THE STEPWISE INCREASING AND DECREASING SHEAR STRESSES CONVERSELY REGULATE THE OSTEOSTATIC POTENTIAL TO SUPPORT OSTEOCLASTOGENESIS

INTRODUCTION Loading induced fluid flow shear stress is an important regulator of bone cell function. In vitro studies have shown that shear stress in the range of 5 to 30 dyn/cm² modulates osteoblastic osteogenesis (bone formation) and osteoclastogenesis (bone resorption) under unidirectional steady, pulsatile, or oscillatory flow conditions [1, 3]. Although the applied waveform of the shear stress could be dynamic in the oscillatory and pulsatile flows, the peak and average shear stress magnitudes have been kept constant in most previous studies. Experiments have shown that bone cells lost their sensitivity during continuous loading, and the sensitivity can be recovered by inserting rest periods during the loading sessions [1]. These results suggest that, in addition to the loading magnitude and frequency, the loading history may have great influence on the cellular response. In this study, we aimed to elucidate how a gradually increased or decreased shear stress regulates osteoblast functions such as their potential to support osteoclast formation. A stepwise increasing loading waveform was designed to mimic the in vivo flow condition where one gradually increases the physical activity levels as in sports warm up processes. In parallel, a stepwise decreasing load waveform was used to mimic the condition when one gradually decreases the physical activity level from extraneous exercise. It is well established that osteoblasts modulate osteoclasts formation (osteoclastogenesis) by secreting nuclear factor kappa B (NF-κ B) ligand (RANKL) and osteoprotegerin (OPG) factors [2], and this process is regulated by fluid flow [3]. To test our hypothesis that the osteostatic potential in supporting osteoclastogenesis is inhibited with increasing shear stress and promoted with decreasing shear stress, we subjected primary osteoblasts to the stepwise increasing and decreasing shear stress regimens and examined the temporal changes of the resulting RANKL and OPG gene expression.

METHODS Primary osteoblasts were harvested from newborn Wistar rat calvaria asceptically. Cells were cultured with DMEM/F12 containing penicillin (100 U/ml), streptomycin (100 mg/ml) and 0.5% l-glutamine (Hyclone) and 10% heat-inactivated fetal calf serum (Hyclone) in a 95% air-5% CO2 humidified environment at 37 °C. Cells from passages 2 or 3 were used in all experiments and confirmed to be osteoblastic phenotype by examining cell morphology and molecular markers such as alkaline phosphatase.

Stepwise loading regimens Confluent cells seeded on serum coated glass slides were subjected to fluid shear through a parallel plate flow chamber, driven by a peristaltic pump (Longer Peristaltic Pump, Hebei, China) with an adjustable flow rate. Except the pump, the system was placed in an incubator during the flow experiments. Five experimental groups (three replicates per group) were used: two groups were subjected to a stepwise increasing shear stress with a 5 dyn/cm² increment every 2 or 4 hours; the other two groups were subjected to a stepwise decreasing fluid shear with a 5 dyn/cm² decrease every 2 or 4 hours; and the static incubated cells were used as controls.

RANKL and OPG gene expression After shearing experiments, total cell RNA was extracted and reverse transcribed to cDNA. Real-time PCR (Thermo Hybaid, UK) reactions were performed in the presence of specific primer pairs, which were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database). PCR conditions were 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C for 35 cycles. Aliquots (10 µL) removed from the PCR mixture was electrophoresed on a 2% agarose gel and stained with ethidium bromide (Sigma). The intensity of amplified bands was analyzed by the MAC BAS software and normalized with β-actin. Each RNA sample was analyzed thrice.

Data processing and statistical analysis Normalized RANKL and OPG RNA expression and the RANKL/OPG ratios between the loaded groups versus the static controls were reported and compared using student’s two-tailed t-test for paired samples. A p-value less than 0.05 indicates statistical significance.

RESULTS Compared with static controls, the stepwise increasing shear stress of 6 or 12 hours significantly decreased the gene expression of RANKL and increased OPG in primary osteoblasts, resulting in a significant twofold to fourfold decrease of the RANKL/OPG ratio (Fig. 1). In contrast, the stepwise decreasing shear of the same time periods significantly increased RANKL and decreased OPG, resulting in a twofold to 2.5-fold increase of the RANKL/OPG ratio (Fig. 1). There was no significant difference between the 6 and 12 hour stimulation.

DISCUSSION Although it is well established that living bone adapts to mechanical loading, it remains less clear how the cells perceive the loading signals and how the tissue responses are formulated. Recent in vivo and in vitro experiments suggest that bone may have “memory” capability, and loading history affects the response, such as periodic insertion of rest periods during loading session increases the anabolic response [1]. Our data using a novel stepwise shearing protocol show that osteoblasts responded differentially when the loading magnitude was gradually changed, suggesting that the cells can recognize the temporal gradient of the fluid shear. The cellular RANKL/OPG expression, an index of the potential of osteoblasts to support osteoclastogenesis, was varied with the temporal changes of the fluid stress during the 6 or 12 hours stimulation period. The increasing shear stress results agree with previous studies where loaded cells displayed suppressed osteoclastogenesis potentials [3]. However, with a temporally decreasing shear stress, we showed here that even with the presence of loading, the osteoclastogenesis support potential of osteoblasts was in fact increased during the experimental period. This result is compatible with the in vivo observation that declining physical activity is associated with decreased bone mass, as seen in previously active athletes. In summary, the stepwise fluid shear regimen appears to be a useful tool to probe cellular response to more complicated loading stimulations as found in vivo.

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Fig. 1. The RANKL and OPG gene expressions and RANKL/OPG ratios of primary osteoblasts under 6 and 12 hours of increasing (▲) and decreasing (△) shear stress relative to static control. Notes: * indicates significance vs. the static control; # indicates significance vs. the same period of decreasing shear stress.