Effects of verapamil on the immediate-early gene expression of bone marrow mesenchymal stem cells stimulated by mechanical strain in vitro

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Background:
To study the effects of verapamil on the immediate-early genes (IEGs) expression of bone marrow mesenchymal stem cells (MSCs) stimulated by cyclic mechanical strain, in order to deduce the role of calcium ion channel in the cell signaling responses of MSCs to mechanical strain.

Material/Methods:
MSCs were isolated and cultured, and the passage of 3–6 MSCs were stimulated by mechanical strain and pretreated with or without verapamil. After that, flow cytometry was used to measure the fluorescence intensity of intracellular Ca^{2+} immediately. The expression of early-response genes/proteins (c-fos, c-jun and c-myc) were examined by RT-PCR, immunohistochemistry and Western blot.

Results:
Intracellular Ca^{2+} concentration of MSCs significantly changed when stimulated by cyclic strain, and the expression of c-fos, c-jun and c-myc remarkably increased in both mRNA and protein levels, while verapamil pre-treatment partially inhibited these effects (P<0.01).

Conclusions:
The changes of the intracellular calcium concentration of MSCs induced by mechanical strain, dependent on the regulation of calcium channel activation, might play a role in the early response of MSCs to cyclic strain.

Key words: mesenchymal stem cells  mechanical strain  c-fos  c-jun  c-myc  Verapamil

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Background

Mechanical loading is crucial for the regulation of various tissues and cells. Mesenchymal stem cells (MSCs) are the cellular basis of bone remodeling and osteoblast differentiation in vivo [1]. Previous studies in vitro showed that appropriate strain could promote MSCs proliferation and osteoblast differentiation [2,3], while overloaded strain could damage MSCs [4]. However, there is great difference in the cell biological effects when stimulated by different strains [5], and the signal transduction pathway of the responses of MSCs to mechanical strain is still not clear.

Recent studies on cell mechanics in vitro demonstrated that cells produce or activate various second or third messengers after mechanical strain, and various signal molecules interact with each other, constructing the network regulation to activate transcription factors. Then the signals will be transmitted from extra-cellular to intracellular environments, and the expression and distribution of relevant genes or proteins will be initiated or regulated to induce series of biological effects [6]. The early cell response to mechanical strain is complicated [7]. Calcium ion, one of the most important intracellular second messengers, regulates various physiological activities [8]. Intracellular calcium is composed of combined calcium and free calcium. Commonly, most of intracellular calcium is combined calcium (99.9%), distributed in the nuclear, mitochondria, endoplasmic reticulum and membrane, while free calcium is rare. The intracellular calcium significantly increases when the cell is stimulated by physical or chemical factors. As an important second messenger, calcium is combined with its main receptor, calmodulin (CaM), and the Ca²⁺/CaM compound can regulate the activity of several enzymes. Walker et al. [9] reported that the increase of the intracellular calcium concentration of MSCs is related to the up-regulation of matrix proteins such as osteocalcin, osteopontin. Hutcheson et al. [10] found that strain-dependent accumulation of intracellular Ca²⁺ was correlated with apoptosis in aortic valve interstitial cells (AVICs), and that those findings indicate early mechano-transductive events that may initiate aortic valve (AV) calcification pathways.

C-fos, c-jun and c-myc are important intermediate products of cell responses to mechanical signals. These genes were activated transiently and rapidly in response to a variety of cellular stimuli. They represent a standing response mechanism that is activated at the transcription level in the first round of response to stimuli, before any new proteins are synthesized. These genes, called immediate-early genes (IEGs) [11], can activate downstream proteases and cytokines and initiate biological effects such as cell proliferation and differentiation.

Verapamil is an L-type calcium channel blocker [12]. In the present study, we observed the expression of Ca²⁺ and c-fos, c-jun, c-myc after pretreatment with Verapamil on the MSCs stimulated by cyclic strain, in order to explore the dependence of calcium ion channel activation on the early response of MSCs.

Material and Methods

Apparatus and reagents: DMEM-LG culture medium (Gibco), fetal bovine serum (Hyclone), Verapamil (Alexis), Trizol (Sigma), Trypsin and agarose (Ameresco), RT and PCR kit (TaKaRa), Calcium ion probe fluo-4/AM (Invitrogen), c-fos, c-jun, c-myc primary antibody (Santa Cruz), Primers (Shanghai Yingjun gene biological technology Co., LTD), cell protein extraction agents, BCA Protein Assay Kit, etc; Main experimental instruments included inverted phase-contrast microscope (OLYMPUS, Japan), flow cytometry (BD, USA), ultraviolet spectrophotometer (SHIMADZU 1240, Japan), PCR instrument (Eppendorf, USA), vertical electrophoresis cells, and electrophorator.

Cell culture

MSCs were acquired from patients with congenital hip dysplasia undergoing pelvic osteotomy (n=10, 3–9 years old, mean age 5.2, with the signed informed consent of their parents). The institutional ethics committee approved the procedure and written informed consent was obtained from each patient. During surgery, samples were collected by iliac crest puncture. MSCs were isolated using Percoll solution gradient centrifugation (Sigma, St. Louis, MO) and were seeded into DMEM-LG medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/mL penicillin and 100 U/mL streptomycin (North China Pharmaceutical Factory, Beijing, China) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Culture medium was replaced and cells were passaged as necessary. The second generation of MSCs was harvested to identify CD34, CD44 and CD105 antigens via flow cytometry (BD, USA), ultraviolet spectrophotometer (SHIMADZU 1240, Japan), PCR instrument (Eppendorf, USA), vertical electrophoresis cells, and electrophorator.

Effect of stretch and Verapamil on intracellular Ca²⁺

The passage 3-6 MSCs were inoculated in 6-well cell culture silicon plate (Flexcell, USA) at a density of 1.5×10^5/2 ml per well,
and the cells adhered the next day. After completely covering the culture medium membrane, the cells were treated with Verapamil 30 min before strain in the concentration of 20 µmol/l as shown in Table 1. The mechanics parameter is sinusoidal wave at 1 Hz for 12%. The cells in different groups came from the same source. A no-stimulation group was conducted under the same conditions except for the mechanical strain stimulation. All the experiments were conducted in the incubator. The intracellular calcium was detected immediately after stimulation. After washing twice by extracellular fluid, the fluo-4/AM was added and incubated for 30 min at 37°C, protected from light. The supernatant was then removed and cells were washed 3 times to eliminate the rudimental probe. Cells were collected after trypsinization and centrifugation, and then cell suspensions were prepared. The fluorescence density was detected by flow cytometry in 488 nm.

Effect of stretch and Verapamil on the intracellular c-fos, c-jun and c-myc expression

MSCs were cultivated in conditioned silicone dishes. After the cells covered approximately 80%, they were respectively loaded with strain at 12% intensity for 0.5 h, 1 h, 2 h, and 4 h. The samples were then collected to detect the gene expression of c-fos, c-jun and c-myc. In the above experiments, the frequency of the strain is sinusoidal wave at 1 Hz. A no-stimulation group was conducted under the same conditions, except for the mechanical strain stimulation, and all the experiments were conducted in the incubator. Other cells were divided into 4 groups: A (strain–, V–), B (strain–, V+), C (strain+, V+) and D (strain+, V–). The strain group was loaded cyclic strain for 1 h and the other parameters were as above. Samples were then collected to detect the expression of c-fos, c-jun and c-myc by RT-PCR, immunochemistry, and Western blot. The sequences of PCR primers were shown in Table 2. The information of primers came from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/LocusLink). Primers were synthesized by Yingjun Company (China). RNAs were extracted by Trizol, and RT-PCR was conducted. The PCR products were electrophoresed in agarose gel, scanned with ultraviolet transilluminator (GelDocXR) and the bands were analyzed. After the cell experiment, the membranes were washed 3 times, fixed by 4% paraformaldehyde for 30 min at 37°C, protected from light. The supernatant was then removed and cells were washed 3 times to eliminate the rudimental probe. Cells were collected after trypsinization and centrifugation, and then cell suspensions were prepared. The fluorescence density was detected by flow cytometry in 488 nm.

Table 1. Groups and treatments.

| Groups | Stimulation times | Verapamil pretreatment (−/+) | Groups | Stimulation times | Verapamil pretreatment (−/+) |
|--------|-------------------|-----------------------------|--------|-------------------|-----------------------------|
| a0     | 0 min             | +                           | b0     | 0 min             | −                           |
| a1     | 1 min             | +                           | b1     | 1 min             | −                           |
| a3     | 3 min             | +                           | b3     | 3 min             | −                           |
| a5     | 5 min             | +                           | b5     | 5 min             | −                           |
| a10    | 10 min            | +                            | b10    | 10 min            | −                           |

Table 2. Primers design of c-fos, c-jun, c-myc and GAPDH.

| Gene   | Primer sequences                                                         | PCR product | Annealing temperature |
|--------|--------------------------------------------------------------------------|-------------|----------------------|
| c-fos  | Sense: 5’ TCCGAAGGGAAAGGAATA 3’ Antisense: 5’ TGATAGAAGGCCAGCATATACACGCATGAGGACCCAGAT 3’ | 476bp       | 52°C                 |
| c-jun  | Sense: 5’ AGGAGAGCAGCAGCATACCC 3’ Antisense: 5’ TGGTCGAGACTGGGAGGC 3’ | 564bp       | 56°C                 |
| c-myc  | Sense: 5’ ATCATCCGGAGCTGATGTTGG 3’ Antisense: 5’ GGGTGGAGAGGTTGTC 3’ | 496bp       | 54°C                 |
| GAPDH  | Sense: 5-GAAGGTGAAGGTCGGAGGTCA-3 Antisense: 5-GAAGATGAGGTTGAGGATTTC-3 | 226bp       | 52°C                 |
Equal amounts of protein (20 µg) of each sample were loaded per well. Electrophoresis was performed. Proteins were transferred overnight, blocked for 2 h, and then incubated for 24 h at 4°C with the first antibody (1:1000). After rinsing 3 times in TBS-T(10 mmol/L Tris-Cl+100 mmol/L NaCl+0.1% Tween20), the sections were incubated with secondary antibody (1:500) for 1 h at room temperature in a blocking solution of 3% normal goat serum in 0.01 M PBS with 0.3% Triton-X 100 (NGST). After 3 rinses in TBS-T, the band was visualized by chemiluminescence reagents with Hyperfilm ECL films.

Statistical analysis

Using SPSS10.0 statistical software, single-factor analysis of variance was performed. A value of P<0.05 was considered statistically significant.

Results

Morphology of the MSCs under microscope

The primary MSCs adhered to the flask wall 2-4 days after seeding, and were fibroblast-like and spindle-shaped. Clustered, rapid cell proliferation was observed after the first medium replacement at days 3 and 4. After multiple medium replacements and PBS washing, hematopoietic cells were gradually eliminated. At days 12–14, cell confluence reached 80%. After passage, the growth rate of the MSCs increased and confluence was achieved after 7 to 10 days. The cells were slender, spindle-shaped, and were spirally distributed until cell fusion. The immunopositive rates of CD34, CD44, and CD105 of the MSCs were 2.3, 97.5, and 96.1%, respectively, which is consistent with the known phenotypic characteristics of MSCs [15]. Cells were inoculated in 6-well cell culture plates covered with silicone. After 48 h, cell confluence reached 80%. The cell morphology showed no significant difference among groups, and no cast-off cells or ruptured cells were detected.

Effect of various stimuli on the intracellular Ca^{2+}

As shown in Figure 1, after mechanical strain application in different times, the fluorescence intensity of the intracellular Ca^{2+} increased in different degrees. The intracellular Ca^{2+} concentration increased at 1 min after stretch, and the fluorescence intensity was 164.63% of the control group. This increase was more apparent at 3 and 5 min, with the fluorescence intensity of 200.06% and 211.21%, respectively, compared with the control group. The magnitude of the up-regulation of the Ca^{2+} concentration showed a decreasing tendency after 10 min, with the intensity of 175.91% of the control. Pretreatment with calcium channel blocker Verapamil 30 min before strain partially blocked the increase of intracellular Ca^{2+}. But along with the time extension, the blocking effect became weaker, with the intensity of 133.62%, 140.27%, 146.77%, and 148.31%, respectively, of the control.

Expression of c-fos, c-jun and c-myc of MSCs by RT-PCR

As indicated by Figure 2A, 2B, there was little or no expression of c-fos, c-jun and c-myc in the no-strain group. After short-term (0.5 h, 1 h, 2 h, 4 h) loading of the strain, gene expression of the c-fos, c-jun and c-myc significantly increased. With the time extension the changes showed the tendency was increase-keeping on-reversion. But there was some difference in the time course and density, of which the increase of c-myc lasted for a long time. After 4 h, the expression of c-fos, c-jun and c-myc recovered to the former level.

As demonstrated in Figure 2C, 2D, after loading with the strain for 1 h, the gene expression of the c-fos, c-jun and c-myc significantly increased. Pretreatment with Verapamil partially blocked the increased expression, with the inhibition of 44.29%, 22.18% and 49.26%, respectively. The inhibition of c-fos, c-jun and c-myc was comparatively apparent.

Expression of c-fos, c-jun and c-myc of MSCs by immunochemistry

The immunochemical staining of MSCs cytoplasm and nucleus without strain (with or without Verapamil pretreatment) was mild, and the staining of c-fos, c-jun and c-myc was similar. The nuclear staining became brown after strain for 1 h. The staining was attenuated by Verapamil pretreatment, with deep staining at the edge of the nuclear membrane (Figure 3).

Protein expression of c-fos, c-jun and c-myc of MSCs by Western blot

As demonstrated in Figure 4, there was little or no expression of c-fos, c-jun and c-myc in the no-strain group, with or without
Verapamil. After loading with the strain for 1 h, the expression of the c-fos, c-jun and c-myc significantly increased, and pretreatment with Verapamil partially blocked the up-regulation of the expression.

**Discussion**

The basic theory of the response of the organism to the mechanics is that cells receive mechanical signals in the microenvironment, and then transform the signals into series of intracellular biochemical reactions, including the reception of the strain, production of the biological signals, transduction of the intracellular signals, and changes of gene expression [7,16,17]. Different extracellular signal stimuli had different signal transduction pathways, and several mediators were involved. The present study found that transient strain could significantly increase the fluorescence density of the intracellular Ca^{2+} of MSCs. The effect was time-dependent within a certain range, and the magnitude of the up-regulation of the Ca^{2+} concentration decreased after 10 min. After pre-treatment with Verapamil, the Ca^{2+} concentration still significantly increased very early (1 min), and then the increase became gradual. Verapamil partly blocked the increase of intracellular Ca^{2+} of MSCs, but the effect became weaker over time, thus we deduce that the rapid increase of Ca^{2+} in the very early time could be attributed to the release of Ca^{2+} from the Ca^{2+} pool by the strain stimulus. As well, the direct reception of the membrane stretch sensitivity calcium channel to the mechanical signal could promote changes in Ca^{2+} concentration. The subsequent gradual increase in Ca^{2+} concentration suggests the exhaustion of the Ca^{2+} pool. Also, the internal flow of Ca^{2+} was blocked by the inhibition of the L-type Ca^{2+} channel by Verapamil. These two factors resulted in the development of the “platform stage” of Ca^{2+} increase.

There are 3 cis-acting elements in the promoter of c-fos: cAMP-response element (CRE), serum response element (SRE), and sis-inducible element (SIE). The synergistic reaction of the above elements plays an important role in the correct expression of c-fos [18]. Ordinarily, the phosphorylated sites are phosphorylated by GSK-3 and CK-2, and are dephosphorylated immediately when receiving the growth stimulus signal to remove the inhibition to the cytokines maintaining the rest state of cells, and promote the cell growth, which allows fast induction of c-jun. The c-fos and c-jun proteins consisted of 380...
and 331 amino acid residues, respectively, and were synthesized when the cells were stimulated. The c-fos and c-jun proteins return to the nuclei after phosphorylation to compose the AP-1 transcription factor, which integrated the upstream signals and combined to the AP-1 attachment region with high affinity to affect the expression of the target genes [19,20]. The AP-1 transcription factor may involve the signal transduction and influence the cell proliferation, and could be considered as the early marker of biosynthesis. Many genes (e.g., osteocalcin, osteopontin, type I collagen protein and Runx-2) contain the AP-1 combining sites (TGACTCA) [21,22]. C-myc [23] is different from the gene family of c-fos, c-jun (AP-1 gene family). The C-myc gene product is a nuclear protein related to cell proliferation, which is synthesized in the cytoplasm and composed of an oligomer with another protein such as Max. The nuclear localization signal of c-myc makes it locate in the nucleus, but c-myc will activate or inhibit several gene transcriptions when combined with the specific DNA site, so as to grow or differentiate. In the normal cells without stimulus, the expression of c-fos, c-jun, and c-myc is very low. Some studies [24] reported that the fos protein transiently increased in the osteoblast of perichondrium and periosteum of the skull and femur in newborn mice, suggesting the important role of c-fos in the normal osteoblasts differentiation. Also, immediate-early protein is the inhibitor of immediate-early gene expression. In the case of c-fos, for instance, the reduction of the c-fos gene transcription after induction is attributed to the inhibition of the protein product of c-fos itself. Therefore, in the present study, the immediate-early genes of MSCs significantly increased after transitory mechanical strain, while the genes rapidly returned to the former level with the time extension. This tendency is in accordance with the other studies on other cells [23,25–27].

In the present study, after loading strain for 1 h on MSCs, the expression of c-fos, c-jun, and c-myc significantly increased, which could be partly blocked by Verapamil pretreatment, detected by PCR, immunochemistry and Western blot. Peake et al. [28] found that the immediate-early genes such as c-fos are downstream of the calcium signal pathway, and their concentrations are affected by calcium. Kletsas et al. [29] demonstrated that the c-fos and c-jun expression increased in the human
periodontal ligament (hPDL) osteoblastic cells after straining, and then the AP-1 transcription factor increased to promote the expression of the osteoplastic genes. The mechanical stimulus increased the intracellular calcium concentration, and promoted the combination of Ca$^{2+}$/CaM and CaMK to activate CaMK, which could enhance the transcriptional activity of c-fos through the phosphorylation of Ser133 and is involved in the nuclear reaction with PKA. There are many kinds of calcium channels [30], such as voltage-sensitive calcium channel (mainly as L-type calcium channel), receptor operated calcium channel, and second operated calcium channel. Verapamil, used in the present study, is an L-type calcium channel, which can specifically block the inflow of the Ca$^{2+}$ induced by the change of voltage-sensitive calcium channel stimulated by strain, and therefore partially block the immediate-early protein expression. However, the blocking effect is still only partial, because the increase of intracellular calcium is independent of the sole activation of calcium channel, while Verapamil can only block the biological effect induced by an L-type calcium channel. Peake et al. [28] reported that Ca$^{2+}$ chelator (EDTA) and mechanical strain-sensitive calcium channel blocker could partly inhibit the biological effect of osteoblasts induced by mechanical stimulus. L-type calcium channel nifedipine used by Walker et al. [9] could also partly block the activation of the downstream of calcium pathway. Therefore, we deduce that the increase of intracellular calcium concentration induced by mechanical strain is related to the activation of several calcium channels, the inflow of extracellular Ca$^{2+}$ and the mobilization of intracellular calcium.

Conclusions

Therefore, we it appears that the mechanical strain on MSCs can initiate several calcium channels, induce extracellular Ca$^{2+}$ inflow and/or calcium release from the endoplasmic reticulum to increase the intracellular calcium concentration, mediate the expression of immediate-early genes, and may involve biological effects such as signal transduction and cell proliferation, as well as differentiation. However, the specific course should be further investigated.

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