

CHAPTER **1**

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The capacity of bone tissue to alter its mass and structure in response to mechanical loading has long been recognized (1) but the cellular mechanisms involved remained poorly understood. Bone not only develops as a structure designed specifically for mechanical tasks, but it can adapt during life toward more efficient mechanical performance. The mechanical adaptation of bone is a cellular process and needs a biological system that senses the mechanical loading. The loading information must then be communicated to the effector cells that form new bone or destroy old bone (2, 3).

The *in vivo* operating cell stress derived from bone loading is likely the flow of interstitial fluid along the surface of osteocytes and lining cells (2, 4). The response of bone cells in culture to fluid flow includes prostaglandin (PG) synthesis and expression of prostaglandin G/H synthase inducible cyclooxygenase (COX-2) (5, 6). Cultured bone cells also rapidly produce nitric oxide (NO) in response to fluid flow as a result of activation of endothelial nitric oxide synthase (ecNOS), which enzyme also mediates the adaptive response of bone tissue to mechanical loading (7, 8). Earlier studies have shown that the disruption of the actin-cytoskeleton abolishes the response to stress, suggesting that the cytoskeleton is involved in cellular mechanotransduction (9). The cytoskeleton of the cell also determines its mechanical structure and as such, becomes crucial for the mechanical response of cells to environmental forces. Extracellular matrix receptors such as integrins and CD44 receptors, located in the cellular membrane, are attached to the extracellular matrix as well as to the cytoskeleton. They are prime candidates as mechanotransducers (10). Thus, the ability of cells to respond to mechanical loading is possibly closely related to its mechanical properties and the transfer of forces via intervening proteins linking the internal structure of the cell to its environment.

The influence of forces on cells has been recognized for a long time and the molecular processes involved are now being uncovered. Living bone is an

evident biological system where the interplay of force and metabolic response is exemplified both at the tissue and the cellular level. Forces are believed to be imparted on osteocytes and bone lining cells by the flow of fluid through the lacuno-canalicular system in mechanically loaded bone. Recently, it has been proposed theoretically, that fluid drag through the extra-cellular matrix concentric to the osteocytic processes is able to amplify strain upto two orders of magnitude (11). Theoretical modeling along with *in vitro* studies, exhibit that the range of stress capable of soliciting meaningful physiological response from bone cells are within the range 0.1- 20 Pa (12). The response of bone cells to mechanical stress has been studied using varying techniques for imparting mechanical loads *in vitro* (12, 13).

A number of studies emphasize the role of osteocytes as the professional mechanosensory cells of bone, and the lacuno-canalicular porosity as the structure that mediates mechanosensing (2, 4, 13). Strain-derived flow of interstitial fluid through this porosity seems to mechanically activate the osteocytes, as well as ensure transport of cell signaling molecules and nutrients and waste products. It has also been shown that the rapid production of NO in human bone cells in response to fluid flow results from activation of endothelial cells nitric oxide synthase (ecNOS) (8). These results suggest that the response of bone cells to mechanical stress resembles that of endothelial cells to blood flow (14-16). In the vascular system, changes in arterial diameter occur in response to changes in blood flow rate, in order to ensure a constant vessel tone, and endothelial cells are widely recognized as the mechanosensory cells of this response. The early response of endothelial cells to fluid flow *in vivo* includes the release of NO and prostaglandins (16). Surprisingly therefore, bone tissue seems to use a similar sensory mechanism to detect and amplify mechanical information as does the vascular system. This concept allows an explanation of the local bone gain and loss, as well as remodeling in response to fatigue damage, as processes supervised by mechanosensitive osteocytes.

Microgravity, as occurs under spaceflight, has negative effects on the skeleton, leading to bone loss. Several studies suggest that bone tissue is

directly sensitive to spaceflight conditions. Microgravity provides a unique mechanical environment that might directly affect the ability of cells to sense forces. Hence, the question remains as to how the lack of gravity is detected by bone cells. Could microgravity act directly on the bone cells? Or more precisely, could osteocytes and osteoblasts read the gravitational field change directly? To answer these questions, the fundamental properties of the way bone cells respond to stress in general, have to be addressed.

Therefore, in this thesis we addressed the following specific objectives:

1. To characterize parallel-plate flow chambers for high frequency flow regimes, and to determine how eventual limitations can be reduced for the use of this apparatus for different frequencies.
2. To investigate the response of bone cells to varying rates of fluid shear stress.
3. To investigate whether an initial stress-kick is required for the response of bone cells to varying rates of fluid shear stress.
4. To investigate whether the activation of bone cells by a small periodic loading stimulus is enhanced by broad-frequency, noisy fluid shear stress stimulation.
5. To test whether high frequency vibration stress applied with varying frequencies and amplitudes affects the nitric oxide (NO) and prostaglandin E₂ (PGE₂) production, and mRNA expression for COX-2 by MC3T3-E1 osteoblastic cells.
6. To investigate events occurring at the onset of mechanical stimulation of cells and characterize the chemical and mechanical activation of cells in response to stress by using a two-particle assay for measuring the viscoelastic properties of cells.
7. To test whether near weightlessness decreases the sensitivity of bone cells for mechanical stress through a decrease in early signaling molecules (NO, PGs) that are involved in the mechanical loading-induced osteogenic response.

The main goal of this thesis was to find characteristic properties of the response of bone cells to mechanical stress and to address the effects of microgravity on the mechanosensitivity of bone cells. To reach this goal, bone cells were subjected to stress using fluid flow stimulation at varying rates and regimes with a rich harmonic content in a parallel-plate fluid flow chamber *in vitro* system. Also, the effect of vibration stress on the activation of bone cells was investigated. Finally, mechanosensing at the single cell level was studied by probing the viscoelastic properties of bone cells.

In Chapter 2, the parallel-plate flow chamber was characterized for its use in dynamic flow regimes. This study re-visited a theoretical description of the parallel-plate chamber and derived parameters for designing the parallel-plate chamber for *in vitro* stimulation of cells at arbitrary frequencies, considering non-turbulent fluid flows.

To find specific properties on the way bone cells respond to dynamic stress, in Chapter 3, the response of bone cells to fluid shear stress at different rates was investigated. We compared the release of nitric oxide by bone cells in response to fluid shear stress regimes with frequencies mimicking the physiological condition. Nitric oxide was used as a parameter for bone cell activation, as it is an important signaling molecule for bone formation (17). The amplitude of the fluid shear stress was also varied to investigate the joint effect of frequency and amplitude of stress to the activation of bone cells.

In Chapter 4, we addressed the nature by which bone cells respond to fluid shear stress in a rate-dependent manner. This was done by introducing a pre-treatment phase to the fluid shear stress regimes used to stimulate bone cells, to either induce an initial stress-kick or remove it. By measuring the nitric oxide released by bone cells in response to the regimes with pre-treatment, the ability of bone cells to respond in a rate-dependent manner was put to the test. The response of bone cells to stress with or without an initial stress-kick, determined whether bone cells required overcoming a stress-threshold to respond in a rate-dependent manner.

The study of bone cell response to stress by fluid flow was then extended to regimes superposed with noisy fluid shear stress with a rich harmonic content. In Chapter 5, we addressed the question whether noise enhancement was possible for bone cells, considering the importance of the initial stress condition (i.e., the presence or absence of an initial stress-kick) for a rate dependent response. The possibility of a noise-enhanced response phenomenon was then used to derive other properties on the way bone cells respond to stress. In particular, differences in the response of MLO-Y4 and MC3T3-E1 cells, as models for osteocytes and osteoblasts, provided insights on the threshold mechanisms for specific signaling molecules (i.e., nitric oxide and prostaglandin E₂).

The properties by which bone cells respond to fluid shear stress as investigated in Chapters 3 and 4 in this thesis, provides possible general rules on bone cell mechanosensitivity. To further understand the effect of stress on bone cells in general, bone cells were also subjected to mechanical vibration by translational motion. In Chapter 6, the response of bone cells to vibration stress in terms of a rapid release of nitric oxide, prostaglandin E₂, and mRNA expression for COX-2, was studied.

However, a more complete understanding of the bone cellular response to stress requires a physical picture for finding characteristic properties at the single-cell level. Finally, the mechanical and physiological response of single bone cells was investigated in chapter 7, to find characteristic behaviors for the stress response of single bone cells. To perform this final study, a two-particle microrheology technique was adapted for investigating characteristic single-cell responses to minute forces.

In the course of this study, we had an opportunity for testing the mechanosensitivity of primary bone cells under the influence of microgravity during the Dutch Soyuz Mission, also called DELTA mission (Dutch Expedition for Life Science, Technology and Atmospheric Research; Soyuz craft launched April 19, 2004, flight mission to the International Space Station). The previous work on characterizing the parallel-plate chamber for

dynamic fluid shear stress was used in designing a downscaled version of an *in vitro* system for fluid flow stimulation of bone cells. A downscaled version was necessary considering strict requirements of a space flight experiment. In chapter 8, we describe the ground preparations and protocols for the experiment FLOW on board of the DELTA mission, where we investigated whether the mechanosensitivity of bone cells was decreased by microgravity.

REFERENCES

1. Wolff J 1892 Gesetz der transformation der knochen, Hirschwald, Berlin.
2. Burger EH, Klein-Nulend J 1999 Mechanotransduction in bone - Role of the lacuno-canalicular network. FASEB J 13(Supplement):S101-S112.
3. Smit TH, Burger EH, Huyghe JM 2002 A case for strain-induced fluid flow as a regulator of BMU-coupling and osteonal alignment. J Bone Min Res 17(11):2021-2029.
4. Cowin SC, Moss-Salentijn L, Moss ML 1991 Candidates for the mechanosensory system in bone. J Biomech Eng 113(2):191-197.
5. Ajubi NE, Klein-Nulend J, Alblas MJ, Burger EH, Nijweide PJ 1999 Signal transduction pathways involved in fluid flow-induced PGE2 production by cultured osteocytes. Endoc Metab 276(1):E171-E178.
6. Klein-Nulend J, Burger EH, Semeins CM, Raisz LG, Pilbeam CC 1997 Pulsating fluid flow stimulates prostaglandin release and inducible prostaglandin G/H synthase mRNA expression in primary mouse bone cells. J Bone Miner Res 12(1):45-51.

7. Johnson DL, McAllister TN, Frangos JA 1996 Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. *Am J Physiol* 271(1):E205-E208.
8. Klein-Nulend J, Helfrich MH, Sterck JG, MacPherson H, Joldersma M, Ralston SH, Semeins CM, Burger EH 1998 Nitric oxide response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent. *Biochem Biophys Res Commun* 250(1):108-114.
9. Ajubi NE, Klein-Nulend J, Nijweide PJ, Vrijheid-Lammers T, Alblas MJ, Burger EH 1996 Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes- a cytoskeleton-dependent process. *Biochem Biophys Res Commun* 225(1):62-68.
10. Wang N, Butler JP, Ingber DE 1993 Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260(5111):1124-1127.
11. Han YF, Cowin SC, Schaffler MB, Weinbaum S 2004 Mechanotransduction and strain amplification in osteocyte cell processes. *Proc Natl Acad Sci USA* 101:16689-16694.
12. Brown TD 2000 Techniques for mechanical stimulation of cells in vitro: a review. *J Biomech* 33(1):3-14.
13. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH 1995 Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB J* 9(5):441-445.

14. Frangos JA, Eskin SG, McIntire LV, Ives CL 1985 Flow effects on prostacyclin production by cultured human endothelial cells. *Science* 227(4693):1477-1479.
15. Furchgott RF, Vanhoutte PM 1989 Endothelium-derived relaxing and contracting factors. *FASEB J* 3(9):2007-2018.
16. Hecker M, Mulsch A, Bassenge E, Busse R 1993 Vasoconstriction and increased flow- 2 principal mechanisms of shear stress-dependent endothelial autacoid release. *Am J Physiol* 265(3):H828-H833.
17. Fox SW, Chambers TJ, Chow JWM 1996 Nitric oxide is an early mediator of the induction of bone formation by mechanical stimulation. *Bone* 19(6):687.

