J. Jpn. Soc. Microgravity Appl. Vol. 28 No. 2 2011 (S36–S40)

8th Japan-China-Korea Workshop on Microgravity Sciences
for Asian Microgravity Pre-Symposium

Fibronectin is Involved in Gravity-Sensing of Osteoblast Like Cell

Jingbao LI, Liang WANG, Gang HE, Mingzhi LUO, Airong QIAN and Peng SHANG

Abstract

ECM-integrin-CSK system is normally considered as gravity-sensor of cell and most researches focus on the cytoskeleton (CSK). How about the extracellular matrix (ECM) protein? In previous work, we found that fibronectin (FN) gene expression in MG-63 cells under simulated weightlessness condition was increased significantly compared to other conditions and this result was verified by microarray test. Here the FN's alterations of another human osteoblast like cell hFOB1.19 on the protein level under diverse gravitational condition were investigated. Both cellular FN and soluble FN were detected. More FN was expressed in hFOB1.19 cells medium after co-cultured with RGD-peptide and integrin antibody. These results hint that FN changes in altered gravity maybe involved with the interference of the interaction between FN and integrin.

1. Introduction

Gravity is a universal force and influences everything in nature. Any slight fluctuations and variations in this force can have significant impact on the structure and functions of organisms. The alterations of metabolism and utilization of various nutrients have been observed during both real and simulated space flight. For astronauts during long term space flight, one of the most important medical risks is bone loss [1]. Although human adaptation to the microgravity environment allows astronauts to maintain overall function, the musculoskeletal system rapidly degrades once the force of gravity is disappeared [2].

Extracellular matrix (ECM) is an essential component of stromal microenvironment both from a structural and functional of view [3]. Combined with their receptor integrins to link with cytoskeleton, some extracellular matrix proteins have been shown to contribute to mechanotransduction. Under weightless environment [4], the ECM-integrin interactions and the downstream signal pathways are interfered, which would eventually affect cell structure and function, especially cell fate [5]. On the other hand, the effects of weightlessness on expression and structure of extracellular matrix have been documented widely as well [3,6,7]. Given the complexity of functions and diverse regulations under weightless environment, extracellular matrix has drawn the attention of many researchers as a promising target to uncover the mechanism of human physiological and pathological changes in space.

Fibronectin (FN), an abundant ECM protein, plays crucial roles in cell adhesion, migration, growth and differentiation. At cell surface, the soluble dimer of FN secreted by cell is assembled into insoluble multimeric fibrils, which so-called cellular FN (cFN). The meshwork of interconnected fibrils including the active form of FN provides a dynamic environment for cells [8].

In our previous work, the transcriptional level of fibronectin in osteoblast like MG-63 cells under diamagnetic levitation condition was increased significantly compared to other gravitational conditions and this result was verified by microarray test [9,10]. To investigate the alteration of FN on the protein level under diverse gravitational condition, osteoblast like cell line hFOB1.19 was applied to diamagnetic levitation and 3-D clinostat. Both forms of fibronectin, cellular FN and soluble FN, were detected.

2. Materials and Methods

2.1 Materials

Tissue culture 96-well plate, strips and dish were purchased from Corning Life Sciences. MEM culture medium, CO_2 independent medium and fetal calf serum (FCS) were purchased from GIBCO™ Invitrogen Corporation. RGD- and RAD-peptides were synthesized by ChinaPeptides Co. Integrinβ1 antibody (Chemicon Co.) was used in blocking experiment. Anti-fibronectin monoclonal antibody (Ab-3, Neomarkers, USA) and secondary antibody labeled with IRDye800CW (Rockland, Gilbertsville, PA) was used in in-cell western (ICW) assay. Human FN ELISA kit (EK0349 Boster Inc. China) was applied for soluble FN detection.

2.2 Cell Culture

The human SV40-immortalized normal osteoblast like cell

Key Laboratory for Space Biosciences & Biotechnology, School of Life Science, Northwestern Polytechnical University, Youyi Xilu No.127, Xi'an City, Shaanxi Province, P.C.710072, China
(E-mail: Shangpeng@nwpu.edu.cn)

line hFOB1.19 was purchased from the Cell Collection Center of Shanghai, which obtained the cells originally from American type culture collection (Manassas, VA, USA). Cells were grown in complete MEM culture medium supplemented with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 10% FCS at 37 ℃, 5% CO_2 in a humidified atmosphere. For culturing of the cells in the superconducting magnet, MEM was changed to CO_2 independent medium with 10% FCS and the tissue culture dishes were sealed with parafilm. GIBCO CO_2 independent medium contains a unique phosphate buffering system. Additionally, it has been formulated with components that enhance cellular production and utilization of CO_2 such that an exogenous source of CO_2 is not required for the maintenance of CO_2 dependent cellular functions. A circulating water-bath was used for temperature control during the experiments. For strips and 96-well plate culture, 1 $\times 10^4$ cells (Approx. 80% confluence) were seeded per well. After 12-h attachment, cells in wells were applied in diamagnetic levitation experiments or inhibition experiments. For vessels culture in 3-D clinostat experiment, 4 $\times 10^5$ cells (Approx. 80% confluence) were seeded per vessel.

2.3 Superconducting magnet

A superconducting magnet (JMTA-16T50MF) which can provide large gradient high magnetic field (LGHMF) was made by Japan Superconductor Technology Inc (JASTEC) dependent on the authors' design plan [11]. Maximum magnetic induction intensity (B) is 16.2 tesla (T) and the product of B*dB/dz is 1100 T^2 m^{-1}. The superconducting magnet can provide three gravitational levels (0G, 1G and 2G) and keep stable more than one year. The magnetic field intensity of three gravity levels (0 G, 1 G and 2 G) was 12, 16 and 12 T, respectively. Four groups were designed in this study, namely, 1 G group (normal gravity, 16 T), control group (normal gravity, geomagnetic field), 2 G group (2 G, 12 T) and μg group (0 G, 12 T, diamagnetic levitation). We have developed many kinds of other experiment equipment matching with the superconducting magnet, which could be used to life sciences including utilizing in structure biology, cell biology, microbiology and development biology. In order to keep the temperature at 37℃ for cell culture in the bore of superconducting magnet, the temperature control system was designed and made by authors. In order to deliver the experimental samples and to accurately detect the gravity levels, an adjustable elevating platform and a gravitational detection system were also developed by the authors. The gravitational detection system contains pressure sensors.

Additionally, it has been formulated with components that enhance cellular production and utilization of CO_2 such that an exogenous source of CO_2 is not required for the maintenance of CO_2 dependent cellular functions. A circulating water-bath was used for temperature control during the experiments.

2.4 3-D Clinostat (Double-Axis Clinostat)

3-D Clinostat (or Double-axis clinostat, DAC), manufactured by Center for Space and Applied Research Chinese Academy of Science (CSSAR), was used for cell or tissue engineering [12]. It contains two axes which the frame rotates on the horizontal outer axis, and the cell culture vessel rotates on the changing inner axis. The dimension of clinostat cell culture vessel is 12.3 (length) ×9.8 (width) ×10 cm (height). To start a clinostat experiment, the culture flasks containing hFOB1.19 cells after 12-h attached growth were filled with medium, taking care to avoid air bubbles, and were fixed into the clinostat, which was rotated randomly for 24 h at the speed of 10 rpm. Ground controls were static control cultures kept in the same room as the 3-D clinostat.

2.5 In-cell Western (ICW)

The cellular fibronectin of hFOB1.19 cells were detected as follow [13]: Medium was removed by aspiration and cells were washed once with PBS. Cells were fixed with 0.5% glutaraldehyde in PBS for 20 min at room temperature, and then washed 5 times with PBS containing 0.1 % Triton X-100 (5 min per wash) prior to 1.5 h blocking with 3% bovine serum albumin (BSA) buffer (in PBS). hFOB1.19 cells were subsequently probed with anti-fibronectin monoclonal antibody (Ab-3, Neomarkers, USA 1:200) in blocking buffer; with gentle mixing overnight at 4℃. Cells were then washed 5 times with PBS containing 0.1% Tween-20. Bound antibody was detected with secondary antibody labeled with IRDye800CW (1:2000 in blocking buffer plus 0.2% Tween-20; Rockland, Gilbertsville, PA) for 1 h. After 5 washes with PBS/0.1% Tween-20, the plate was scanned and the captured image of the signal was processed and quantified the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) at 169-μm resolution (medium quality, 3-mm focus offset, intensity setting of 5) for both 700- and 800-nm channels. 800 nm signal stand for the cFN in each well and 700 nm signal for cell number in each well. So the number of 800 nm/ 700 nm means the cFN normalized by cell number. Signal was quantified using Odyssey Infrared Imaging System Application Software version 2.1 (LI-COR Biosciences).

2.6 ELISA

Soluble fibronectin in cell culture medium was measure with human FN ELISA kit (EK0349 Boster Inc. China). The samples and standards were prepared and processed as mentioned in the kit manual.

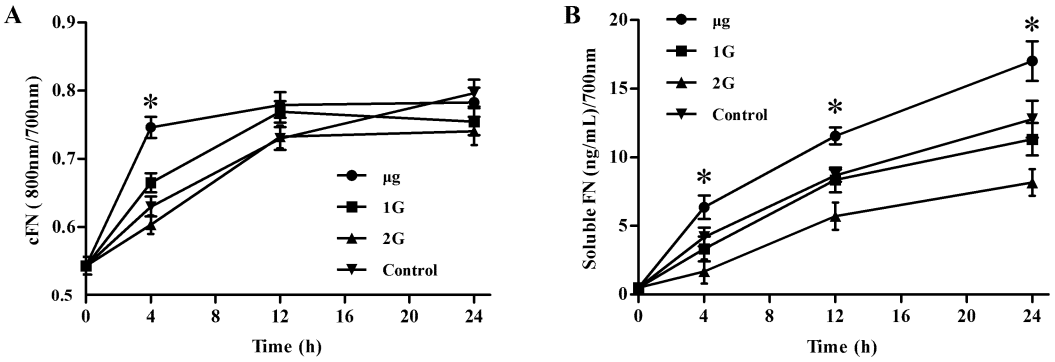

Fig. 1 The effects of gravitational environments formed by large gradient high magnetic field on two forms of fibronectin. (A) The cFN of hFOB1.19 cells from diverse gravitational conditions were detected by ICW; (B) The soluble FN in the condition medium of hFOB1.19 cells from diverse gravitational conditions were detected by ELISA**.** (* for P<0.05, t-student test, n=3)

2.7 Statistical Analysis

Statistically significant differences were determined by Prism statistical software (GraphPad Software Inc). P <0.05 was considered significant in all cases. All data averages or means are accompanied by standard deviations to indicate the amount of variability in the data.

3. Results

3.1 The Effects of Gravitational Environment on Cellular Fibronectin

hFOB1.19 cells in micro wells were cultured in diverse gravitational environments formed by superconducting magnet. No significant growth rate difference was investigated under each gravitational condition. Cellular FN of samples from different time was detected by ICW (Fig. 1A). The samples from simulated weightlessness (μg) had more cFN than other groups at the beginning of treatment (4h). But there is no significant difference between cFN in weightlessness group and other groups as time went on (12h and 24h).

3.2 The Effects of Gravitational Environment on Soluble Fibronectin

cFN is assembled from soluble FN on the surface of cell. The cell culture medium from superconducting magnet was collected and the concentrations of soluble FN were detected by ELISA. As shown in Fig. 1B, samples from weightlessness (μg) always had more soluble FN in 24h. Interestingly, hyper gravity group (2G) showed lower soluble FN than other groups.

3.3 The Effects of 3-D Clinostat on Soluble Fibronectin

Another simulated weightlessness model, 3-D clinostat, was applied to verify the alteration of soluble fibronectin under weightlessness condition. hFOB1.19 cell secreted more FN in clinostat than that in normal culture in every sample time (**Fig. 2**). Compare to the control group, there is no significant cell growth rate difference in 3-D clinostat group.

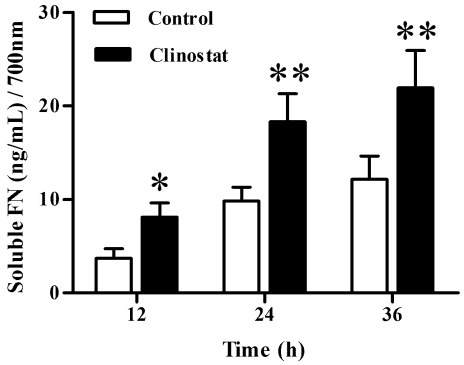

Fig. 2 The effects of 3-D clinostat on soluble FN of hFOB1.19 cells. (* for P<0.05, ** for P<0.01, t-student test**,** n=3)

3.4 Fibronectin Secretion was Affected by Interfering the Interaction between Fibronectin and Integrin

RGD sequence (Arg-Gly-Asp) in Fibronectin molecule can be recognized by integrin, a group of receptor on cell surface. We believe that gravity supply mechanical load to the interaction between fibronectin and its receptor integrin. When the interaction is weakened under physical unloading (microgravity), cells will secret more fibronectin for compensation to strengthen the interaction. In order to verify whether the interference of the fibronectin's interaction with its cell surface receptor, integrin, can affect the expression of fibronectin, RGD-peptide, a FN-integrin binding site peptide was added in the hFOB1.19 cell culture to compete the interaction with integrin. After 24h's culture, the soluble FN was significantly increased with co-cultured with RGD-peptide (**Fig. 3**).

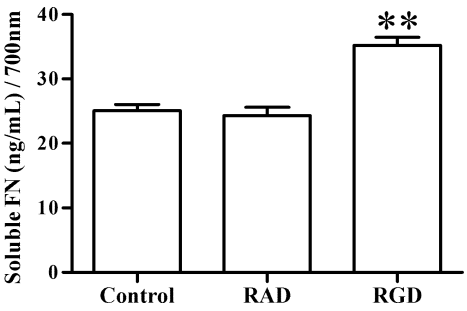

Fig. 3 The effects of binding competition on soluble FN. hFOB1.19 cells in micro wells were co-cultured with RGD-peptide (0.1mg/mL) for 24h and the soluble FN were detected by ELISA. RAD, analogue of RGD was applied as negative control of RGD. (** for P<0.01, t-student test, n=3)

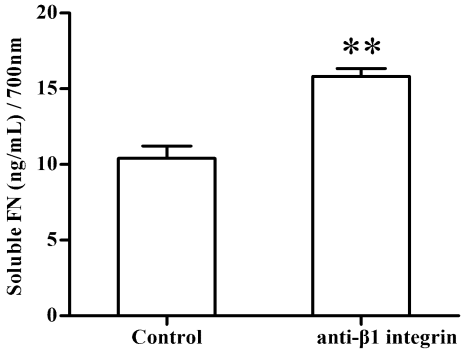

Fig. 4 The effects of integrin blocking on cellular fibronectin. hFOB1.19 cells in micro wells were co cultured with β1integrin antibody (0.5mg/mL) for 24h and the soluble FN were detected by ELISA.(** for P<0.01, t-student test**,** n=3)

On the other hand, to verify whether the interference of the interaction between fibronectin and its cell surface receptor, integrin, can affect the expression of fibronectin, β1 integrin antibody was added in the hFOB1.19 cell culture to block the integrin. After 24h's culture, the expression of FN was significantly increased with co-cultured with β1 integrin antibody (**Fig.4**).

4. Discussion

Cell growth and survival are regulated by several environmental signals, including contact with ECM, other cells, and soluble growth and survival factors. We hypothesize that gravity is a component of the mechanical environment needed for the efficient transduction of cell growth and survival signals from the ECM [14].

The assembly of fibronectin matrix can be considered as chemical reaction process: soluble fibronectin for substrate and cellular fibronectin for product[15]. Soluble fibronectin in culture media can be regarded as extracellular reservoir for cellular fibronectin formation. More soluble fibronectin will benefit cellular fibronectin formation. Integrin plays important role in cellular fibronectin formation (assembly). Once the interaction between integrin and soluble fibronectin is interrupted by physical unloading (microgravity) or by chemically blocking, the formation of cellular will be inhibited. To sustain the regular level of cellular fibronectin, which is crucial to cellular functions, even fate, cell will up-regulate fibronectin expression and enhance the concentration of soluble fibronectin under those specific environments. On the other hand, gravity plays an important role in fibronectin assembly because it strengthens the interaction between integrin and soluble fibronectin. So that FN is involved in the gravity-sensing of osteoblast cell. Besides the gravity, other mechanical factor can affect FN matrix too, such as shear force[16].

FN can be a ligand for a dozen members of the integrin receptor family [17]. Integrins are structurally and functionally related cell-surface heterodimeric receptors that link the ECM with the intracellular cytoskeleton. A large number of different integrins bind to FN, including integrin α5β1, the primary receptor for FN assembly [18]. The FN modulation effects of integrin were proved in this paper by antibody blocking and competitive inhibition. Except for interaction with FN or other ECM proteins outside of cell, a series of signal transduction pathways downstream linked with integrin should be involved in the gravity-sensing and FN alter expression of osteoblast cell. Further research should be performed.

Although the effects of magnetic field on the cells were expected to be removed by comparison of the results from diverse gravitational environments, there still existed controversy about the separating weightless effects from magnetic effects. 3-D clinostat, a relative popular model to mimic weightless effects, was applied in FN research here in order to confirm those results from diamagnetic levitation. The similar results indicated that the reason of FN over-expression is weightlessness. And the validity of diamagnetic levitation to simulate weightless effect was simultaneously proved at least in the FN assembly research here.

Acknowledgement

The Project Supported by National Natural Science Foundation of China (31100667), Natural Science Basic Research Plan in Shaanxi Province of China (Program No. 2011JQ4002) and Northwestern Polytechnical University (NPU) Foundation for Fundamental Research (Grant No. NPU-FFR-JC201162 & NPU-FFR-JC201160). We also thank Dr. Amy Schutz-Geschwender (LI-COR Biosciences) for technical suggestions in ICW experiments.

References

1) Z. Chunqiu, Z. Xizheng, W. Han, H. Daqing and G. Jing: Med. Hypotheses., **67** (2006) 1414.
2) C. Dufour, X. Holy and P. J. Marie: Exp. Cell. Res., **313** (2007) 394.
3) K. K. Parker and D. E. Ingber: Philos. Trans. R. Soc. Lond. B. Biol. Sci., **362** (2007) 1267.
4) V. Vogel and M. P. Sheetz: Curr. Opin. Cell. Biol., **21** (2009) 38.
5) V. E. Meyers, M. Zayzafoon, S. R. Gonda, W. E. Gathings and J. M. McDonald: J. Cell. Biochem., **93** (2004) 697.
6) M. Infanger, P. Kossmehl, M. Shakibaei, J. Bauer, S. Kossmehl-Zorn, A. Cogoli, F. Curcio, A. Oksche, M. Wehland, R. Kreutz, M. Pual and D. Grimm: Cell. Tissue. Res., **324** (2006) 267.
7) M. Infanger, P. Kossmehl, M. Shakibaei, G. Schulze-Tanzil, A. Cogoli, S. Faramarzi, J. Bauer, F. Curcio, M. Paul and D. Grimm: J. Gravit. Physiol., **11** (2004) 169.
8) I. Wierzbicka-Patynowski and J.E. Schwarzbauer: J. Cell. Sci., **116** (2003) 3269.
9) A. Qian, S. Di, X. Gao, W. Zhang, Z. Tian, J. Li, L. Hu, P. Yang, D. Yin and P. Shang: Acta. Biochimica. Et. Biophysica. Sinica., **41** (2009) 561
10) A. Qian, W. Zhang, Y. Weng, Z. Tian, S. Di, P. Yang, D. Yin, L. Hu, Z. Wang, H. Xu and P. Shang: Acta. Astronautica., **63** (2008) 929.
11) A. Qian, D. Yin, P. Yang, B. Jia, W. Zhang and P. Shang: IEEE. Trans. Appl. Supercond., **19** (2009) 42.
12) L. Xiang, F. Qi, D. Dai, C. Li and Y. Jiang: Appl. Biochem. Biotechnol., **162** (2009) 654.
13) J. Li, Z. Wang, G. He, W. Zhang, A. Qian and P. Shang: Anal. Biochem., **398** (2010) 254.
14) A. Andrea and D. H. Kurt: Bone, **38** (2006) 749.
15) I. Wierzbicka-Patynowski, Y. Mao and J.E. Schwarzbauer: J. Cell. Physiol., **210** (2007) 750.
16) A. J. Engler, M. Chan, D. Boettiger and J.E. Schwarzbauer: J. Cell. Sci., **122** (2009) 1647.
17) R. Pankov and K. M. Yamada: J. Cell. Sci., **115** (2002) 3861.
18) A. Askari, P. A.Buckley, A. P. Mould and M. J. Humphries: J. Cell. Sci., **122** (2009) 165.

(Received 21 Sept. 2010; Accepted 12 Jul. 2011)