Cyclic tensile stretch modulates osteogenic differentiation of adipose-derived stem cells via the BMP-2 pathway

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Abstract

Introduction: Mechanical forces play critical roles in the development and remodelling process of bone. As an alternative cell source for bone engineering, adipose-derived stem cells (ASCs) should be fully investigated for their responses to mechanical stress and the mechanisms responsible for osteogenic induction in response to mechanical signals.

Material and methods: We hypothesized that appropriate application of uniaxial cyclic tensile strain to ASCs could increase bone morphogenetic protein-2 (BMP-2) expression and improve osteogenesis of ASCs. To test our hypothesis, ASCs from the same flask of the same donor were subjected to tensile strain with different patterns in order to eliminate the difference of donor site and passage. After surface markers investigation, the osteo-induced ASCs were subjected to uniaxial cyclic tensile stretch with the following two loading patterns: long duration continuous pattern (6 h, 1 HZ, 2000 µε) and short duration consecutive pattern (17 min every day for 10 consecutive days, 1 HZ, 2000 µε). Then osteogenic related genes were analysed by real-time PCR.

Results: The ASCs were positive for the markers STRO-1, CD90 and CD44 and negative for CD34. Cyclic tensile strain of 6 continuous h’ duration significantly increased gene expressions of BMP-2 and Runx2, and depressed OCN mRNA expression. In contrast, mechanical loading of 17 min every day did not significantly affect gene expression of BMP-2, Runx2, OCN or ALP.

Conclusions: We indicate that ASCs may sense mechanical loading in a duration-dependent manner and cyclic tensile stretch may modulate the osteogenic differentiation of ASCs via the BMP-2 signalling pathway.

Key words: adipose-derived stem cells, mechanical stress, osteogenesis, BMP-2.
the signalling pathway of mechanical signals has undergone elaborate investigations [5, 8-11]. In parallel, the responses to mechanical stress of ASCs should be fully understood before they are applied in bone tissue engineering.

Previous investigations suggested that pulsating fluid flow was able to up-regulate some corresponding molecules such as cyclooxygenase-2 gene expression, nitric oxide production, polyamines levels, and paladin [12-14]. However, the osteogenic markers responsible for different patterns of mechanical stress need to be further investigated.

Bone morphogenetic proteins (BMPs) are among the stimulators of bone formation and play a central role in controlling the proliferation and differentiation of osteoblasts. BMPs, especially BMP-2 and BMP-4, exert a biological function by binding to a heterodimeric receptor complex at the cell membrane that transduces the signal into the cell [15]. Runt-related transcription factor 2 (Runx2) acts as a platform through which multiple external signals are integrated, and can be activated by BMP-2 [2]. The expression of Runx2 continues to modulate bone formation by regulating the activity of differentiated BMSCs or osteoblasts [16]. Runx2 induces the expression and synthesis of osteocalcin (OCN), alkaline phosphatase (ALP) and collagen type I (Col-1), which are involved in different stages of osteogenic differentiation [15, 17].

Therefore, the aim of the present study is to investigate the effects of uniaxial cyclic tensile stretch on the osteogenic differentiation of ASCs. We hypothesized that appropriate application of uniaxial cyclic tensile strain to ASCs could increase BMP-2 expression and improve osteogenesis of ASCs.

Material and methods

Adipose-derived stem cells isolation and culture

Adipose-derived stem cells from the same flask of the same donor were subjected to tensile strain with different patterns in order to eliminate the difference of donor site and passage. Adipose-derived stem cells were isolated from 3 male Sprague-Dawley rats less than 4 weeks old, as described by Zuk et al. [18]. The rat was prepared with standard sterile technique to excise the inguinal fat pads, following protocols approved by the IACUC at Sichuan University. The obtained tissue was washed twice with phosphate-buffered saline (PBS, pH 7.4) supplemented with streptomycin sulfate (500 g/ml) and penicillin (600 g/ml) and then incubated in α-modified Eagle’s medium (α-MEM; Gibco, Paisley, UK) and finally minced into small pieces of 0.5 cm³. The fine tissue was digested by 0.05% type 1 collagenase (Sigma, St. Louis, MO) with vigorous shaking for 40 min at 37°C. Floating populations were removed by centrifugation at 1200 rpm (250 g) for 8 min and cells were pelleted. A single-cell suspension was obtained and re-suspended in culture medium composed of α-MEM supplemented with 10% fetal bovine serum (FBS, Gibco, Paisley, UK) and finally seeded on the plastic flask for the final isolation step selected for the plastic adherent populations. Cells were cultured at 37°C in a 90% humidified atmosphere and 5% CO₂ and were used for the following test.

Investigation of surface markers

The cells were analysed by immunofluorescent staining for STRO-1, and immunocytochemical staining for CD34, CD44 and CD90. Briefly, cells in passage 3 were seeded on a 6-well culture plate and grown until they reached 80% confluence. They were washed with PBS 3 times for 5 min each in a shaker and then incubated with 0.2% Triton X-100 in PBS for 20 min at room temperature; this was followed by incubation with 10% goat serum in PBS for 1 h at room temperature. Primary antibodies of STRO-1 (R&D systems, Minneapolis, MN, USA) were applied for 1 h at 37°C, and then washed with PBS in a shaker. FITC-labelled secondary antibody (goat anti-mouse: Zymed, CA, USA) was applied for 1 h at 37°C away from light, then washed with PBS. The slides were then examined with a Leica Inverted Fluorescence Microscope. CD34, CD44 and CD90 (Lab Vision-Neomarkers, Fremont, CA, USA) were analysed by immunocytochemical staining. Briefly, after incubation with primary antibodies, cells were incubated with biotinylated anti-immunoglobulin and reagent using labelled streptavidin-biotin (LSAB) kit peroxidase (Dako, Carpinteria, CA, USA). The peroxidase reaction was visualized with 0.01% H₂O₂ and 3,3’-diaminobenzidine under light microscopy. The nuclei were re-stained with haematoxylin.

Application of cyclic tensile stretch

Force-loading plates were made out of the bottom of the BD Falcon™ 75 cm² cell culture flask (BD Bioscience, CA, USA), 7.8 × 3.8 cm in size and 1.2 mm thick. Adipose-derived stem cells in passage 3 were seeded on the loading plates at a density of 2 × 10⁵ cells per plate and the plates were divided into 10 different groups with 3 samples for each group (Table I). Every plate was kept in glass culture dishes of 9 cm in diameter. When cells were totally attached to the plates 6 h later, the culture medium was replaced with osteogenic medium containing α-MEM supplemented with 10% FBS, 0.1 μM dexamethasone (Sigma), 10 mM glycerol phosphate (Sigma), and 50 mM L-ascorbic acid-2-phosphate (Sigma) [19]. After being osteo-induced for 48 h,
Table I. ASC stretching and sample collection protocol in this study

| Long duration continuous pattern | Short duration consecutive pattern |
|---------------------------------|-----------------------------------|
| Day 1  | Day 3  | Day 7  | Day 10 |
| 6 h cyclic stimulation at 1 Hz.  | 17 min cyclic stimulation at 1 Hz. | 17 min cyclic stimulation at 1 Hz. | 17 min cyclic stimulation at 1 Hz. |
| 6 PCR samples                     | 6 PCR samples                      | 6 PCR samples                      | 6 PCR samples                      |

cells were subjected to uniaxial cyclic tensile stretch by a 4-point bending mechanical loading device [20-22] with the following protocols: long duration continuous pattern and short duration consecutive pattern (Table I). Osteogenic medium was changed every day and the manipulation was done very carefully to prevent contamination. Each experiment was carried out 3 times and all measurements started 2 h after the last strain cycle.

Analysis of mRNA by real-time PCR

Total RNA extraction was started immediately after stretching using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA integrity was verified by 12% agarose gel electrophoresis and its yield and purity were verified by the ratio of A (260)/A (280) using UV-spectroscopic analysis. Then cDNA synthesis was performed by reverse transcriptase (Takara Biotechnology, Co., Ltd. Japan). Preparations of cDNA were checked by spectrophotometry and gel electrophoresis. Primers determined through the established GenBank sequence are listed in Table II. Real-time PCR was run in the ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, USA) using the hot-start DNA Master SYBR Green I Kit (Takara Biotechnology, Co., Ltd. Japan). Preparations of cDNA were checked by spectrophotometry and gel electrophoresis. Primers determined through the established GenBank sequence are listed in Table II. Real-time PCR was run in the ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, USA) using the hot-start DNA Master SYBR Green I Kit (Takara Biotechnology, Co., Ltd. Japan) with the following programme: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melting curve analysis. The specificities of the PCR products were verified by conducting melting curve analyses between 60 and 95°C.

With the LightCycler software, the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene. PCR efficiency (E) was obtained by using the formula E = 10⁻¹/slope. Data were used only if the PCR efficiency calculated was between 1.85 and 2.0. Values of relative gene expression were normalized by the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL) was used for statistical analysis. To quantitatively analyse the gene expression, mean values and standard errors of the mean (SEM) were calculated. A general linear model for repeated measurement analysis was used to study the influence of factors on cell differentiation, and the Bonferroni method was applied for multiple comparisons. Furthermore, the correlation between gene expressions was analysed by Pearson’s test. Differences at a probability of less than 0.05 were considered statistically significant.

Results

Primary culture and phenotypic profile of adipose-derived stem cells

The cell mass of ASCs grew slowly and single colonies could be identified after 4-5 days of culture, finally reaching 80% confluence after 7-10 days in primary culture. However, ASCs grew at a rapid rate in the second passage and presented a homogeneous elongated spindle and polygonal fibroblastic shape.

We used the 3rd passage of ASCs to investigate the CD phenotypes and STRO-1 phenotype. As we expected, CD44 (Figure 1B) and CD90 (Figure 1C) were expressed on ASCs. STRO-1, a marker for cells with multi-lineage potential, was also expressed (Figure 1D). In contrast, CD34, which represents the

Table II. Real-time PCR primer description

| Symbol | Sense (5'→3') | Antisense (5'→3') | Genbank accession | Product size [bp] |
|--------|--------------|------------------|-------------------|------------------|
| GAPDH  | TATGACTCTACCACCGCAGGAAT | ATACTCAGCACCACGATCACC | NM_017008         | 138              |
| OCN    | GACCCTCTCTCCTGTCCTCTCCTG | CACCTACTGGCCTCTGTGTTT | NM_013414         | 124              |
| ALP    | CCTAGCACAACACGACTCCCTCCTA | GTCAGTCAGTTGGTCCGATTC | NM_013059         | 138              |
| Runx2  | TTCTGCAAGGGTCTCTATAGCTTC | CCTTCCATAGCAGTGTAACACC | XM_0010669 | 150              |
| BMP-2  | GTGAGGATTAGCAGGCTTGGGC | CTCGGGTTGAGGATGCTG | NM_017178         | 109              |
haematopoietic lineage marker, was negative [23] (Figure 1A). Although some studies have found that CD34 was present on human ASCs early in passage, which is a phenotypic characteristic different from BMSCs [23, 24], species variation may exist, as studies of murine adipose tissue demonstrated that the CD-34 negative SVF populations were also enriched for ASCs [25].

Different loading patterns had different effects on the orientation of cells. When subjected to the short duration of 17 min every day for 10 days, the cells presented a particular orientation away from the direction of the long axis of the plate from the 4th day of stretching (Figure 2A). In contrast, cells subjected to 6 h of stretching and the control group showed no particular orientation (Figure 2B).

To investigate the effect of mechanical strain on the osteogenic differentiation process of ASCs, the ALP and OCN mRNA expression was quantitatively analysed. Alkaline phosphatase overall was slowly increased by stress loading, while the control group maintained a steady level. However, there is no significant difference between loading groups and controls (Figure 3A). Overall, cyclic tensile stretch for 17 consecutive min did not significantly change the mRNA expression of OCN. Analysis of the between-subject effects showed that stretching significantly decreased OCN mRNA expression ($p < 0.05$) (Figure 3B). Seventeen min duration of loading for various days showed a peak expression of ALP and OCN on the 7th day in the loading group (Figure 3).

Cyclic tension strain up-regulated the osteogenic regulation genes

To investigate the molecular mechanism during the osteogenic process of ASCs treated with cyclic tension strain and osteogenic medium, we determined the mRNA level of BMP, a potential osteogenic differentiation factor, in vitro, and Runx2, the molecular biomarker in the osteogenic process and an important transcription factor that regulates osteogenic differentiation.

The within-subjects effects analysis indicated that loading time had a significant effect on BMP-2 mRNA expression. Duration of 6 h’ loading up-regulated the BMP-2 expression significantly compared with the short time loading duration of 17 min for various days; in addition, analysis of between-subject effects showed that mechanical
loading had a significant influence on BMP-2 expression; 6 h' stretching up-regulated the BMP-2 expression three-fold more than the static group (Figure 4A).

The gene expression of Runx2 was observed at all time points in osteo-induced and mechanical stress loading condition. Figure 4B demonstrates that 17 min loading did not have a significant effect on Runx2 mRNA expression, whereas loading for 6 h resulted in an approximately 5-fold increase in Runx2 expression vs. controls. In addition, 6 h' loading had a significant effect on Runx2 expression vs. all times points of 17-min loading. Further statistical results showed that there was a significant correlation between levels of mRNA expression of BMP-2 and Runx2 in the same sample (p < 0.05), as assessed by Pearson's correlation tests.

Discussion

The results of phenotypic detection of ASCs is similar to most previous studies showing that the ASCs have similar phenotypes as BMSCs, and we have already proved the pluripotency of cells in our

Figure 2. Effects of cyclic tensile stretch on the orientation of ASCs. ASCs of passage 3 were seeded at 2 × 10^5 per plate and cultured in osteogenic medium. A) After being subjected to cyclic tensile stretch for 4 days (17 min a day, 1 Hz, 2000 με), ASCs presented 90% confluence and an orientation perpendicular to the strain axis. B) Cells in static controls and 6 h duration group oriented randomly (magnification, ×100)

Figure 3. Effects of cyclic tensile stretch on osteogenic differentiation marker genes of ASCs. ALP (A) and OCN (B) gene expression are shown in different mechanical loading duration (6 h continuous loading and 17 min every day for consecutive 1 d, 3 d, 7 d, and 10 d). Bars represent mean ± SEM; n = 3; Significant differences among the groups are noted by "p < 0.05"
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former experiments [26]. Thereby ASCs could provide a promising alternative cell source for bone tissue engineering. To date, many papers have focused on the effects of mechanical stress on osteocytes, osteoblasts, BMSCs or the BMSC cell line [4, 7, 27, 28]. However, only a few reports thus far have addressed this issue for ASCs [12-14]. Hence more investigations should be carried out on the mechanosensitivity of ASCs and its underlying mechanism.

Unfortunately, identification of mRNA markers characterizing the differentiation of ASCs towards an osteogenic lineage is further complicated by the known variability of cells from different individuals, different harvesting methods, and different passage of ASCs [29, 30]. Therefore, we use the same passage of the same explants from the same donor animal in order to have identical characteristics in all groups from the very beginning.

To detect the regulatory mechanism of mechanical stretch in the osteogenic differentiation of ASCs, cells exposed to two patterns of cyclic tensile stretch were subjected to analysis of BMP-2 and Runx2 mRNA expression and their correlation. BMP-2 has been proved to play a central role in the proliferation and differentiation of BMSCs [31]. The way that BMP-2 modulates osteogenic differentiation in MSCs varies widely, and one possible mechanism is the interaction with specific transcription factor Runx2, which has been indicated to function downstream of BMPs [17]. The presence of some cis-acting elements, which respond to the strain force in the promoter region of BMP-2, has been suggested on Runx2 [32]. Furthermore, it is indicated that BMP-2 could be enhanced by mechanical tension-stress in bone formation during distraction osteogenesis and could further induce in situ bone formation by a paracrine and autocrine mechanism [33]. Susperregui et al. [34] further found that uniaxial cyclic tensile strain alone was sufficient to increase expression of BMP-2 in the absence of chemical factors. We observed for the first time that cyclic tensile strain increased BMP-2 gene expression threefold and Runx2 fivefold after 6 h of mechanical loading. Moreover, the level of BMP-2 mRNA expression obtained a significant correlation with Runx2 mRNA expression. This suggests that cyclic tensile strain affects early differentiation marker Runx2 in ASCs, and it is possible that elevated expression of Runx2 in ASCs is a consequence of the autocrine effect of BMP-2.

Different studies have demonstrated that mechanical loading could affect ALP and OCN gene expression. Increased ALP activity has been used in many studies as an indicator of osteoblastic differentiation of MSCs [18]. However, previous papers have also reported that osteoblastic subclones exhibited mineralization without increased ALP mRNA activity [35]. In addition, depressed ALP activity has been found after mechanical loading in bone-like cells, human osteoblasts and ASCs [3, 12, 36]. Furthermore, ALP has been reported to be biphasic in MSCs, with decreased activity prior to mineralization, and reaching the first peak concentration on day 14 during mechanical loading in BMSCs [37]. Osteocalcin is thought to be up-regulated at the onset of mineralization [18]. However, after cyclic mechanical loading of a long period or high magnitude, OCN gene expression or concentration was decreased [7, 38, 39], which is in accordance with the result in our study (Figure 3B). We also found that ALP gene expression was not significantly influenced under cyclic tensile stretch. Nevertheless, ALP gene expression holds a steady level in the osteogenic medium whereas after cyclic tensile stretch for 17 min every day ALP activity tends to increase and reach a peak on the 7th day of mechanical loading. Thus, the trend of ALP is probably to increase by cyclic tension, and it may increase to a significantly high level in the later stage of osteogenesis.

We have also observed that mechanosensitivity of ASCs varies with the patterns of cyclic uniaxial mechanical stretch. Two different durations of mechanical loading, namely continuous 6 h for 1 day and 17 consecutive min for 10 days, were applied at a fixed frequency of 1 Hz and settled magnitude of 2000 µε. The latter loading pattern was expected to simulate a physiological oral masticatory condition [40]. The results indicated that the short duration of 17 min mechanical loading lasting for 10 days changes the orientation of ASCs, whereas the mechanical loading of long duration has significant effects on BMP-2, Runx2 and OCN gene expression. The particular change of orientation perpendicular to the long axis of stretching is consistent with the observations reported by Koike et al. [7] and Zhang et al. [41], who found that BMSCs responded to uniaxial cyclic stretch stimulation by reorganizing their orientations close to 100-110 degrees after a prolonged stimulation. Mechanical loading time is one of the determining parameters advocated by Kaspar et al. [3]. Most studies reach a consensus that the mechanical responsiveness of MSCs presents a type-[42], magnitude-[7], frequency-[43], cycle number-[3], and differentiation-[4] dependent manner; however, the most appropriate combination of the mechanical parameters is still obscure. One possibility is the different conditions in vitro and in vivo, that is, cells in vivo did not receive the same strain signal as in the cell stretching system since the bone matrix filters the physical load, and, moreover, the in vivo biomechanical condition is too complex to be
simulated by in vitro mechanical loading models or algorithm models [44, 45].

We have to point out that one limitation of this study is the loading plate cultured in the semi-open culture dish, and the frequent change of culture location between culture dish and loading dish made it vulnerable to fungal contamination. This shortened the loading time to a significant degree and limited the loading days (17 min of daily loading) to less than 2 weeks. Despite its limitation, this study does suggest that cyclic strain could affect the osteogenesis of ASCs and the long duration of 6 h is more likely to have this effect.

In conclusion, we found that ASCs are sensitive to cyclic tensile strain. Cyclic tensile strain of 6 continuous h duration rather than 17 min of consecutive stretching could significantly increase gene expression of BMP-2 and Runx2 and depress OCN mRNA expression. We indicate that ASCs may sense mechanical loading in a duration-dependent manner and cyclic tensile strain may modulate the osteogenic differentiation of ASCs via the BMP-2 signalling pathway.

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