing FSS from 0.3 to 1.5 Pa for over 6 h significantly enhances the proliferation rate of PDL cells by 50% relative to the control group (0 Pa). Oscillating fluid flow (1 Pa, 1 Hz) can protect cells from apoptosis with or without TNF-α, and the reduction of FSS cannot
inhibit the apoptosis of osteoblast-like MLO-Y4 cells \(^23\). All the above findings support our results that FSS inhibits MSC apoptosis.

The second finding of this study is that physiological level FSS promotes the osteogenic differentiation of MSCs, which is consistent with the results of some previous studies. Kim et al. found that FSS induces the osteogenic differentiation of MSCs \(^24\). Yourek et al. showed that MSCs’ ALP activity is significantly increased in the osteogenic medium after 4 or 8 days of FSS exposure, and osteopontin expression is also higher than that in control groups \(^25\). Previous studies have shown that FSS can enhance ALP activity and upregulate osteogenic genes, such as OCN, COL I, and runt-associated transcription factor 2 (Runx2) \(^26\).

The relationship between apoptosis and osteogenic differentiation was investigated by double staining single cells. The results showed that the apoptosis and osteogenic differentiation of MSCs are mutually exclusive. In our previous study, individual MSCs revealed a reciprocal relationship between osteogenesis and apoptosis during culture in different adhesive micropatterned islands \(^27\). The MSCs in large areas and low circularity prefer to differentiate into osteoblasts, whereas those in small areas may undergo apoptosis.

Some previous studies showed that YAP/TAZ is an important signaling mediator of the substrate stiffness-induced fate of stem cells, and the force applied to the nucleus directly drives YAP nuclear translocation by decreasing the mechanical restriction on molecular transport in nuclear pores \(^28,29\). However, our experimental results showed that FSS inhibits the nuclear localization of YAP in MSCs. The spread shape, area, and nuclear area of the cells are not changed after exposure to a physiological level of FSS for 3 days. Thus, we assume that the cytoskeleton tension and the forces applied to the nucleus are not changed, and the decrease of YAP nuclear transfer induced by FSS is mediated by other mechanisms. YAP1 coactivator is an essential regulator of cell proliferation \(^30\), and many studies showed that YAP is a pre-apoptotic regulator of mammalian cells \(^31\). When YAP is translocated to the nucleus, it binds to P73 in the nucleus, thereby promoting the transcription of downstream pro-apoptotic genes and inducing apoptosis \(^32\). A recent study found that FSS upregulates the expression of Piezo1 to promote the translocation of YAP to the nucleus, thereby inducing the apoptosis of suspending tumor cells. In contrast, FSS promotes YAP translocation to the cytoplasm in adherent tumor cells \(^33\). This finding is consistent with our present results, i.e., FSS promotes the translocation of YAP to the cytoplasm from the nucleus and decreases the apoptosis level. Further studies should be performed to clarify the mechanism underlying FSS-regulated MSC apoptosis.

**Methods**

**Cell culture**

Mouse MSCs (Invitrogen, US) within passage 10 were cultured in α-MEM medium (Hyclone, UT) supplemented with 10% (v/v) fetal bovine serum (Gibco, NY) and 1% (v/v) penicillin and streptomycin...
(Invitrogen, CA) under humidified conditions at 37 °C and 5% CO₂. After reaching 70% confluence, the cells were treated with 2% trypsin/EDTA solution (Invitrogen, CA). The suspended cells were seeded on the bottom of 6-well plates at a density of 5×10³ cells/cm². After seeding for 1 h, the culture medium was gently removed and changed every 3 days. All methods were carried out in accordance with relevant guidelines and regulations.

**Application of fluid shear stress**

In our previous study, we designed a cone-plate flow chamber based on 6-well plates, in which FSS was uniformly distributed on the annular region of the well bottom. In the present study, the cone-plate flow chamber was further modified to apply FSS independently to each well (Fig. 1A). The device consisted of a stationary plate underneath a rotating cone (Fig. 1B). The distance between the cone's tip and plate surface was controlled by placing a silicon membrane (Fig. 1C). Wall FSS exerted on the cells was controlled by specifying the cone's rotation speed. Cone was fabricated according to hydrodynamic calculation, in which a uniform wall FSS field was provided on the cell surface. MSCs were cultured on the bottom of a 6-well plate. MSCs were treated with a specific FSS thrice a day for 1 h each time.

**Numerical simulation**

Using COMSOL Multiphysics software, FSS was simulated by setting the parameters of device and the angular rate of the cone in accordance with the method used in the previous study. For the cone-plate flow chamber model, the cone's generatrix was machined as polyline to establish a uniform wall FSS field on the plate surface. The maximum radius of the cone was 15 mm, and its vertical distance to the tip was 1.3 mm. The gap between the cone's tip and the plate surface was 0.2 mm. The radius of a well for the 6-well culture plate was 17 mm. A no-slip boundary condition was assumed for all rigid surfaces in the model except for the cone. A free surface boundary condition was used for the upper fluid surface within the well.

Navier–Stokes equations were used to define the flow behavior of viscous fluids. Incompressible viscous fluid was assumed to have a density of 1×10³ kg/m³ and a viscosity of 1×10⁻³ Pa·s. An iterative method was used to solve the equations for steady flow, and convergence was identified when the relative tolerance was less than 0.001.

During numerical simulation, Reynolds number was computed as defined by Sdougos et al. According to the calculated Reynolds number, fluid flow in the cone-plate should be assumed as steady laminar flow, and FSS is calculated according to the following equation:

\[ \tau = \mu \frac{\partial v}{\partial z} \]

We first calculated the shear strain rate \( \gamma = \frac{dv}{dz} \) along the direction close to the plate surface based on numerical simulation results and then obtained FSS by multiplying the shear strain rate with the viscosity.
coefficient $\mu$. All rigid surfaces in the model were assumed to be non-slip boundary conditions. Upper flow surface in the well was adopted as an open boundary condition, and the rotating cone was adopted as a sliding boundary condition. MATLAB and Origin software were used for data processing.

**Immunofluorescence staining**

After incubation in a 6-well plate for 3 days, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, rinsed with phosphate buffer saline twice, permeabilized with 0.2% Triton-X100 for 10 min, and blocked with 1% bovine serum albumin for 60 min at room temperature. In addition, the cells were labeled with primary antibody against alkaline phosphatase (ALP, Santa Cruz Biotechnology, Inc., USA), type I collagen (COL I, Santa Cruz Biotechnology, Inc., USA), osteocalcin (OCN, Santa Cruz Biotechnology, Inc., USA), or YAP (ABclonal, CN) at a 1:200 dilution overnight at 4 °C. Second antibody labeling was performed in the dark for 60 min at 37 °C. The nuclei were stained with 0.1% Hoechst (Invitrogen, USA) for 10 min. The cells were double-stained with an apoptosis assay kit (One Step TUNEL Apoptosis Assay Kit, Beyotime, CA) and ALP-conjugated TRITC. After fixation and permeabilization, the cells were incubated in the dark with a TUNEL reaction mixture for 60 min at 37 °C.

**Image analysis**

For mechanical experiments, MSCs were chosen from annular regions with inner and outer diameters of 5 and 12 mm, respectively, where the wall FSS appears uniform. The spreading area and shape of a single cell were obtained from bright-field image. Its nuclear area was acquired from Hoechst-staining image. Bright-field or fluorescence images were analyzed using ImageJ software.

**Statistical analysis**

Statistical data were expressed as mean ± standard error of mean. Three independent experiments were performed with at least 50 cells for each group. One-way analysis of variance (ANOVA) with Turkey’s post hoc test for multiple comparisons was performed for statistical analysis by using Origin software. The osteogenesis ratio of three osteogenic markers was calculated by chi-square test. The mean values of different groups were regarded as significantly different when $p < 0.05$.

**Declarations**

**Acknowledgments**

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**Conflict of interest statement**

The authors declare no conflict of interest.
Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

References


Figures

Figure 1

Cone-plate device for applying FSS to cells. (A) The photograph of cone-plate flow chamber system. (B) The side-view photo of the flow chamber. (C) The schematic graph of the flow chamber. Finite element analysis of wall FSS in cone-plate flow chamber. (D, F) Wall FSS distribution on the bottom plate for 36 and 80 rad/s angular velocity of the cone, respectively. (E, G) Radial distribution of wall FSS for 36 and 80 rad/s angular velocity of the cone, respectively.
Figure 2

Apoptosis of individual MSCs under static condition or FSS exposure for 3 days. (A) Typical photographs of TUNEL staining. Scale bar, 50 μm. (B) Fluorescence intensity of TUNEL staining under static condition and FSS exposure. *, p<0.05.

Figure 3

ALP expression of individual MSCs cultured under static condition or FSS exposure for 3 days. (A) Typical fluorescent photographs of ALP. Scale bar, 50 μm. (B) Fluorescence intensity of ALP. (C) Osteogenesis ratio. *, p<0.05.

Figure 4

OCN expression in individual MSCs cultured under static condition or FSS exposure for 3 days. (A) Typical fluorescent photos of OCN. Scale bar, 50 μm. (B) Fluorescence intensity of OCN. (C) Osteogenesis ratio. *, p<0.05.
Figure 5

COL I expression in individual MSCs cultured under static condition or FSS exposure for 3 days. (A) Typical fluorescent photos of COL I. Scale bar, 50 μm. (B) Fluorescence intensity of COL I. (C) Osteogenesis ratio. *, p<0.05.

Figure 6

Relationship between ALP expression and apoptosis in MSCs cultured under static condition or FSS exposure for 3 days. (A) Scatter plots of ALP and apoptosis. A dot represents the fluorescent intensity of ALP and the level of apoptosis of a single cell. (B) Mean fluorescence intensity of ALP and apoptosis of each group. Four quadrants in B were divided according to the values of the cells under static condition.
Figure 7

Nuclear localization of YAP in MSCs under static condition or FSS exposure for 3 days. (A) Typical fluorescence photos of YAP. Scale bar, 50 μm. (B) Percentage of YAP nuclear translocation under static condition and FSS exposure. *, p<0.05.