CHAPTER 9

GENERAL DISCUSSION

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Mechanotransduction in bone

Bone is an obvious biological system that exemplifies the interplay of mechanical stress and adaptive response both at the tissue and cellular levels (1-4). It is currently thought that osteocytes do not directly sense the loading of the bone matrix, but rather respond to the strain-induced flow of interstitial fluid along the network of osteocytes (5, 6). Several studies suggested that the rate of the applied mechanical strain is related to bone formation rather than the magnitude of strain (for example see (7, 8)). Low magnitude (< 10 με), high frequency (10 - 100 Hz) loading has been shown to be capable of stimulating bone growth and inhibiting disuse osteoporosis (9). Also, high-amplitude, low-frequency stimuli are quite rare in the activities of daily life, whereas high-frequency, low-amplitude stimuli are quite common (10). High rates of loading also increased bone mass and strength after jumping exercises in middle-aged osteopenic ovariectomized rats (11). The rate of loading seems to be a decisive factor in bone formation and maintenance. However, the underlying physical picture for understanding how the rate of stress might relate to a meaningful physiological response remains poorly understood.

Rate-dependent response by bone cells

In this thesis we found that bone cells release increased amounts of NO linearly correlated to the rate of fluid shear stress. This supports the notion that bone formation *in vivo* is stimulated by dynamic rather than static loads (12), and that low-magnitude, high-frequency mechanical stimuli may be as stimulatory as high-amplitude, low-frequency stimuli. This rate-dependent response was found to occur, provided that the cells are “kicked” in a pre-conditioned state (13). The finding that the bone cell’s response to fluid shear stress is rate dependent provides an explanation why adaptive bone formation can occur despite the sporadic occurrence of high-amplitude strains in daily life (14). Based on theoretical analysis, it was shown that strain-induced flow in the

canalicular system, in turn induces fluid drag across the extra-cellular matrix on osteocyte processes, that are amplified to two-orders of magnitude (15, 16). This also provides an explanation for sustained bone formation despite the sporadic occurrence of high amplitude strains in normal physiological loading conditions. The theoretical approach leads to an *extra*-cellular mechanism for amplifying stress, whereas, experimental investigations leading to a rate-dependent response, provided a cellular basis for understanding the osteogenic adaptation of bone to mechanical loading. Further understanding of how bone cells respond to stress at the cellular level might provide a deeper insight on how maintained bone health copes with low amounts of high amplitude loading. A phenomenological interpretation underlines the importance of both the rate-dependence, and the requirement of an initial stress-kick, for the stress response of bone cells. The phenomenon is that bone is able to sustain itself despite the sporadic occurrence of meager strains, whereas, bone cells are known *in vitro* to require stresses imparting higher strains. However, it might be possible that sporadic bouts of responses in terms of signaling molecule release, could account for sustained bone health. A normal person is in a condition of unloading during sleep. However, this does not necessarily support a predicament of bone loss. Thus, the theoretical attempt to explain strain amplification via extra-cellular matrix drag, while being important for understanding fluid shear stress stimulation, might not fully support an understanding of sustained bone metabolism despite normal conditions of low strains.

Implications of threshold activation: enhanced response to stochastic stress

As bone exhibits the property of an adaptive response to mechanical stress, various ways of imparting stress to bone has been shown to achieve enhanced bone formation. For instance, there is reason to believe that muscular activity might be related for stimulating strain on bone despite low magnitudes. Strong evidence for the relation between muscle and bone was shown for 778 healthy Argentinians by correlating “whole body bone mineral content”, as indicator

for bone strength, and “lean body mass”, as indicator for muscle strength (17). This undermines a possible excessive requirement for exercise to induce high enough strain magnitudes for stimulating bone cells. Another example is the use of low-magnitude high frequency loading, which has been shown to stimulate osteogenic response from various species (9). One other example is the use of low intensity pulsed ultrasound, which has been shown to have osteogenic benefits (18-20). Techniques of imparting stress by vibrating plates or ultrasound seem to exhibit effective stimulation of cells, however the underlying physical picture of the transfer of forces to the cellular effectors despite the soft tissue barrier, is not straightforward. The possible medical benefits of these techniques might overshadow the importance of understanding the underlying mechanisms of how they might work. However, it is only by a deeper understanding that further benefits could be achieved. The processes by which bone cells are stimulated by vibrating plates, ultrasound, or even musculatory vibration might not be directly the result of a force transfer to the cells themselves. As mentioned, intervening soft-tissue might attenuate the assault of vibratory stress from an already meager source.

We have shown the property that the bone cell response to stress is enhanced by noise. The nitric oxide released by bone cells reached a maximum at the application of an optimum noise-level by fluid shear stress. No distinct peak response was conclusive for the prostaglandin E_2 response. Our study used noise by fluid shear stress stimulation to find differences in the response of MLO-Y4 and MC3T3-E1 cells as models for osteocytes and osteoblasts, respectively. The results indicated differences in threshold-activation for NO and PGE_2 production for both cell types. A peak response is indicative of a small difference between the input signal and the threshold. Hence these results suggest that at low stress conditions with noise, MLO-Y4 cells could have a peak PGE_2 response, while MC3T3-E1 cells, a peak NO response. At conditions of high stress with noise, MLO-Y4 cells could have a peak NO response, while MC3T3-E1 cells, a PGE_2 response. It is possible that *in vivo*, osteoclasts are driven to be active close to osteocytic regions at low stress

conditions with noise. On the other hand, osteoclasts are driven to be active near osteoblastic regions at high stress conditions with noise. Clearly, low stress might promote bone loss; however, high stress seems to promote the activity of osteoclasts near osteoblasts. This supports the notion that high stresses ultimately leading to bone microdamage and osteocyte apoptosis, initiate bone remodelling (21). Whereas microdamage promotes low fluid fluid flow and osteocyte apoptosis, explaining the recruitment of osteoclasts (21, 22), very high stress by itself stimulates osteoclasts via PGE_2 upregulation. Furthermore, the possible role of noisy or stochastic stress in the mechanical adaptation of bone provides a partial explanation for the role of obscure effects of indirect stress applications to stimulate bone formation. The threshold-activation property of bone cells indicate a capacity for enhanced response at stress conditions obscured even by minute stress sources, as from musculatory vibration, ultrasound, or vibratory motion, typified by low-magnitude high frequency loading.

Stress, cellular deformation, mechanosensing, and mechano-activity

Recent studies on the osteogenic activity of bone cells investigated the effects of stress using varying techniques (fluid flow, substrate strain, hydrostatic pressure, vibration stress). In these studies, the magnitude (and rate) of stress was shown to correlate with the cellular response, in terms of signaling molecules. This correlation was suggested to be deformation-dependent. Relating the effects of fluid flow and substrate straining have shown that the former induces higher release of signaling molecules (23, 24). A numerical study confirmed that the cellular deformation caused by stress induced by fluid flow is fundamentally different from that induced by substrate straining (25). Fluid shear stress has a larger overturning effect on the bone cells, while the effect of substrate strain is focused on cell-substrate attachments. These recent results confirm the importance of investigating how cells deform in response to stress for understanding the corresponding physiological response of cells.

The collaboration between experimental investigation and theoretical analysis of the response of bone cells to stress has proven effective for advanced understanding of underlying processes in bone mechanotransduction. The role of osteocytes, as the mechanosensors in bone *par excellance*, has been elucidated in the past years with more detail. Computational models for cells have previously treated the cytoplasm and cytoskeleton as a continuum (26). Recognizing the importance of the cytoskeleton has led to more recent approaches that treat the cytoskeleton more closely to its physical reality, as consistent of interconnected fibers.

Using a two-particle *in vitro* assay for measuring the viscoelasiticity of cells, with the recently derived two-particle microrheology, we probed the mechano-activity and mechanosensitivity of various bone cell types and fibroblasts. Mechano-activity is characterized by the induction of force traction on attachment sites by cells, and mechanosensitivity is the ability of cells to sense forces. We found that osteocytic cell types (primary osteocytes from chicken and MLO-Y4 cells) induce a relatively higher traction force on attached particles than osteoblastic cells (primary osteoblasts from chicken and MC3T3-E1 cells). Fibroblastic cells (CCL-224) are even more mechano-active compared to MLO-Y4 cells, which explains the propensity of fibroblasts for motility *in vitro*. In our two-particle *in vitro* assay, MLO-Y4 cells release nitric oxide simultaneous with increasing force application. Another typical response to increasing force application is the induction of force traction on the attached beads by cells, simultaneous with morphological adaptation from a spherical to a polar shape defining ends at the attachment points. It is clear that force traction, morphology change, and possibly the release of signaling molecules are all related in similar pathways, in response to environmental stress conditions.

Towards quantitative description of bone cell mechanosensitivity

We have outlined above two discernible properties of bone cell response to fluid shear stress, which are, rate-dependence and threshold-activation. The

rate-dependent response was interestingly demonstrated by bone cells in a broad range of frequencies induced by vibration stress, showing a distinctive correlation to the acceleration rate (chapter 6). This rate-dependent response showed an anti-correlated release of NO and PGE_2. As mentioned in the preceding sections, these signaling molecules modulate the activity of osteoclasts. Hence, loading frequency can fine-tune the localized recruitment and inhibition of osteoclasts. The loading rate is believed to be the important parameter for stimulating bone formation (27, 28). However, the underlying cellular mechanisms are now slowly being revealed. By recognizing the mathematical precision of bone mechanical adaptation, as Wolff did, the science of understanding the mechanobiology of bone has been subject also to numerical simulations (29, 30). The final local effects on bone, under conditions of loading are further understood by having a quantifiable interplay between cell types, due to the resulting release of signaling molecules. Complex systems are generally exhibiting power-law phenomena that might prove useful in characterizing behaviors of biological systems (for a review on complex biological systems and scale invariance phenomena see (31)). A power-law description is typical for complex systems, which are generally exhibiting scale invariance. Power-laws and scale invariance have been used in studying phase transitions in materials and in describing systems that spontaneously self-arrange (31).

We can derive an empirical power-law for bone cells. Based on our results, we propose an empirical quantifiable property of bone cells, that is, the correlation of the accumulated amount of signaling molecule to the cell's experienced frequency of stress. Thus,

$$[M] \propto s(\frac{\sigma - \theta}{\sigma})\omega^{\beta} \qquad [1]$$

where s is the normalized sigmoid function to enforce threshold-activation, with a threshold θ, and the experienced stress σ. $[M]$ is the accumulated amount of the signaling molecule M and the frequency $f = \omega/(2\pi)$, and β determines the power-law relation between stress and response. Equation [1] incorporates in a

power law, the property of bone cells that the released signaling molecules, is related to the frequency of stress, above a threshold. In Chapter 3, we showed that $\beta = 1$ for fluid shear stress stimulation, and in chapter 5, we showed that $\beta = \pm 3$ for translational vibration stress acting on attached bone cells. Thus, β is a property associated with the type of stress. Whereas $\beta = 1$ for contact stress (*e.g.,* fluid shear stress, for low frequencies, < 10 Hz), $\beta = \pm 3$ for stress induced by body forces (*e.g.,* motion of the nucleus through cytoplasm, for high frequencies, > 10 Hz). The absolute value of β is then useful for finding possible new mechanisms by which bone cells sense loading at different frequency ranges. In Chapter 6 we have also shown that the release of NO and PGE_2 anti-correlated. We showed that for NO, $\beta > 0$ and for PGE_2, $\beta < 0$, since NO released positively correlated with ω^3, while PGE_2 released negatively correlated with ω^3. Thus, β is also a property of the specific signaling molecule. The sign of β for the associated signaling molecule indicates upregulation ($\beta > 0$) or downregulation ($\beta < 0$) in relation to the loading frequency.

The threshold activation, enforced by the sigmoid function we introduced in [1] was partially demonstrated in Chapter 4, where we showed that without an initial stress-kick, no rate dependent response occurred. Threshold-activation was also demonstrated by the possibility of stochastic resonance in the way bone cells respond to fluid shear stress. Note however, that the accumulated signaling molecule released [*M*] is the sum of all molecules released by a population of cells. Also, the threshold for NO and PGE_2 were demonstrated to be unequal. Thus, the threshold θ, is also a property of the cell population under question in relation to a specific signaling molecule. The threshold is reached when a critical number of cells have produced enough amounts of signaling molecules [*M*]. Further proof of this assumption can be demonstrated by showing that single cells do not have the same stress thresholds for releasing a measurable amount of signaling molecules. From our results in Chapter 7, we have only shown the possibility that the release of signaling molecules might be related to a change in the mechanical properties of the cell. Physiological

conditions are probably influenced by the activity of collaborating aggregates of cells. Hence, experimental results suggesting power-laws on cell cultures *in vitro* might not necessarily translate to single cell properties but considering that a biological system is complex, the property of scale invariance, might suggest that single cells posses similar power-laws.

The validity of equation [1] is anticipated to be limited to a range of frequencies possibly below 100 Hz, for an actual mechanical loading regime on the cell. In chapter 6 we used frequencies 5, 30, 60 and 100 Hz and found a linear correlation between the total NO and PGE_2 released by bone cells, with ω^3. In chapter 7 we have shown that bone cells are basically elastic until ~ 10 Hz, having a viscoelastic transition between 10-100 Hz, beyond which, a viscoelastic stiffening occurs. Transmembrane proteins and ultimately, the cell cytoskeleton might be related to mechanosensing by cells (32). Since the mechanical properties of cells depend on the cytoskeleton, changes in cell compliance might be a direct indication of cell mechanosensitive properties. Thus, a linear relation between the physiological responses of bone cells to ω^β, is possibly related to changes in the viscoelasticity of cells.

Implications for the extreme condition of unloading: microgravity

We have shown that bone cells are responsive to dynamic stress. This supports findings that the rate rather than the magnitude of loading is the important parameter for osteogenic properties of mechanical loading to bone (33). This insight has powerful implications on the local activity of osteocytes for directing the mechanical adaptation of bone. We have also shown that the released signaling molecules of bone cells are related to the frequency of stress in an empirical relation. Since the activation of bone cells is highly dependent on the frequency of experienced stress, bone cells might be capable of responding to very meager amounts of strain, after overcoming a stress threshold. Thus, under extreme conditions of unloading, it might be possible to counteract the onslaught of bone loss by sporadic bouts of high impact loading. There is a large amount of data on the catabolic effects to bone of prolonged

unloading (34-36), and suggested counteractive remedies or preventive measures for bone loss, which are both based on exercise or pharmacological applications (37-39). Regardless of the counteractive procedure for bone loss, the approach has to eventually target the bone cells. It has been suggested that imbalances in the activity of bone cells for directing bone resorption or formation contributes to bone loss (34). We have shown here relations between what we consider fundamental parameters of mechanotransduction in bone, which are the applied stress, the amount of signaling molecules released by osteocytes, and their possible roles for directing the local activity of osteoclasts and osteoblasts. Bone loss can be understood as resulting from a disturbance of the homeostasis of these parameters. However, since these parameters are closely related as we have shown, it might be possible to restore their homeostasis despite extreme conditions of unloading.

Studies by Tabony et al. (40-42) have shown that microtubule self-organization *in vitro* is gravity-dependent and that this self-organization affects the transport of intra-cellular particles. Since, a reorganization of microtubules might affect the cell viscoelastic properties, gravity or the loss of gravity might affect the way cells sense forces. To address the underlying cellular mechanisms for studies on bone loss in relation to microgravity, Cowin (43) addressed the question whether bone cells are able to read the changes in gravitational field or detect this indirectly via contact stresses. It would seem that the light weight of cells, undermines the role of gravity on cellular behavior. Thus, the question remains on how cells might detect microgravity directly. We probed the activity of bone cells by measuring the forces induced by cells on attached fibronectin-coated beads (chapter 7). We showed that the force fluctuation $<ff^*>$ was proportional to ω^{-2}, which is a signature for continuums with slowly evolving internal processes (44). Although we probed the traction forces induced by cells on particles outside the cell, this force fluctuation is indicative of intra-cellular processes. The power-law for cellular force fluctuation is an indication for the diffusive properties of intra-cellular particles or organelles. Reaction-diffusion processes govern the relation

between microtubule re-organization, and possibly intra-cellular transport, which might be affected by gravity (41, 45). Thus, intra-cellular transport might be crucial for mechanosensing. Hence, cells might be able to detect changes in the gravitational field directly by the gain or loss of gravitational forces influencing the re-structuring of self-organizing polymers inside cells, thereby influencing intra-cellular transport. Thus, it is possible that the signature for force fluctuations inside cells might change under microgravity influencing bone cell mechanosensitivity as indicated by a changed release of signaling molecules.

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