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Pressure simulation of orthodontic force in osteoblasts: a pilot study

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Structured Abstract

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Objectives – To elucidate the *RUNX2* gene expression induction in human osteoblasts after mechanical loading.

Design – Using a stringent pulse-chase protocol human osteoblasts were exposed to centrifugal pressure force for 30 and 90 min. Untreated control cells were processed in parallel. Before, and at defined times after centrifugation, total RNA was isolated. *RUNX2* gene expression was measured using real-time quantitative reverse transcriptase polymerase chain reaction. The stress/control ratio was used to illustrate possible stimulatory or diminishing effects of force application.

Results – Immediately after 30 min of force application the *RUNX2* gene expression was induced by a factor of 1.7 ± 0.14 as compared with the negative control. This induction decreased rapidly and reached its pre-load levels within 30 min. Longer force applications (up to 90 min) did not change the *RUNX2* gene expression.

Conclusion – In mature osteoblasts centrifugal pressure force stimulates *RUNX2* gene expression within a narrow time frame: loading of mature cells results in a temporary increase of *RUNX2* expression and a fast downregulation back to its pre-load expression level. With this pilot study the gene expression behavior after mechanical stimuli could be determined with a simple laboratory setup.

Key words: mechanical load simulation; orthodontic tooth movement; osteoblasts; *RUNX2*

Introduction

The skeleton is an efficient feedback-controlled, steady-state system that continuously integrates signals and responses. The local structural adaptation of

bones to mechanical loads is the basis for almost all orthodontic and orthopedic procedures. Increased mechanical loads stimulate bone formation and suppress resorption, inducing osteoblast activity, whereas unloading has the opposite effect and stimulates bone degradation by osteoclasts (1). Hunter described this remodeling adaptation over 200 years ago: 'bone moves out of the way of pressure' (cited in 2).

The *Runt*-related transcription factor 2 (*RUNX2*), is one of the major genes responsible for bone development and bone homeostasis. Its function is essential for the differentiation of osteoblasts from undifferentiated progenitor cells. *RUNX2* transcripts are translated into protein at sites of bone and cartilage formation (3). Similar distribution patterns during intramembranous and enchondral ossification in human fetal oro-craniofacial tissues has been reported (3). Its involvement was elucidated as mutations in the *RUNX2* gene cause cleidocranial dysplasia (CCD; MIM 119600) (4, 5). This syndrome is characterized by an overshooting development of tooth germs in the permanent dentition and a dysplastic bone formation particularly in the craniofacial skeleton, including brachycephaly, delayed closure of fontanels and sutures, and hypoplasia of the midface (6, 7). In mice, homozygous loss of the *RUNX2* gene leads to a complete absence of enchondral and membranous ossification because of the lack of osteoblast differentiation (8). Therefore, this gene is recognized as the master gene for bone development and is also part of major signal transduction pathways (9). Major osteoblast-specific genes, e.g. osteocalcin, alkaline phosphatase, and the type-I collagens contain RUNX2 binding sites in the regulatory parts of their genes (9–11). In addition to its role in osteoblast differentiation, it is postulated that *RUNX2* is also involved in bone matrix deposition in differentiated osteoblasts (12). The expression of *RUNX2* itself is also regulated through factors influencing osteoblast differentiation, e.g. the BMP growth factor group. Additionally, RUNX2 binding sites are found in the *RUNX2* gene itself, emphasizing an autoregulatory feedback mechanism controlling its gene expression (12, 13).

The effects of mechanical load/unloading on bony tissue were investigated in different animal, cell and organ culture approaches. Mechanical loading consisting of tension force (14), stretching (15), intermittent hydrostatic compression (16), micro- (17, 18), and hypergravity (19) have all been described using *in vivo* and *in vitro* systems (18, 20, 21). Recently, Ziros and co-workers (22) showed an increase in the *RUNX2* expression level after 30 min of tension type of loading in a setting using periodontal-ligament fibroblasts. *RUNX2* protein synthesis was first observed after 3 h of loading, reaching its maximum after 6 h. This coincides with modifications in the RUNX2 protein through phosphorylation, which is catalyzed by stimulated mitogen-activated protein kinases (MAPK) (22). In contrast to mechanical load application, immobilization leads to *RUNX2* inactivation and suppression of the osteoblastic phenotype (23). After 24 h of simulated microgravity, alkaline phosphatase and osteocalcin, markers of osteoblast differentiation, were suppressed (23).

Orthodontic force leads to alveolar bone resorption at the pressure areas and bone apposition at the tension regions. This force affects both the extracellular matrix (ECM) and the cells. However, the cells respond both to force, probably via specific mechano-signal transduction pathways, and to the changes (tissue injury) in the ECM. Only an *in vitro* cell culture model can evaluate the direct cellular response to force excluding cell–ECM interactions. In such a model, using human periodontal ligament fibroblasts (hPDLF), we previously showed that pressure type of force achieved by centrifugation, which simulates orthodontic force, increased the mRNA levels of matrix metalloproteinase 1 (MMP-1) and tropoelastin (24, 25).

The objective of this study was to utilize this *in vitro* model on the dominant cellular component of the bony structure and monitor possible changes in *RUNX2* gene expression in osteoblasts subjected to pressure force.

Materials and methods

Cells and cell culture

Normal human osteoblasts were supplied by Cambrex (Verviers, Belgium) as cryopreserved cultures in the second passage. The cells were expanded in plastic flasks in OGM medium provided by the manufacturer and following their instructions (Cambrex). Briefly, OGM medium was supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/ml ascorbic acid, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cultivation and feeding was done according to the

manufacturer's instructions. Osteoblasts of the fourth passage were seeded into 12-well plates at 38 000 cells/well. The plates were ready for stimulation after the cells reached confluence.

Pressure force simulation

Pressure was achieved through centrifugation according to Redlich et al. (18) using the following pulse-chase protocol. The plates (described above) were centrifuged (= pulse) at 200 × *g* for 30 min or 90 min, respectively using a Hettich Rotixa/P centrifuge (Hettich, Tuttlingen, Germany) equipped with micro titer plates swing-out buckets (Fig. 1). This model is based on application of a constant centrifugal pressure force at a magnitude of 40.3 g/cm^2, which resembles clinical orthodontic force (26). The calculation of force (24) is based on the equation:

$$P = m \times r \times \mathrm{RPM}^2 \times \pi^2/(A \times 9.8 \times 900),$$

with P, pressure per cm^2 of cells (kg), m, mass of medium (0.00076 kg), r, radius (0.208 m), RPM, revolution/min (930), and A, area of contact between medium and cells (3.8 cm^2). Cell cultures grown under identical conditions were used as negative controls and remained unloaded. Immediately before (T_0, pre-load time period) and at defined points in time after centrifugation (1.5, 2.5, 4.5, 8.5, 16.5, and 32.5 min, respectively; post-load time period) total RNA was isolated from experimental and control cells as described below. From each plate two wells were randomly chosen for the determination of the cell count and cell viability with the trypane blue exclusion test.

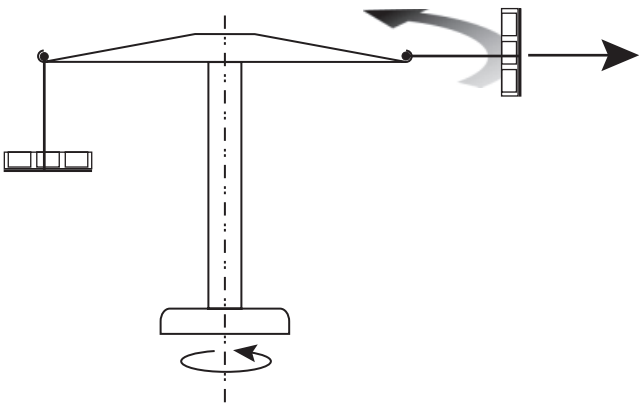

Fig. 1. Schematic drawing of the experimental setup for hypergravity simulation through centrifugation. Side view of a centrifuge. Left, the buckets in resting position. During centrifugation (right) the buckets swing out because of centrifugal force (thick arrows).

RNA isolation

Total RNA was isolated using the guanidinium thiocyanate-based 'RNeasy Mini Kit' (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following modifications: 10 mM dithiotreitol was used instead of β-mercaptoethanol. For cell lysis the culture medium was aspirated at defined chase times after centrifugation and 350 μl RTS buffer (Qiagen) supplemented by dithiotreitol (DTT) medium was applied. After 5 min the content of each well was collected in separate centrifugation tubes and the wells were washed with 250 μl RTS/DTT medium. Both fractions were combined and stored at −70°C until further processing. RNA isolation commenced using the above-mentioned 'RNeasy Mini Kit'. 'QIAshredder columns' (Qiagen) were used to disrupt high molecular genomic DNA, which was then removed through on-column digestion with 'RNase-free DNase Set' (Qiagen) during the subsequent purification procedure. The purified RNA was eluted with RNase-free water and the yield was measured using an UV-photometer.

Real-time quantitative RT-PCR

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the 'QuantiTect SYBR Green One-Step RT-PCR-Kit' (Qiagen) on a 'Gene-Cycler 2000' (Corbett Research, Sydney, Australia) according to the manufacturer's instructions. In short, each PCR reaction consisted of 10 ng of total RNA, the appropriate amounts of the Taq DNA polymerase and reverse transcriptase (RT) enzyme mixtures and 0.5 μM of each PCR primer (see Table 1) in a reaction volume of 25 μl. The RT-PCR was started with 30 min of cDNA synthesis at 50°C. This was followed by a denaturating step of 15 min at 95°C in order to inactivate the RT-enzyme mix and to activate the Taq DNA polymerase mixture. Thirty-five PCR cycles were done, each consisting of 15 s denaturation at 94°C, 30 s primer annealing at the primer specific temperatures (Table 1), 30 s primer extension at 72°C, and 15 s fluorescence based quantification at temperatures 5°C below the melting point of the respective amplicons (Table 1). The PCR was finished with an additional 10 min elongation phase at 72°C. The specificity of the PCR product was verified by adding a

Table 1. **Specifications of the PCR primers used in the quantitative real-time RT-PCR. Shown are the gene-specific primer sequences for the forward (f) and reverse (r) primers of *RUNX2* (31) and *GAPDH* (32), and their specific reaction conditions**

Gene of interest	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Data acquisition temperature (°C)	Amplicon length (bp)	GenBank accession number
RUNX2	f: ACT TTC TCC AGG AAG ACT GC	50	80	295	AH005498
	r: GCT GTT GTT GCT GTT GCT GT				
GAPDH	f: ACC ACA GTC CAT GCC ATC AC	58	80	452	X01677
	r: TCC ACC ACC CTG TTG CTG TA				

melting curve analysis between 60°C and 95°C. Each PCR setup contained a 'no-template control', the gene-specific quantification standards, the T_0 samples and stress and control samples of corresponding chase time. Real-time products were analyzed on 2% agarose gels to confirm the size of the PCR fragment and thereby primer specificity.

Quantification standards were produced as follows: The appropriate genes were amplified from total RNA as described. The PCR fragments were separated by agarose gel electrophoresis. Gene specific PCR fragments, as determined by the size of the PCR fragment, were excised and purified using the 'MinElute Gel Extraction Kit' (Qiagen). The purified products were quantified photometrically and diluted over a functional range of concentrations (five orders of magnitude).

Data analysis

The experiments were performed at least twice from different human donors of primary cells. Each chase time was represented by at least two independent samples. For each gene the quantifications were normalized against the T_0 samples of the respective gene under surveillance. Arithmetic mean and standard deviation were calculated for each chase time.

Results

In order to simulate the effect of orthodontic tooth movement on bone cells, isolated human osteoblasts were exposed to a centrifugal pressure force for 30 or 90 min. Before (= pre-load) and at defined times after force application (= post-load) total RNA was isolated. Gene expression of *RUNX2* was measured using quantitative real-time RT-PCR. Unstimulated cells grown under identical conditions and processed in parallel were used as negative controls. The results of the force applications were measured in comparison with the corresponding negative controls.

In all experiments the trypan blue exclusion test confirmed a cell vitality of at least 90% after application of centrifugal pressure. The application of centrifugal force for 90 min induced no significant response of the *RUNX2* gene over a period of 32 min post-load (Fig. 2b). Only after 30 min of force application a stimulatory effect was observed (Fig. 2a): 2.5 min post-load an increase in *RUNX2* expression by 1.7 ± 0.14 was detectable. This increase diminished rapidly within the next 2 min. During the next 30 min post-load the *RUNX2* expression reached its pre-centrifugal level. The stress vs. control ratio in Fig. 2 was chosen to emphasize the *RUNX2* alteration after 30 min of pressure force application onto human osteoblasts *in vitro*.

Discussion

Bone development and bone homeostasis depend on mechanical load. While unloading or immobilization results in disuse osteopenia or osteoporosis, loading promotes bone development and formation. Based on the current literature, we concluded that the *Runt*-related transcription factor 2 (*RUNX2*) responds to mechanical stimuli in accordance with its prominent function in osteogenesis. Consequently, based on previous work (24, 25), we simulated pressure mechanical load on mature osteoblasts *in vitro* using a centrifugal gravity field of $200 \times g$ and varying load times. This force corresponds to 40.3 g/cm^2, a value achieved with typical orthodontic treatment (26, 27). The pressure caused a transient increase in *RUNX2* gene expression,

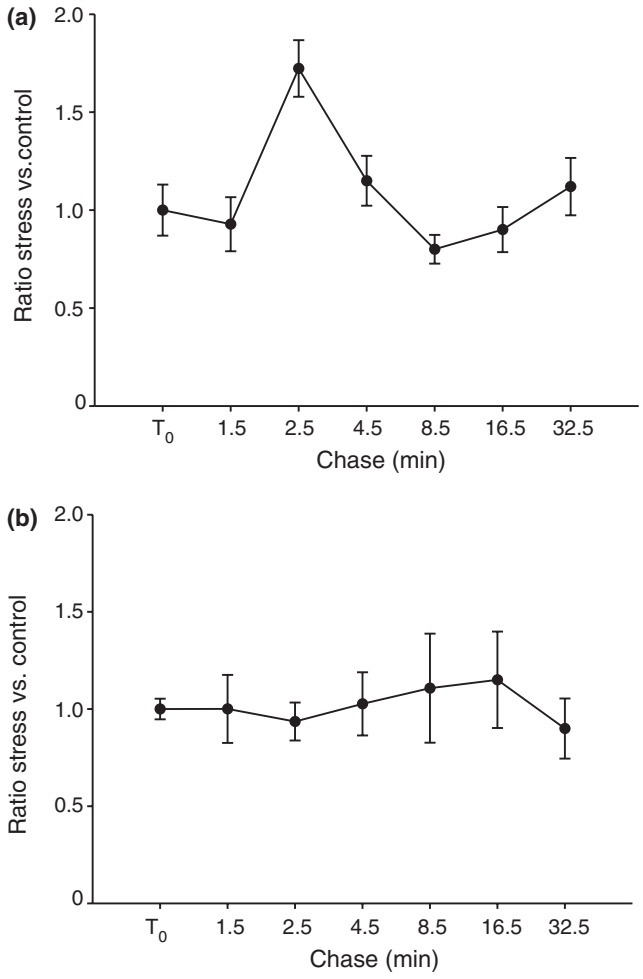

Fig. 2. RUNX2 gene expression after 30 min (a) and 90 min (b) centrifugation at 200 × *g* in isolated human osteoblasts. The determined absolute concentrations were normalized against T_0. Shown is the ratio between stress and control of a representative experiment at different times post-load. Mean and SD were calculated from at least two independent repeats.

which decreased rapidly towards its pre-pressure level. Longer (90 min) periods of mechanical pressure did not affect *RUNX2* mRNA levels. This is the first report relating *in vitro* pressure loading to *RUNX2* gene expression demonstrating that within the time limits of this model *RUNX2* showed little affection by the force.

Mechanical loads should be differentiated into pressure (compression) and tension (stretching) type. Recently, the effect of tension on *RUNX2* in hPDLF cells was described by Ziros and co-workers (22). The authors showed that *RUNX2* gene expression increased significantly after 30 min of application of tension and remained elevated even after 12 h of tension. The striking differences between this study and that of Ziros et al. (22) can be attributed to:

1 Different type of loading (pressure vs. tension).
2 Different type of cells (osteoblasts vs. PDL fibroblasts, probably prior to cell differentiation towards an osteoblastic phenotype).
3 Duration of force application (short period vs. extended period).

It should be emphasized however, that both studies found high responsiveness of *RUNX2* gene expression to external forces. Further pressure experiments of longer duration of centrifugation are required to fully elucidate *RUNX2* response to force.

Ziros and co-workers (22) also reported, that longer tension application times not only induced mRNA translation but also involved transcriptional regulation. They first observed RUNX2 protein synthesis after 3 h of loading, with its maximum after 6 h. *RUNX2* DNA binding activity reached its highest level after 6 h loading (22). This coincides with modifications of the RUNX2 protein through phosphorylation, catalyzed by stimulated mitogen-activated protein kinases (22).

Additionally, the autoregulatory feedback mechanism of the *RUNX2* gene plays a crucial role in this context (12). The *RUNX2* gene contains RUNX2 binding sites in its regulatory parts showing inhibitory or stimulatory effects on gene expression (13). They contribute to the auto suppression effects of the RUNX2 protein (13). Mature osteoblasts show a significant *RUNX2* gene expression level. In accordance with the autoregulatory feedback mechanism, additional cell stimulation will therefore result in a temporary increase in *RUNX2* expression and an immediate down regulation back to the previous expression level, corresponding to its current maturation state. Figure 2 supports this interpretation and describes regulatory mechanisms which down-regulate overshooting reactions.

The results of the present study partially support the well documented *in vivo* effect of orthodontic force. Namely, pressure type of force, which leads to bone resorption in the direction of tooth movement and bone apposition at the tension aspect of the force. The findings that *RUNX2* is almost always kept to its pre-pressure level will facilitate bone resorption at the orthodontic pressure aspect. This is further supported by the work of Ozawa et al. (28), who showed that continuous applied compressive pressure on osteoblast-like cells *in vitro* caused a

reversible decrease in alkaline phosphatase activity as well as the production and secretion of PGE2, which, in turn, inhibits differentiation of osteoblasts. The effect of tension on the other hand (22), which caused up-regulation of *RUNX2* gene expression will enhance new bone formation at the tension aspect of tooth movement.

For clinicians it is critical to understand the effects of orthodontic tooth movement, or mechanical load in general, at the molecular level, which would lead to the establishment of more sophisticated treatment procedures in the future. In the long term, evidence based molecular genetics will contribute to a more detailed understanding of the biological effects of orthodontic force. The equilibrium of bone formation and resorption in orthodontic tooth movement is a result of multiple defaulted values. Each influence has to take into account the acting element that can be directly influenced by the clinician (characteristics such as a function of force, duration, magnitude, frequency, and application) and the reacting element (e.g. given anatomical situation, periodontal health, etc.). As results of *in vitro* simulations can only represent single variables, their conclusion must be regarded as elements of a complex patchwork (29, 30). In this pilot study only the duration of pressure was varied, which caused an immediate gene response. Knowledge of each additional variable will reduce the use of standard values when calculating individual optimized force applications.

Conclusions

Application of centrifugal pressure force on osteoblasts cell culture stimulates *RUNX2* gene expression over a narrow time frame, which caused a short temporary increase in the expression of this gene with a rapid downregulation back to its previous expression level. With this pilot study the gene expression behavior after mechanical stimuli could be determined with a simple laboratory setup.

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