Seventh International Bone Fluid Flow Workshop

Translational Bone Fluid Flow

September 20–21, 2005
New York City

Sponsors:
The City College of New York
Department of Biomedical Engineering
National Science Foundation
The Whitaker Foundation
OrthoLogic

Program and Abstracts
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New York Center for Biomedical Engineering
Departments of Biomedical and Mechanical Engineering
The City College of New York
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• Susannah Fritton, Ph.D., The City College of New York
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• Yi-Xian Qin, Ph.D., State University of New York, Stony Brook
• Shelly Weinbaum, Ph.D., The City College of New York

Welcome

Fluid movement in bone has been studied for more than two decades. These studies have been both theoretical and experimental and have been accomplished at the organ, tissue, cellular, and molecular level to determine the effects of fluid movement on the resident cell population. The clinical translation of the accumulated studies is now being considered.

The goals of the International Bone Fluid Flow (IBFF) Workshops are to explore the biological and physical mechanisms of fluid flow through bone and to stimulate interdisciplinary interaction among Workshop participants, particularly at the student and young investigator level. Workshop participants include biologists, biomedical engineers, and clinicians whose principal interest is in bone research.

The First Bone Fluid Flow workshop was held in September 1997 at The City College of New York. In the intervening eight years, five additional workshops have been held, both in the United States and Europe, and it is our great pleasure, on the occasion of the Seventh IBFF Workshop, to once again serve as host to this event, which has been held annually since 2001. A list of previous IBFF Workshops is at the back of this program.

The focus of the Seventh International Bone Fluid Flow Workshop is on the role of fluid flow in bone physiology, in bone tissue engineering, and in the translation to clinical practice. This includes the cellular and molecular response to bone fluid flow and imaging and experimental approaches to determine the flow. The program consists of 14 invited lectures and 13 student presentations, with ample time for discussion and some social functions. Like its predecessors, the current IBFF Workshop is designed to provide an interdisciplinary forum at this exciting stage of the research, the stage at which the participants in the field of bone fluid flow are unveiling the mechanism of cellular stimulation by fluid flow, bone fluid flow imaging, and translation to the clinic.

Welcome to the Seventh International Bone Fluid Flow Workshop!

Steve Cowin
The City College of New York

Contents

Welcome from Workshop Organizer Steve Cowin ........................................... 4
Program ........................................................................................................... 6
Abstracts ......................................................................................................... 9
Workshop Organizers .................................................................................... 37
International Bone Fluid Flow Workshops, 1997–2005 ............................... 38
Sponsors ........................................................................................................... 39
Program

8:00–8:30 AM Breakfast & Registration

8:30 AM
Welcome: Steve Cowin, City College of New York

8:35 AM
Presenter: Susannah Fritton, City College of New York
Osteocyte lacunar and canalicular structure
SP Fritton, T Beno, C Ciani, SB Doty

9:00 AM
Presenter: Lynda Bonewald, University of Missouri at Kansas City
Osteocytes are dynamic, not passive cells in their response to strain and remodeling of their microenvironment
LF Bonewald, J Feng, SE Harris, JX Jiang, S Kotha, N Lane, D Nicoletta, E Sprague

9:25 AM
Presenter: Liyun Wang, Mount Sinai School of Medicine, New York
Investigating the structure of osteocytic pericellular matrix based on diffusion measurements in the bone lacunar-canalicular system
L Wang, Y Wang, Y Han, RJ Majeska, MB Schaffler

10:00 AM
Presenter: R. B. Patel, Case Western Reserve University
Measuring permeability and anisotropy effects of cortical bone using FRAP
RB Patel, JM O’Leary, ML Knothe Tate

10:10 AM
Presenter: Uday Chippada, Rutgers University
SMD studies of effects of structural water on the stiffness of collagen
U Chippada, D Zhang

10:20 –10:45 AM Break

10:45 AM
Presenter: Sheldon Weinbaum, City College of New York
Mechanotransduction and strain amplification in bone cell processes
Y Han, S Cowin, M Schaffler, S Weinbaum

11:10 AM
Presenter: Theo Smit, Vrije Universiteit Medical Centre, Amsterdam
Bone apposition in a remodeling osteon is related to matrix shear strain, not fluid flow
TH Smit, RGM Breuls

11:35 AM
Presenter: Jenneke Klein-Nulend, ACTA-Vrije Universiteit, Amsterdam
Nitric oxide production by bone cells in response to mechanical vibration
J Klein-Nulend, RG Bacabac

12:00 PM
Presenter: R. G. Bacabac, Vrije Universiteit
Characterizing the viscoelasticity and probing the mechano-activity of bone cells by two-particle microrheology
RG Bacabac, DS Mizuno, ThH Smit, JWVA van Loon, F MacKintosh, CF Schmidt, J Klein-Nulend

12:10 PM
Presenter: Vikram Sreedharan, Rutgers University
Electrical response of a circular cell monolayer to an axisymmetric, low-frequency, oscillatory extracellular electrical field
V Sreedharan, D Zhang

12:20 PM
Presenter: Eric J. Anderson, Case Western Reserve University
Fluid modeling of the lacunocanalicular network and imparted cellular-level forces
EJ Anderson, ML Knothe Tate

12:30 PM
Presenter: Agnès Rémont, University of Paris
Mixture theory for multiphase flow: application to bone interstitial fluid
A Rémont, S Nall, S Cowin

12:40 –1:30 PM Lunch

1:30 PM
Presenter: Christopher Jacobs, Stanford University
Bone cell mechanotransduction via loading-induced oscillatory fluid flow
C Jacobs, J Rodriguez, N Batra, L You, Y Li, J You, C Yellowley, H Donahue

1:55 PM
Presenter: Jean Jiang, University of Texas Health Science Center, San Antonio
Fluid flow shear stress induces the assembly of Cx43-forming hemichannels responsible for the release of PGE2 in osteocytes
PP Cherian, AJ Siller-Jackson, S Burra, S Gu, LF Bonewald, E Sprague, JX Jiang

2:20 PM
Presenter: Henry Donahue, Pennsylvania State University
Fluid flow activation of gap junctional communication and hemichannel activity in bone cells
HJ Donahue, AF Taylor, MM Saunders, DC Genetos, RC Riddle

2:45 PM
Presenter: X. Edward Guo, Columbia University
Substrate modulation of osteoblast modulus and response to mechanical stimuli
E Takai, KD Costa, CT Hung, XE Guo

3:10 PM
Presenter: Carmen Huesa, University of Aberdeen
Role of caveolin-1 in regulation of eNOS activation in osteoblastic cells
C Huesa, M Helfrich, R Aspden

3:20 PM
Presenter: Kenneth A. Myers, University of Calgary
Effect of 1α,25 dihydroxyvitamin D3 treatment on the shear stress mechanosensitivity of an osteoblast-like cell line
KA Myers, NG Shrive, DA Hart
Osteocyte lacunar and canalicular structure

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Introduction
Interstitial fluid movement through the lacunar-canalicular porosity, the porosity that houses osteocytes and their processes, is believed to be involved in bone’s mechanosensory system. The permeability of bone, which has a great influence on interstitial fluid flow, is largely determined by bone’s microstructure. While many two-dimensional cross-sections of osteocyte lacunae and canaliculi can be found in the literature, there is very little quantification of the three-dimensional osteocyte architecture and canaliculi orientation. To better understand the pathway of interstitial fluid flow and to more accurately quantify bone permeability, it is important to have accurate three-dimensional structural measurements of the lacunar-canalicular porosity.

Methods
Bone samples were taken from the tibia of six Sprague Dawley rats. After fixation, bone blocks were stained with basic fuchsin and thin sections were scanned using confocal microscopy (Zeiss LSM 510) with a 63x objective lens. From z-stacks (3-D reconstructions of collections of scanned images at different focal depths), several microstructural parameters of the lacunar-canalicular porosity were measured, including the osteocyte lacunar density, the length of a cuboidal unit cell of bone matrix surrounding the osteocyte lacuna, the osteocyte lacunar axis lengths, the number of canaliculi emanating from each lacuna, and the distribution of canaliculi in three dimensions.

Results
The number of lacunae per unit volume was found to be 80,600/mm³, which corresponds to a mean volume of 1.24 x 10⁻⁵ mm³ of bone matrix associated with an individual osteocyte lacuna, giving a side length of 23.2 ± 0.7 μm for a cuboidal periodic unit cell surrounding the lacuna. The lacunae resembled triaxial ellipsoids with major, intermediate, and minor axes lengths of 17.6 ± 0.3 μm, 6.1 ± 0.3 μm, and 4.0 ± 0.2 μm, respectively. The number of canaliculi emanating directly from each lacuna and the number of canaliculi intersecting all faces of the cuboidal unit cell was 52.5 and 116, respectively, so that the number of canaliculi added from branching within the unit cell was approximately 63 per lacuna.

Discussion
Confocal images revealed that osteocyte lacunae and interconnecting canaliculi could be highly visualized in the rat tibia. Our measurements provide a more complete characterization of the lacunar-canalicular microstructure, thus improving the microstructural parameters that we will input into the previously developed bone permeability model [1, 2] that calculates the local permeability coefficients of the lacunar-canalicular porosity. Accurate determination of bone permeability is critical to better understand the involvement of interstitial fluid flow in bone’s mechanotransduction mechanism and in osteocyte nutrition.

References
Osteocytes are dynamic, not passive cells in their response to strain and remodeling of their microenvironment

LF Bonewald, J Feng, SE Harris, JX Jiang, S Kotha, N Lane, D Nicolella, E Sprague

The matrix producing osteoblast is highly metabolically active compared to the osteocyte. This is probably due to the fact that osteoblasts exist as matrix producing cells for only days or a few weeks and must generate several times their volume in matrix while the encapsulated osteocyte is viable for years or decades. However, the osteocyte is not an inactive cell, especially in response to mechanical strain. While fairly well accepted and documented that the osteocyte can send biochemical signals in response to mechanical strain, studies addressing osteocyte modification of its lacunae have been controversial. New studies suggest that the osteocyte is a dynamic, active cell that can modify its microenvironment. Holmbeck and colleagues have found that osteocytes in mice lacking MT-1 MMP, a matrix metalloproteinase that cleaves collagen, fail to form canaliculi (J. Cell Sci. 2005). Our preliminary data, like that of Holmbeck and co-workers, shows that the number and branching of dendrites increases with age. Data showing increased expression of a molecule thought to play a role in dendrite formation, E11, in osteocytes with loading also supports this hypothesis. Recent experiments show that osteocyte lacunae in mice treated with the Glucocorticoid (prednisolone) are enlarged and the surrounding matrix is hypomineralized. Lacunae around osteocytes in mice subjected to loading label with fluorochrome dyes. These data suggest that the osteocyte is a dynamic cell that can generate processes even when embedded in mineralized tissue and can also alter its microenvironment (e.g. enlarge or reduce the size of its surrounding lacunae).

These studies have implications with regards to type and magnitude of fluid flow shear stress moving through the lacunae and canaliculi of osteocytes. It could be inferred that if the osteocyte can modify the size of its lacunae, it can modify the diameter of its canaliculi. Generation of additional canaliculi with their internal dendritic processes and removal of matrix from lacunae and canaliculi would dramatically modify bone fluid flow. For the same rate of bone fluid flow within a canaliculi, a narrower canal diameter would increase shear stress, while widening the canal would decrease shear stress. Widening of canals and lacunae in addition to increasing numbers and branching of canaliculi would lead to reduced shear stress. This may explain why the aging skeleton is less responsive to strain. Osteocytic response in diseased physiological states could play a role in pathological changes in bone.

Investigating the structure of osteocytic pericellular matrix based on diffusion measurements in the bone lacunar-canalicular system

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Abstract

The pericellular matrix surrounding osteocytes and their cell processes has been found to govern the permeability of the lacunar-canalicular system to fluid and solute transport, which is essential for osteocyte survival and function as mechanical sensors. Using our newly developed approach based on Fluorescence Recovery After Photobleaching (FRAP) and mathematical modeling, the diffusion coefficient of fluorescein (MW = 376) between individual osteocyte lacunae was determined to be $3.0 \pm 0.5 \times 10^{-6}$ cm$^2$/sec, which is 60% of its diffusion coefficient in water and is similar to diffusion coefficients measured for comparably sized molecules in cartilage. This reduced diffusion through the osteocytic pericellular matrix was analyzed using the fiber matrix theory, which has been applied to study the structure of glyocalyx of the endothelial cells. We found that the diffusion of fluorescein in bone was consistent with the presence of an osteocytic pericellular matrix whose structure resembles that proposed for the endothelial glyocalyx, where instead of extended GAG side chains, spherical clusters (radius 5-6 nm) are attached to core proteins such that the clusters repeat every 20 nm in three dimensions [Squire et al., (2001) J. Struct. Biol. 136, 239-255].
Measuring permeability and anisotropy effects of cortical bone using FRAP

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Introduction
The specific aims of this study were threefold, (i) to measure the currently unknown diffusion constants of various size macromolecules through the lacunocanalicular network, (ii) to compare differences in permeability in the transverse and longitudinal planes in cortical bone, and (iii) to compare permeability across length scales including the matrix microporosity, cellular syncytium, and “bulk tissue” permeability.

Methods
Diffusion constants were calculated based on FRAP experiments conducted on a laser scanning confocal microscope (SP2 AOBs, Leica Microsystems, Mannheim Germany). Longitudinal and transverse sections were obtained from the mid-diaphysis of a fresh bovine femur cortex. Samples were soaked in tracer solution until sample was saturated. Acid Yellow Fluorescein free dye (Sigma) and fluorescein conjugated dextran molecules of 3, 10, 40, and 70 kDa tracer molecules were chosen to achieve our aims. Bleach regions included tissue, cell, and subcellular length scale areas. Ten measurements were made per sample (n = 3); for each sample, diffusion constants were reported as the mean ± standard error of these ten measurements.

Results
The measured diffusion coefficient decreased exponentially with increasing molecular weight (Fig. 1). Significant differences were also observed in coronal and transverse diffusion constants (Fig. 2) for small molecular weight (MW) free dye (300 Da), but not for the larger MW dyes. The permeability of cortical bone is highly dependent on length scale measured.

Discussion
These studies show, for the first time to our knowledge, that the permeability of cortical bone is not only dependent on the size of the molecule being transported but also on the direction of transport, and the length scale of the system through which transport occurs. Of particular interest was the observation that the low pass filter function of bone’s molecular sieve shows anisotropy, whereby small molecules (300 Da) are transported more rapidly in the longitudinal plane than in the transverse plane. This anisotropy may confer an additional means by which low molecular weight substances are transported preferentially, e.g. in the direction of growth during bone modeling.

Intercellular signaling in osteocytes after mechanical stimulation

A Vatsa,1 D Mizuno,2 TH Smit,3 CF Schmidt,2 FC MacKintosh,4 J Klein-Nulend1 1Osteoporosis, 2Orthopaedic Research Center, The Cleveland Clinic Foundation, Cleveland, OH 3Dept. of Clinical Physics & Engineering, VU University Medical Center; 4Theoretical Physics, Vrie University

Introduction
It is believed that osteocytes are the mechanosensors in bone (1). If bones are mechanically loaded, the resulting shear stress caused by strain-derived interstitial fluid flow can activate them. Activated osteocytes produce signaling molecules like nitric oxide (NO), which modulate the activity of osteoblasts and osteoclasts thereby orchestrating bone adaptation to mechanical loading. This single-cell level mechanosensing and intercellular signaling is essential for bone adaptation (2). We studied how a mechanically stimulated single osteocyte functions in a social context via intercellular communication, by monitoring changes in intracellular NO production, using DAR 4M AM chromophore, in the surrounding osteocytes.

Methods
Osteocyte culture: MLO-Y4 osteocyte-like cells (kindly donated by Dr LF Bonewald) were seeded at 2x10³ cells/cover slip (700mm²) and incubated in D-MEM with 10% serum overnight at 37°C at 5% CO₂ in air. NO: MLO-Y4 cells were loaded with 10μM DAR-4M-AM chromophore in D-MEM for 2h at RT. Then medium was changed to D-MEM. DAR-4M-AM is membrane-permeable and reacts with NO to form a stable, fluorescent compound intracellularly. Mechanical stimulation: A single cell was subjected to a localized oscillatory mechanical stimulus (10-20nN) using an Eppendorf micromanipulator. Imaging: Fluorescence images were recorded using rhodamine filter (excitation λ= 554nm; emission λ=572nm). Fluorescence was quantified w. Scion Image software.

Results
Mechanical stimulation of a single osteocyte increased fluorescence intensity in surrounding osteocytes (Fig. 1), indicating upregulation of NO production.

Discussion
NO production is essential for mechanical loading-induced bone formation in vivo (3). Hence, NO production in response to mechanical stimulation is a meaningful parameter for measuring bone cell activation. Our technique allows real-time monitoring of chemical signaling at the single osteocyte level. Here we show that a single mechanically stimulated osteocyte activates its surrounding osteocytes via direct and/or indirect intercellular signaling, as indicated by upregulation of intracellular NO production in these cells. This likely contributes to the dynamic processes involved in bone adaptation.

Figure 1. Fluorescence images showing intracellular NO production in MLO-Y4 osteocytes (A) before, and (B) after mechanical stimulation of a single osteocyte (encircled). (C) Fluorescence intensity of 4 individual osteocytes in the vicinity of the mechanically stimulated osteocyte (dashed line: stimulation of single osteocyte).

Acknowledgement
Supported by NWO - ALW/FOM, project # 01F8B28/2.

References
SMD studies of effects of structural water on the stiffness of collagen
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Introduction
Collagen type-I is the most abundant structural protein in tendon, skin and bone. It is a major
determinant of the mechanical behavior of these connective tissues. Structural water plays an
important role in the stiffness and flexibility of collagen

Methods
Steered Molecular Dynamics (SMD) on the stretching and bending of collagen type I (Protein Data Bank entry code 1QSU) was performed using laterally packed triple helices. 11 triple
helices for sliding and 7 triple helices for the bending simulations were staggered and then
merged to form a single PDB file. Sliding and bending were done for two cases: “with” and
“without” structural water, using a constant velocity protocol. The results from these two cases
were compared. For the sliding simulations, the central triple helix was pulled while keeping the
surrounding molecules fixed. For the bending simulations, the C-terminus of all the molecules
were fixed, while a lateral force was applied perpendicular to the axis joining the Cα atoms of the
C-terminus and the N-terminus (see Figure 1).

Results
Simulations were performed using different pulling speeds, and the trend of the results is the
same for all simulations. From the force-displacement graphs, it was evident that the collagen
fibril is more flexible in the presence of structural water. From the force-deflection curves of the
bending simulation, a larger force is required to deflect the molecules in the absence of structural
water.

Discussion
The structural water molecules were thought to provide stability to the collagen structure by
forming water-bridged hydrogen bonds. But Holmgren et al experimentally proved that the extra
stability was due to an inductive effect. This paper discusses about the effect of structural water.
Structural water acts as a shield to the triple helices, preventing them from unwinding and
forming direct inter-helical bonds, thus decreasing the mechanical resistance of the molecule to
relative sliding. And during bending, the structural water molecules act as a lubricant between
the layers of triple helices, thus increasing the flexibility of the crystal.

Figure 1. (a) Crystal packing looking down the helix axis; (b) 3 perspectives of a same model showing the 11 adjacent triple helices; (c) sliding simulation; (d) bending simulation.

Mechanotransduction and strain amplification in bone cell processes
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Introduction
A paradox in bone tissue is that tissue-level strains due to animal and human locomotion are too small to
initiate intracellular chemical responses directly. A model was recently proposed in You et al. (2001) J.
Biomech. 34, 1375-1386 to resolve this paradox, which predicts that the fluid flow through the
pericellular matrix in the lacunar-canalicular porosity due to mechanical loading can induce strains in the
actin filament bundles of the cytoskeleton that are more than an order of magnitude larger than tissue
level strains. In this study, we greatly refine this model by using the latest ultrastructural data for the cell
process cytoskeleton, the tethering elements that attach the process to the canaliculal wall (You et al.

Methods
We have constructed a much more realistic 3-D model for the osteocytic process and have used large
deformation “elastica” theory for finite EI to predict the deformed shape of the tethering elements and the
hoop strain on the actin filament bundle. The model for the actin filament bundle is based on a 19
filament central bundle that is cross-linked by fimbrin and surrounded by a double helical coil of brush
border myosin I which advances 37.5 nm in each revolution. In the model the tethering fibers in the
pericellular matrix, when subject to hydrodynamic drag, transmit a tensile force across the cell process
membrane via membrane proteins that are directly linked to the myosin I helices. A detailed analysis of
the stress distribution in this complicated structure is presented and the strains induced on the outer
filaments of the actin filament bundle are calculated.

Results
Our model predicts a cell process that is three times stiffer than in the previous study by You et al. (2001)
and that the effective Young’s modulus of the cell process is 600 times greater than the cell body. Despite
its stiffness the model predicts hoop strains on the cell process which are one to two orders of magnitude
greater than whole tissue strains. These cellular level strains are >0.5 percent for tissue level strains >
1000 microstrain at 1 Hz and > 250 microstrain at frequencies > 10 Hz. This suggests that only rather
large tissue strains in the physiological range can produce a stimulatory response and that without the
strain amplification mechanism bone cells would not be able to detect the small whole tissue strains that
are produced by mechanical loading in vivo.

Discussion
The foregoing results provide a reasonable explanation of the results of You et al. (2000) J.
Biomech. Eng. 122, 387-393 and other investigators who have shown in vitro that cells grown on elastic substrates
do not elicit chemical signals when subject to strains of <0.5 percent, whole tissue strains that would
cause bone fracture. Furthermore, it would appear, in view of the large difference in the elastic modulus of
the cell process and the cell body, that when cells are subject to fluid shear stress in vitro it is the cell
body that is producing the observed chemical responses such as the release of Ca ions, PGE, and other
second messengers. In contrast, in vivo one anticipates that the cell process is the sensing element of the
cell. Therefore, we propose that this strain amplification model provides a more likely hypothesis for the
excitation of osteocytes than the fluid shear hypothesis previously proposed in Weinbaum et al. (1994),
J. Biomech. 27, 339-360.
Bone apposition in a remodeling osteon is related to matrix shear strain, not fluid flow

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Introduction
Osteoblast activity in a remodeling osteon is characterized by a rapid apposition of bone at the start of refilling process, which progressively slows down as the osteon is filled. In line with earlier work, we hypothesize that osteoblast activity is related to the local stimulation of osteocytes. The question addressed here is whether the osteocytes are steered by interstitial fluid flow or by deformation of the bone matrix.

Methods
We used Bio’s theory in a biphasic finite element model of a remodeling osteon (Fig.1) to calculate fluid flow and matrix shear strain rate under the loading regime of a walking cycle.

Figure 1: Axysymmetric FE mesh of a tunneling osteon. The arrows indicate the longitudinal loading direction.

Results
Along the whole surface of the closing cone, the magnitude of fluid flow was equal, following the external dynamic loading regime (Fig.2A). So, fluid flow amplitudes did not change during the refilling process. On the other hand, local shear strain rates in the bone matrix gradually diminished as refilling progressed (Fig.2B); this correlated linearly to the decreasing bone apposition activity by osteoblasts (graph not shown).

Figure 2: (A) Fluid flow, and (B) shear strain rate of the bone matrix in the closing cone as a function of time during the complete walking cycle, taken at two positions (see insert): near the reversal zone (black), and at the base of the cone (blue).

Discussion
Osteoblast refilling in a remodeling osteon thus is related to the local mechanical strain environment in the closing cone. Matrix shear strain seems to be the relevant mechanical stimulus controlling bone apposition rather than fluid flow.

Nitril oxide production by bone cells in response to mechanical vibration

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INTRODUCTION
Bones are able to adapt their mass and structure to the demands of mechanical loading. Furthermore, it has been suggested that the rate rather than the magnitude alone of the applied loading stimulus correlates to bone formation in vivo [1]. In bone, mechanical loading is likely transferred to the cells by strain-induced fluid flow through the lacuno-canaliculair system [2]. We have recently found in another study that the NO production by MC3T3-E1 cells was linearly dependent to the rate of fluid shear stress, which depended on both magnitude and frequency [3]. In that study however, the applied fluid shear stress was only up to a maximum of 9 Hz. Using animal models, low magnitude (< 10 μm/s) high-frequency (10 - 100 Hz) mechanical stimuli have been shown to be capable of stimulating bone growth by doubling bone formation rates and inhibiting disuse osteoporosis [4]. Thus, it would seem that higher frequencies are also stimulatory to bone cells. Regardless of frequency range, the applied rate of loading seems to be a decisive factor in bone formation and maintenance. However, the nature by which bone cells respond to mechanical vibration is unknown. We studied the response of bone cells to mechanical vibration at a wide frequency range (5 Hz up to 100 Hz), at different magnitudes. We tested whether vibration stress applied with varying frequencies and amplitudes affects the nitric oxide (NO) production by MC3T3-E1 osteoblast-like cells.

MATERIALS AND METHODS:
Bone cell culture: For vibration stress treatment, MC3T3-E1 cells were plated in 24-wells plates, at 4 x 10³ cells/cm², in CO₂-independent α-MEM medium with 2% FBS, and incubated for 5 min in the presence of mechanical vibration at varying frequencies (5Hz, 30Hz, 60Hz, 100Hz) and amplitudes (see Table 1). Vibration stress was implemented on attached cells by sinusoidal displacement of the 24-wells plate along the cells’ plane of attachment using a voltage controlled linear actuator. Conditioned medium was sampled after 5 min of vibration stress treatment to measure accumulated NO in medium produced by MC3T3-E1 cells.

NO: The conditioned medium was assayed for NO, which was measured as nitrite (NO₂⁻) accumulation in the conditioned medium, using Griess reagent. Data from separate experiments were collected and expressed as mean total amount of NO in nmol units.

RESULTS:
NO production in rapid response (within 5 min) to treatment with vibration stress linearly correlated with the applied maximum acceleration rate, which is third order in time-dimension (Figure 1A; R < 0.027, R² > 0.97). However, NO production did not linearly correlate with the applied maximum velocity, which is first order in time-dimension (Figure 1B).

DISCUSSION:
NO production is an essential step for mechanical loading-induced bone formation as observed in rats in vivo [5]. Hence, NO production in response to mechanical vibration is a meaningful parameter for measuring bone cell activation. NO production by bone cells linearly correlated with the maximum applied acceleration rate, which is third order or cubic in time dimension (see Table 1). This suggests that the bone cell response to vibration stress treatment is highly dependent on the applied frequency of loading regardless of the applied amplitude. The response however, did not linearly correlate with the applied maximum velocity, which is a joint effect of the applied amplitude and frequency, both at first order. This corresponds to our earlier finding that bone cell response is linear to the applied fluid shear stress rate (also third order in time dimension) [3]. Interestingly, bone cells do respond to high frequency vibration stress (i.e., 100 Hz) although fluid shear stresses in vivo might involve lower frequencies [6]. In this study we have not considered in detail the physical differences of the effects of fluid shear stress or vibration stress on bone cell deformation. However complicated the transfer of forces at the cellular level might be, in terms of fluid shear stress or vibration stress, our results imply that the joint effect of the frequency and amplitude of loading might play similar roles for different types of stresses on bone cells. Our results suggest that the joint effect of the frequency (at third order) and amplitude (at first order) of loading correlates to the biochemical response of bone cells that contribute to sustained bone metabolism. Furthermore, this response might involve mechanisms that contribute
to a specific behavior of bone cells in response to vibration stress enabling recognition of high frequency loading.

Table 1. Data of applied vibration stress

<table>
<thead>
<tr>
<th>Regime</th>
<th>5 Hz</th>
<th>30 Hz</th>
<th>60 Hz</th>
<th>100 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (mm)</td>
<td>5</td>
<td>4.5</td>
<td>1.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Maximum acceleration rate (m/s² x 10³)</td>
<td>0.15</td>
<td>30.1</td>
<td>93.8</td>
<td>186</td>
</tr>
<tr>
<td>Maximum velocity (m/s)</td>
<td>0.15</td>
<td>0.85</td>
<td>0.66</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Maximum velocity = amplitude x frequency x (2π)
Maximum acceleration = amplitude x frequency² x (2π)²

Figure 1. Effect of vibration stress on NO production by bone cells. A. Bone cells respond linearly to the applied maximum acceleration rate of vibration stress immediately after 5 min. B. The response to mechanical vibration does not correlate linearly to the applied maximum velocity (see also Table 1). Values are mean total amount ± SEM.

Characterizing the viscoelasticity and probing the mechano-activity of bone cells by two-particle microrheology

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Introduction

We have shown earlier that the nitric oxide (NO) response of bone cells to fluid shear stress (at 5 and 9 Hz) and vibration stress (5-100 Hz) correlated with the applied stress-rate [1,2]. This suggests that bone cellular metabolic activity and mechanical properties are related. To understand the relation between cellular metabolic activities and mechanical properties, a physical portrait of cell viscoelasticity is needed. Therefore, here we develop a novel application of two-particle microrheology [3] to characterize the viscoelasticity and probe the mechano-activity of bone cells.

Methods

MLO-Y4 osteocytes (kindly provided by Dr. L. F. Bonewald, MC3T3-E1 osteoblasts, and CCL-224 fibroblasts were incubated at 22°C and 37°C, using CO₂-independent medium. Fibronectin-coated polystyrene spheres were optically trapped and maneuvered to capture a cell (fig. 1). The cell stiffness was measured by perturbing one of the spheres (fig. 1). Cell mechano-activity was characterized as the difference in the cell compliance from actively deforming the cell (active mode), and passively monitoring the fluctuations of the two spheres (passive mode). Cell mechano-activity was also characterized by morphological changes and the external force fluctuations induced by the cells on the attached spheres.

Results

MLO-Y4 cells were relatively less stiff compared to MC3T3-E1 cells. MLO-Y4 cells showed an elastic plateau < 20Hz, a viscoelastic transition from 20-100Hz, and viscoelastic stiffening > 100Hz. MLO-Y4 cells exhibited morphological changes, and increased attachment area with the fibronectin-coated spheres. CCL-224 cells showed highest mechano-activity, having highest difference in compliance measured by passive and active modes. The external force fluctuation was found proportional to ω² (i.e., ΔF/ω = ω²).

Discussion

Previously, we showed that osteocytes are more mechano-sensitive than osteoblasts. The differences in viscoelastic modulus between MLO-Y4 and MC3T3-E1 cells suggest a close relation between the mechanical properties and metabolic activity of these cells. Furthermore, the viscoelastic property changes in MLO-Y4 cells might relate to the stress-rate dependent release of NO. Continued morphological adaptation by MLO-Y4 cells during sphere attachment, supports the notion that morphology and stress induction by cells are related. High mechano-activity by CCL-224 might indicate faster metabolism necessary for mobility. Finally, the proportionality between the external force fluctuation with ω², indicates a quantifiable signature for non-thermal related activity by bone cells. This signature is expected in continuums with slowly evolving internal processes [4]. Our results show novel correlations between mechanosensitivity, mechano-activity, and the viscoelasticity of bone cells, furthering insight for the osteogenic response of bone cells to stress.

Acknowledgement

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References


Fig. 1. MLO-Y4 force-displacement curve.
Electrical response of a circular cell monolayer to an axisymmetric, low-frequency, oscillatory extracellular electrical field

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Introduction

The interaction between the exogenous electromagnetic fields (EMFs) and biological cells and tissues is a contentious subject. Currently, there is no consensus on its mechanism of influence on biological entities and on the extent of its impact. Current spread in an axisymmetric cell monolayer is solved in this work.

Methods

In this work, the governing equation for the distribution of intracellular potential within a 2 – D cell monolayer induced by an externally applied, oscillatory, axisymmetric electric field is derived by homogenizing the confluent, gap-junction-coupled, cell monolayer as a circular, gigantic, unified cell.

The nondimensional governing equation for intracellular voltage \( V_i \) is

\[
\frac{2}{r^2} \frac{d}{dr} \left( r^2 \frac{dV_i}{dr} \right) + \frac{1}{r} \frac{dV_i}{dr} + V_i = \frac{dV}{dt} + V_i.
\]

And the boundary conditions, are

\[
(V_i^+ - V_i^-) + \left( \frac{\partial V_i^+}{\partial r} - \frac{\partial V_i^-}{\partial r} \right) = \frac{2z}{\xi} \left( \frac{1}{r} \frac{dV}{dt} \right), \text{ at } z = r_i,
\]

\[
(V_i^+ - V_i^-) + \left( \frac{\partial V_i^+}{\partial r} - \frac{\partial V_i^-}{\partial r} \right) = \frac{2z}{\xi} \left( \frac{1}{r} \frac{dV}{dt} \right), \text{ at } z = 1.
\]

Results

This equation is solved both analytically and numerically by finite element (FEMLAB), with both solutions match each other perfectly. The solution is further discussed in terms of current spreading in a layer of epidermal cells underneath an electrode in the current-stimulated wound healing, and the electrical response of gap-junction-connected osteocytes lying on the cross-section of an osteon in bone under the influence of strain-generated streaming potentials (SGPs).

Discussion

A layer of osteocytes falling on a representative cross-section of an osteon. Under oscillatory loading, an oscillatory, axisymmetric flow field is generated in the osteon. Coincidently, an oscillatory axisymmetric streaming potential field is also generated which serves as the extracellular electrical potential to the coupled osteocytes.

Figure 1. Dependence of the intracellular potential \( V_i \) at the outer rim on the parameters \( \xi, 0, \text{ and } \xi \). (a) When \( V_o \) is spatially linear; (b) When \( V_o \) is spatially parabolic.

Fluid modeling of the lacunocanalicular network and imparded cellular-level forces

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Introduction

Osteocytes are thought to be mechanosensors, where transmission of mechanical signals can occur via fluid pressure and shear stresses imparted by fluid moving through the lacunocanalicular system due to load-induced fluid flow. These fluid pathways and impared forces have been described through computational modeling of idealized osteocyte geometry where a simplified lacunocanalicular pathway showed differences in the body and surface forces imparted to the osteocyte cell body and process (Anderson et al. 2005). This computational study gives insight into the diverse mechanical environment of the osteocyte in idealized conditions and examines the sensitivity to more physiologic geometry and loading conditions.

Methods

In order to investigate the fluid environment at the level of the osteocyte, computational models of the lacunocanalicular network were created and flow simulations were performed using a computational fluid dynamics package (CFD-ACE, CFDRC). Models were created with physiologically-based dimensions for (1) a lacuna with three canaliculi of simplified geometry, (2) an anatomic-baased lacuna with multiple canaliculi, and (3) canaliculi of straight and tortuous geometries, where flow was induced via pressure gradient and values of velocity profile, pressure, and shear stress on the osteocyte surface were evaluated.

Results

For the single lacuna with both two and three canaliculi, differences in impared forces were found between the osteocyte cell body and process, where processes are exposed to higher gradients of shear stress and the cell body is exposed to a sustained hydrodynamic pressure (Fig. 1a). In the anatomic-ba-based model, we again see sustained pressure within the lacuna and high gradients in the canalicul channels (Fig. 1b). In order to understand the effect of physiologic geometry or tortuosity on flow the change in shear stress was calculated on the process surface due to geometrical changes in the canaliculus (Fig. 1c).

![Fig. 1](image)

Discussion

Using computational modeling to investigate the fluid environment of the lacunocanalicular network, it has been shown that the osteocyte cell body and process experience a difference in impared forces for idealized geometry. Furthermore, when anatomic-ba-based models are created with a more physiologic geometry, it is again shown that the mechanical stimulation at the cellular level varies between the canalicul and lacunar cavities. Limitations due to microporosity, cell deformation, and flow resistance due to the existence of the pericellular matrix (detailed in Han et al. 2004) would definitely have an effect on cellular level flow regimes, however as complexity is increased in computational modeling of the lacunocanalicular network, it can be expected that this qualitative relationship between what forces the process and cell body experience would still exist.
Introduction

Interstitial fluid mechanical behavior in bone has received little attention by itself. Fluid flow phenomena occur at a smaller scale than pores. Physical phenomena that do not influence macroscopic flow could be of interest at the microscopic scale for their interaction with cells and fluid in the intercellular matrix. Thus, physical phenomena need to be described at the fluid scale to account for physical interactions that could be ignored at the macroscopic scale.

Methods

To account for the multiphasic nature of interstitial fluid, it is modeled as a mixture of fluids with physical interactions that could be ignored at the macroscale. Membrane. Thus, physical phenomena need to be described at the fluid scale to account for physical phenomena that do not influence flow. We have recently found that disruption of the cytoskeleton and interfering with integrin function both have dramatic effects on intracellular calcium signaling, suggesting that mechanical stressing of the cytoskeleton is a key component.

Results

Equations for fluid behavior as a mixture and each of its constituents are obtained. They show that physical phenomena such as electro-osmosis and diffusion could intervene while convection drives the flow when hydraulic pressure gradients occur.

Discussion

This new model allows one to include interaction between phases within interstitial fluid. Estimation of different physical phenomena leads to a better understanding of what could influence fluid flow through bone tissue. Some experimental data on composition of bone fluid and its variations and also on chemical exchanges are needed to precise boundary conditions to solve the equations set.

Physical phenomena influencing fluid flow within the lacuno-canalicular porosity

Bone cell mechanotransduction via loading-induced oscillatory fluid flow

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BACKGROUND

Bone cells occupy fluid filled voids (lacunae) in the mineralized matrix and interconnected by small tubes (canaliculi). As the bone matrix is cyclically loaded, fluid flows in the lacuno-canalicular network from regions of high matrix strain to low matrix strain and back in an oscillatory fashion. Although, it has been demonstrated that bone cells respond to steady and pulsatile fluid flow we have focused on oscillatory flow profiles incorporating a direction reversal since this is what bone cells are expected to experience in vivo. We have shown that oscillatory fluid flow is indeed a potent signal for bone cells in terms of intracellular calcium signaling (Jacobs et al., 1998) and that there appears to be important differences between the response to oscillatory flow and flows that do not incorporate a reversal of flow direction. We have characterized the biochemical signal pathway activated by oscillatory fluid flow as involving IP3 mediated calcium signaling and MAP kinase signaling leading to osteogenic gene regulation (You et al., 2001). We also found that although chemotransport due to oscillatory flow does potentiate the response, fluid induced shear stress is critical to the effect (Haut Donahue, 2003). Finally, we found that PGE2 release (critical to bone adaption to mechanical loading) occurs in response to oscillatory fluid flow independent of intracellular calcium signaling, but does involve membrane associated extracellular proteoglycans (Reilly et al., in press; Saunders et al., 2003) suggesting that at least two cellular signaling pathways are activated by oscillatory fluid flow.

DYNAMIC VS STEADY FLOW AND THE CYTOSKELETON

Interestingly, we have found that in contrast to unidirectional flow, the signaling pathway activated by oscillatory fluid flow does not involve the stretch activated membrane calcium channel. We speculate that one reason for this might be the viscoelastic mechanical nature of cells. Chronic unidirectional flow is likely to result in much larger cellular deformations than short-term reversing flow, thereby activating different cellular signaling pathways, perhaps associated with fracture healing. We have also recently found that while an hour of unidirectional flow leads to the formation of actin stress fibers in the cytoskeleton of bone cells (Pavalko et al., 1998), this does not occur with reversing oscillatory flow, supporting the view that cellular viscoelasticity may be an important consideration for mechanotransduction. However, we have recently found that disruption of the cytoskeleton and interfering with integrin function both have dramatic effects on intracellular calcium signaling, suggesting that mechanical stressing of the cytoskeleton is a key component.

We have also found that for a short period after stimulation, bone cells exhibit a diminished sensitivity (Donahue et al., 2003). This is consistent with the in vivo observation that the osteogenic effect of loading is dramatically increased when rest periods are periodically inserted (Robling et al., 2000). It is also suggestive that optimal osteogenic loading patterns might be identified through in vivo approaches.

STEM CELLS AND OSTEOPROGENITORS

Mechanical disease is known to result in decreased numbers of bone forming cells. Thus, loading induced fluid flow may be an important regulator of osteoprogenitors as well as mature bone cells. In our recent work, we have found that oscillatory fluid flow does indeed increase the proliferation rate of bone marrow stromal cultures as well as the expression of markers of osteogenic differentiation. We are currently employing a gene array approach to better characterize this effect.

REFERENCES

Fluid flow shear stress induces the assembly of Cx43-forming hemichannels responsible for the release of PGE$_2$ in osteocytes

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Introduction: Mechanosensing bone osteocytes express large amounts of connexin (Cx) 43, yet gap junctions are only active at the small tips of their dendritic processes, suggesting another function for Cx43. Previous studies have shown that both primary osteocytes and the osteocyte-like MLO-Y4 cells respond to fluid flow shear stress by releasing intracellular prostaglandin E$_2$ (PGE$_2$).

Methods: Cells were treated with fluid flow shear stress at 16 dynes/cm$^2$ in the absence and presence of 18 β-glyceroldehyde (β-GA), carbenoxolone or Cx43-hemichannel blocking antibody. Hemichannel activity was analyzed by dye-uptake analysis. Immunofluorescence, sucrose gradient, biotinylation and detergent-extraction were used to analyze the assembly and surface expression of Cx43-hemichannels.

Results: Cells plated at lower densities release more PGE$_2$ than cells plated at higher densities. This response was significantly reduced by Cx43 antisense and by the gap junction and hemichannel inhibitors, β-GA and carbenoxolone, even in cells without physical contact, suggesting the involvement of Cx43-hemichannels. Inhibitors of other channels, such as the purinergic receptor P2X$_7$ and the prostaglandin transporter PGT, had no effect on PGE$_2$ release. Shear stress induced the opening of hemichannels in primary osteocytes and MLO-Y4 cells, and this opening was inhibited by an antibody specific for Cx43-hemichannels. In the presence of fluid flow shear stress, Cx43 protein redistributes and migrates towards the plasma membrane. There was an increase in the connexon (hexameric) forms of Cx43 after fluid flow shear stress. Surface expression of Cx43 was also increased by shear stress. Shear stress rendered the Cx43 located at the cell surface more resistant to Triton-X-100 extraction, suggesting the formation of detergent-insoluble protein plaques, similar to previously reported gap junctional plaques.

Discussion: These results suggest fluid flow shear stress induces the assembly and translocation of Cx43 to the membrane surface and that un-opposed hemichannels formed by Cx43 serve as a novel portal for the release of PGE$_2$ in response to mechanical strain.

Fluid flow activation of gap junctional communication and hemichannel activity in bone cells

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Introduction: The mechanism by which bone cell networks perceive, integrate and respond to their biophysical environment is not known. We have proposed that gap junctional intercellular communication (GJIC) and release of nucleotides, specifically adenosine triphosphate (ATP), from osteogenic or osteoblastic cells are both essential to maximize bone cell response to the physical environment. Our central hypothesis is that biophysical signals, such as fluid flow, stimulate osteoblast proliferation and differentiation via a mechanism involving mobilization of cytosolic Ca$^{2+}$, activation of GJIC and release of ATP through gap junction (GJ) hemichannels.

Methods: We utilized a novel co-culture fluid flow apparatus to examine whether osteocytes exposed to mechanical signals communicate proliferation, differentiation and osteogenic signals to osteoblastic cells, a dogma of bone cell biology with surprisingly little experimental support. We also examined the role of GJ hemichannels in mechanotransduction on fluid flow activation of osteoblasts and the role of fluid flow in inducing hMSC differentiation.

Results and Discussion: Osteocytic MLO-Y4 and osteoblastic hFOB 1.19 cells were cultured on opposite sides of perforated membranes (tracer studies confirmed a migration rate through the membrane of less than 2%). This system enabled us to apply physiological levels of fluid shear to MLO-Y4 cells while permitting them to be in direct contact with hFOB 1.19 cells that are not themselves exposed to flow induced shear stress. Dye transfer analysis with calcine-AM showed that MLO-Y4 cells are coupled via gap junctions to hFOB 1.19 cells cultured on the opposite side of the membrane. GJIC between MLO-Y4 and hFOB 1.19 cells was completely blocked by the application 30μM eGx to the culture system.

MLO-Y4 cells were exposed to flow sufficient to induce a shear stress of 5 dynes/cm$^2$ for 1 hour and post incubated the co-cultures for 2 hours at 37°C. Under these conditions a highly significant increase in alkaline phosphatase activity was detected in the hFOB 1.19 cells in contact with flowed MLO-Y4, compared to un-flowed control co-cultures (P<0.01). Interestingly, when hFOB1.19 were exposed either to direct flow or to conditioned media from flowed MLO-Y4 they did not display an increased alkaline phosphatase activity. We also demonstrated that MLO-Y4 cells exposed to fluid flow display Lucifer yellow dye uptake indicative of activated GJ hemichannels. Interestingly, fluid flow did not activate hemichannels in osteoblastic MC3T3-E1 cells. Fluid flow activated hemichannel activity and ATP release were significantly attenuated in cells transfected with Cx43 siRNA suggesting that flow activated hemichannels composed of Cx43 mediate ATP release. We also found that oscillatory fluid flow induced a rapid, flow rate-dependent increase in [Ca$^{2+}$], that triggered a 116% increase in proliferation of human mesenchymal stem cells (hMSC) and resulted in increases in steady state levels of osteocalcin, type I collagen and osteopontin mRNA. Exposure to ATP also resulted in an increase in hMSC proliferation and apyrase inhibited flow induced proliferation. These results suggest that fluid flow stimulates hMSC proliferation and differentiation, perhaps through release of ATP.
INTRODUCTION

Osteoblasts in vivo exist in close contact with the bone matrix, which is rich in various extracellular matrix (ECM) proteins such as type I collagen and fibronectin (FN). It is well known that the nature of adhesion of osteoblasts to various ECM proteins modulates bone cell functions. For example, osteoblasts adhered to substrates such as FN, that bind collagen via integrins, display distinct cytoskeletal structures compared to osteoblasts on substrates that promote non-integrin mediated adhesion (Cao et al., 2002). Osteoblast adhesion to different ECM proteins may lead to alterations in the mechanical properties of the osteoblasts since the f-actin and microtubule cytoskeleton have been shown to be mechanically stiffened by the stiffness of the ECM (Mastrogiacomo et al., 2000). It has also been demonstrated that the transmission of mechanical signals in bone cells may depend on intact cytoskeleton (Beddington and Karsenti, 2003). Thus cell responses to mechanical stimuli are likely to be modulated by differences in mechanical perturbation of the cytoskeleton and focal adhesion proteins depending on the ECM substrates. However, there are limited studies examining osteoblast mechanical properties on different ECM substrates, or examining osteoblast response to mechanical stimuli when adhered to different substrates (Karsenti, 2003). In this study, we have examined the changes in cytoskeletal organization and elastic modulus of osteoblasts upon adhesion to FN or glass, and the contribution of the f-actin and microtubule cytoskeleton to cell stiffness. We have also examined changes in prostaglandin E2 (PGE2) release in response to fluid shear stress of osteoblasts adhered to these substrates, since PGE2 has been shown to play an important role in mechanotransduction, e.g. (Goligorsky et al., 2004).

METHODS

Culture of Osteoblast Cells and Culture Glass slides were coated with 1.0 mg/ml FN or phosphate-buffered saline (PBS, bare glass), incubated for 90 minutes at 37°C, washed with PBS containing 0.2% BSA, and then rinsed with PBS. Osteoblast-like MC3T3-E1 cells were detached using trypsin/EDTA, resuspended in 0.2% MEM with 2% charcoal-stripped fetal bovine serum, and allowed to recover for 45 minutes at 37°C. The cells were then plated onto the coated slides at a density of 5x10^4 cells/cm^2, and allowed to attach for 24 hours at 37°C.

Cell Modulus Measurements: An atomic force microscope (AFM; Bio-Rad, Hercules, CA) with a pyramid-tipped cantilever probe (~50nm tip radius) was used to measure the elastic modulus of MC3T3-E1 cells on FN or glass (n=21) and glass (n=17) slides, respectively. We have also examined changes in PGE2 (PGE2) release in response to fluid shear stress of osteoblasts adhered to these substrates, since PGE2 has been shown to play an important role in mechanotransduction, e.g. (Goligorsky et al., 2004).

RESULTS

Osteoblasts plated on FN had a significantly higher Eapp of 1.9±0.8kPa compared to 0.9±0.4kPa for those on glass, as measured by AFM indentation (ANOVA, p<0.01). Figure 1A). Cytoskeletal staining showed f-actin accumulation at the substrate-osteoblast interface, and similar microtubule networks on both substrates (not shown). Distraction of f-actin reduced Eapp of the osteoblasts plated on FN by ~60% (paired t-test, p<0.04), to the level of osteoblasts plated on glass. GTP-γS stimulation of cytoskeleton by treatment with DMSO (vehicle) did not affect Eapp. Osteoblasts plated on FN also released 65% more PGE2 per mg DNA, as response to fluid shear stress, compared to osteoblasts plated on glass (2x10^4). Osteoblasts that were not subjected to shear did not show in increase in PGE2 secretion regardless of the substrate to which they were adhered.

Our results confirm earlier observations that eNOS in osteoblasts is expressed at relatively low levels, even though the enzyme endothelial NO synthase (eNOS) responsible for NO production in response to strain in bone is activated. However, the mechanism by which the enzyme eNOS is activated is still not well understood. In endothelial cells eNOS is localised in caveolae in the plasma membrane where it is bound to caveolin-1 and held in an inactive state until dissociation following shear stress and activation of eNOS. So far, there are few reports on the presence of eNOS in osteoblast cells and there are no reports on the relative presence and numbers of caveolae in osteoblasts and osteocytes. In order to understand better how eNOS might be regulated and why osteocytes respond better to strain than osteoblasts we investigated the abundance of eNOS, caveolin-1 and -2 proteins and caveola in osteoblast cells.

METHODS

We used the cell lines MC3T3 (early osteoblasts), MLO-Y4 (ostecocyte-like) and 3T3 (fibroblasts) and HUVEC (primary endothelial cells) and primary mouse calvarial osteoblasts. Cells for immunofluorescence studies were grown to 50% confluency on glass slides (MC3T3, 3T3 and primary osteoblasts), or on collagen-coated glass slides (MLO-Y4). Cells, grown to confluency on collagen-coated glass slides (2.2 x 10^4 cells/cm^2), were subjected to a pulsatile fluid flow using a FlexFlow™ system using shear stresses 0, 0.1, 0.7, 1.4 and 2.1 Pa, fixed in 4% paraformaldehyde in phosphate buffer, pre-stained and stained with antibodies to caveolin-1 and -2 and eNOS. Cultured cells for electron microscopy were fixed in 2.5 % glutaraldehyde in phosphate buffer, post-stained in osmium tetroxide and embedded in epon. Ultrathin sections were examined in a Philips CM10 microscope and caveola counted in individual cells. 40 cells were counted per cell type by independent observers. Results are expressed as number of caveolae per mm cell membrane.

RESULTS

Immunostaining for eNOS revealed only cytoplasmic staining and no indication of association with the plasma membrane. The ultrastructural analysis revealed that MLO-Y4 cells had the highest number of caveolae (1.05±0.09 per um), compared with MC3T3 (0.05±0.04) and primary osteoblasts (0.02±0.04). Western blotting and immunostaining showed a lower concentration of caveolin-1 in MLO-Y4 than in MC3T3, or primary osteoblasts, whereas the opposite was seen for caveolin-2. eNOS was undetectable by Western blotting in the insoluble fraction of the osteoblastic cells and not found in whole cell lysates. By immunostaining caveolin-1 was associated with the plasma membrane as well as being present in the cytosol, whereas caveolin-2 was mainly cytoplasmic (probably Golgi). After application of shear stress up to 60 minutes, we found no re-localisation, at least as seen by immunostaining, in any of the osteoblastic cell types.

DISCUSSION

Our results confirm earlier observations that eNOS in osteoblasts is expressed at relatively low levels, even though the enzyme can be rapidly induced by shear stress and produce considerable amounts of NO. We found no colocalisation of eNOS and caveolin-1 on the plasma membrane and further studies are underway to analyse by immuno-EM whether eNOS and caveolin-1 localise in the Golgi during protein processing. Clear differences were seen in the pattern of caveola between different osteoblastic populations with the highest number in the osteocyte-like cells. So far, our studies have failed to clarify how eNOS is activated in osteoblast cells, but reveal an interesting increase in caveola number in more differentiated osteoblastic cells, which may have relevance for mechanosensing.
Effect of 1α,25 dihydroxyvitamin D3 treatment on the shear stress mechanosensitivity of an osteoblast-like cell line
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Introduction
In the past, shear stress has been shown to stimulate differentiation of in vitro osteoblast cultures. However, the effects of differentiation on osteoblast sensitivity to shear have not been investigated to a significant degree. This could be an important area of research, in light of recent evidence that osteoblast mechanosensitivity to other modes of stress is altered in a more differentiated phenotype. In this study, we investigated how sensitivity to shear stress changes with differentiation, in an osteoblast-like cell line. Osteoblast differentiation was effected by treatment with 1α,25 dihydroxyvitamin D3 (1,25(OH)2 D3). This naturally occurring hormone has been shown to produce a more differentiated phenotype in osteoblast-like cell cultures.

Methods
Cells from an osteoblast-like cell line, MG-63, were cultured on glass microscope slides, to the point of 60-70% confluency. Once cultures reached the desired cell density, growth media was replaced by one of two serum-free conditions: [A] Dulbecco’s Modified Eagle’s Medium (DMEM); [B] DMEM + 50 nM 1,25(OH)2 D3. These conditions were maintained for a period of 20 hours, immediately prior to loading. The loading protocol was as follows: shear stress applied cyclically, at 0.5 Hz, with amplitude of 10 dynes/cm2. Load was applied in this manner for 1 minute, followed by a 14 minute, unloaded, rest period. This intermittent loading cycle was repeated continuously for 4, 8 and 12 hour experiments. Immediately after loading, messenger RNA was collected and analyzed using RT-PCR techniques and human-specific reagents. The expression of a subset of genes related to cellular differentiation and proliferation, as well as matrix biosynthesis and degradation was assessed.

Results
The transcriptional response for a subset of genes was significantly different in treated cultures. For example, expression of Runx2 (a differentiation marker) was not significantly elevated in untreated cultures, when compared to an unloaded, untreated control. However, significant increases were observed in the loaded, treated cultures when compared to the unloaded, treated controls.

Discussion
1,25(OH)2 D3-treated cells exhibit an altered response to shear stress compared to less differentiated cells. This may suggest that as osteoblast phenotype changes with differentiation, sensitivity to shear is altered in concert.


Bone has the capacity to alter its mass and structure to its mechanical environment. It is generally accepted that osteocytes are the mechanosensitive bone cells regulating the remodeling process. When bone is loaded, extracellular fluid is squeezed through the thin layer of non-mineralized matrix surrounding the osteocytes toward the bone surface. This flow produces fluid shear stress at the osteocyte membrane that activates the osteocyte. Absence of loading results in local stasis of extracellular fluid in the canalicular network and induces regulated osteocyte death, i.e. apoptosis. It has been observed that apoptotic osteocytes are often in contact with osteoclasts (Bronckers et al., J. Bone Miner Res 1996) and therefore we suggest that osteocyte apoptosis plays a key role in the bone remodeling event. Tumor Necrosis Factor-α is a pro-inflammatory catabolic cytokine with apoptotic potency. In bone, it stimulates osteoclastogenesis and inhibits osteoblast function. Elevated levels are found in bone diseases like osteoporosis and periodontitis. Here we investigated if TNF-α affected apoptosis in osteocytes (OCY), osteoblasts (OB) and periosteal fibroblasts (PF), and whether mechanical loading could affect this process. The loading protocol was as follows: shear stress applied cyclically, at 0.5 Hz, with amplitude of 20 hours, immediately prior to loading. The transcriptional response for a subset of genes was significantly different in treated cultures. For example, expression of Runx2 (a differentiation marker) was not significantly elevated in 1α,25 dihydroxyvitamin D3 (1,25(OH)2 D3). This naturally occurring hormone has been shown to produce a more differentiated phenotype in osteoblast-like cell cultures.

Methods
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Discussion
1,25(OH)2 D3-treated cells exhibit an altered response to shear stress compared to less differentiated cells. This may suggest that as osteoblast phenotype changes with differentiation, sensitivity to shear is altered in concert.

TNF-α-induced apoptosis of osteocytes is inhibited by mechanical loading
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Bone has the capacity to alter its mass and structure to its mechanical environment. It is generally accepted that osteocytes are the mechanosensitive bone cells regulating the remodeling process. When bone is loaded, extracellular fluid is squeezed through the thin layer of non-mineralized matrix surrounding the osteocytes toward the bone surface. This flow produces fluid shear stress at the osteocyte membrane that activates the osteocyte. Absence of loading results in local stasis of extracellular fluid in the canalicular network and induces regulated osteocyte death, i.e. apoptosis. It has been observed that apoptotic osteocytes are often in contact with osteoclasts (Bronckers et al., J. Bone Miner Res 1996) and therefore we suggest that osteocyte apoptosis plays a key role in the bone remodeling event. Tumor Necrosis Factor-α is a pro-inflammatory catabolic cytokine with apoptotic potency. In bone, it stimulates osteoclastogenesis and inhibits osteoblast function. Elevated levels are found in bone diseases like osteoporosis and periodontitis. Here we investigated if TNF-α affected apoptosis in osteocytes (OCY), osteoblasts (OB) and periosteal fibroblasts (PF), and whether mechanical loading could affect this process.

The loading protocol was as follows: shear stress applied cyclically, at 0.5 Hz, with amplitude of 20 hours, immediately prior to loading. The transcriptional response for a subset of genes was significantly different in treated cultures. For example, expression of Runx2 (a differentiation marker) was not significantly elevated in untreated cultures, when compared to an unloaded, untreated control. However, significant increases were observed in the loaded, treated cultures when compared to the unloaded, treated controls.

Discussion
1,25(OH)2 D3-treated cells exhibit an altered response to shear stress compared to less differentiated cells. This may suggest that as osteoblast phenotype changes with differentiation, sensitivity to shear is altered in concert.

Figure 1: Effect of TNF-α on caspase 3/7 activity

Figure 2: Effect of PFF on caspase 3/7 activity
Skeletal muscle dynamics generated fluid flow in bone and its role in adaptation

INTRODUCTION: Exercise such as muscle contraction appears to increase blood flow to the skeletal tissues, i.e., bone and muscle. These evidences imply that bone fluid flow induced by muscle dynamics may play an important role in regulating fluid flow through coupling of muscle and bone via microvascular system. We propose that musculo-dynamics induced by physiologic muscle contraction can significantly induce fluid flow and enhance perfusion in bone, which may act as a mediator in initiating and regulating osteolastic adaptation. Using oscillatory pressurized marrow fluid flow stimulus, the physiological fluid stimuli was found to initiate new bone formation and reduce intracortical bone pores caused by disease, even in the absence of direct tissue strain [1]. The objectives for this work were to evaluate (a) the role of muscle contraction served as a dynamic pump in regulation of intramedullary pressure (Imp), (b) the response of cortical perfusion enhanced by dynamic muscle contraction, and (c) the in vivo adaptive response to dynamic skeletal muscle contraction adjacent to bone.

METHOD: Experimental setting of muscle induced Imp: An experiment with total of three 9-month old rats was performed. Rats were anesthetized using standard isoflurane inhalation. A micro cardiovascular pressure transducer was inserted through the chest and sealed with a cap. An electronic muscle stimulus was connected to the electrodes and muscle contraction was applied to the skeletal muscle adjacent to the right femur with frequencies of 1, 2, 3, 5, 7, 10, 25, 35, 45 and 60 Hz with the same electrical magnitude.

In vitro study: A bimodal suspension (HLS) rat model is developed to generate intramedullary fluid flow in femur via dynamic muscle contraction. HLS preparation was performed in 8 female rats, 6 months old (n=8 experimental, n=4 control). Daily fluid flow loading was administrated by a low voltage, extremely small current and battery powered electronic muscle stimulator at left femur with frequency of 10 Hz, 5 min/day, for 3 weeks.

RESULT: Skeletal muscle contraction significantly generates fluid flow pressure in the marrow cavity, in which low magnitude muscle contraction increased the Imp on the order of 8%/1.4 mmHg (1 Hz), 8.7%/0.5 mmHg (2.5 Hz), 9.3%/4.7 mmHg (25 Hz), and 18%/0.1 (45 Hz). Heart rate alone induced 2 mmHg Imp.

Muscle contraction improved short term perfusion in bone. Fluorescent tracer in the experimental bone was stained in the Haversian region and was 3x greater than the control bone, in which the number of stained Haversian canals was 127%/44 [14.3%/4.6 (mm²)] for normalized to bone cross sectional area] in loaded femur and 42%/3.5 in the control. The perfusion in the tibia at the loading was also increased from 36%/8.3 (control) to 58%/18.0 (experimental). There was no strain in the lumen-canalicular area with 30 x (0.2 micron in diameter) loading. Micro-CT images showed that 5 min, 10 Hz, 3 weeks of loading did not result in significant structural changes in the experimental group.

Dynamic histomorphometry analysis indicated significant trabecular bone remodeling, in which the ratio of labeled vs. bone surface (LS/BS) has more than 50% increase in the experimental femur compared to contralateral control. Double labels have shown in the experimental group, but not in the sham control, in which mineral deposition rate (MAR) was at 1.5 µm/day. There is no significant evidence of bone resorption at the periosteal and endosteal surfaces in both experimental and sham control femurs after 3 weeks.

Discussion: These results suggest that dynamic fluid flow contraction in muscle may generate hyper-tension in the skeletal nutrient vessel and induce Imp, and may substantially influence the fluid flow in bone. Physiologic and dynamic muscle contractions may serve as a non-invasive source for generating Imp, which can further drive fluid flow through bone. This may help to devise a biomechanical based intervention for treating osteoporosis, muscle atrophy, and accelerating fracture healing. [This work is kindly supported by the NIAMS (AR049286), US Army (USAMRAA) (DAMD17-02-1-0218) and the Whitaker Foundation (RG-00024)].


Evidence for muscle pump-generated intramedullary pressure as a driving force contributor to bone interstitial fluid flow

INTRODUCTION: The muscle pump lowers the intramedullary pressure (Imp) in the long bones, which may generate a normal range of fluid flow that may help to maintain bone homeostasis. Both the lower and higher Imp have been demonstrated to be associated with bone loss and bone gain, respectively. However, the role of muscle pump-generated fluid flow in bone physiology remains unknown.

METHOD: A novel technique was developed to measure bone fluid flow in vivo in the femur of rats. The technique involves the injection of a small volume of a saline solution containing a fluorescent dye into the bone marrow cavity and the measurement of the fluorescence intensity at different time points. The fluid flow rate was calculated using the Stokes-Einstein equation.

RESULT: The results showed that the fluid flow rate in the femur of rats increased significantly when the muscle pump was activated. The fluid flow rate reached a peak at 5 minutes after the injection of the saline solution and then decreased gradually. The fluid flow rate was found to be higher in the femur of rats with a higher muscle pump activity.

DISCUSSION: These results suggest that the muscle pump-generated fluid flow in bone may play an important role in maintaining bone homeostasis. Further studies are needed to investigate the effects of different muscle pump activities on bone physiology.


Modulation of cell differentiation in bone tissue engineering constructs cultured in a flow perfusion bioreactor

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Bone tissue engineering provides an alternative treatment toward healing large bone defects arising from maladies such as trauma, tumor resection or birth defect. Promising tissue engineering strategies have involved both singular and integrated approaches to bone regeneration utilizing various combinations of scaffolds, osteogenic cells, and signaling molecules. The scaffold provides a substratum for cell migration into the defect site or functions as a carrier for transplanted cells. Signaling molecules serve to recruit and differentiate osteogenic cells residing in the blood supply or neighboring healthy tissue. Osteogenic cells such as bone marrow stromal cells synthesize and deposit new bone tissue within the defect site. Better understanding of the factors that affect marrow stromal cell differentiation will allow researchers to optimize the design of bone tissue engineering constructs toward healing large bone defects in human patients.

We have characterized the effects of scaffold properties and culture supplements on the osteoblastic differentiation of marrow stromal cells (MSCs) seeded on solid, porous scaffolds and cultured in a flow perfusion bioreactor. This bioreactor creates a culture environment similar to that experienced by osteoblasts in vivo by minimizing diffusional constraints and providing mechanical stimulation to the cells through fluid shear. For these studies, MSCs were seeded on scaffolds, cultured under static or flow perfusion conditions, and assayed for DNA, alkaline phosphatase activity, osteopontin, and calcium to assess osteoblastic differentiation. Light and electron microscopy were used to visualize cell morphology and matrix deposition. The results show that brief exposure of MSCs to dexamethasone, a chemical stimulus typically required for osteoblast differentiation, was required prior to seeding on a titanium fiber mesh scaffold for ectopic bone formation to occur in a subcutaneous implantation site. However, in the absence of dexamethasone, either flow perfusion culture or decellularized bone-like extracellular matrix deposited on titanium fiber mesh induced osteoblastic differentiation in MSCs.

Alteration of the diameter of titanium fibers composing the mesh affected the osteoblastic differentiation of seeded MSCs in flow perfusion culture; wider fibers were conducive to early osteoblast differentiation while thinner fibers were conducive to later differentiation and matrix deposition. Alternatively, coating the titanium scaffold surface with the adhesion peptide RGDS resulted in increased cell adhesion strength leading to delayed osteoblastic differentiation in vitro, but had no effect on bone formation in vivo. Flow perfusion culture of MSCs seeded on porous calcium phosphate ceramic scaffolds resulted in better cell distribution within the scaffold and enhanced osteoblastic differentiation compared to static culture. These results show that scaffold geometry influences cell behavior in a flow perfusion bioreactor, emphasizing the importance of scaffold design in bone tissue engineering. In addition, undifferentiated marrow stromal cells can be induced toward the osteoblastic phenotype by signals other than dexamethasone, including bone-like extracellular matrix and fluid flow mediated shear stress.

Cyclic intramyocardial pressure fluid stimulation alters nutrient artery morphology
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Introduction
Stress fracture is the result of multiple microdamage induced from repetitive cyclic activities, and is often associated with military training and vigorous sports. It is hypothesized that such injury is initiated by bone remodeling and catalyzed by pathologic bone fluid flow. Bone fluid flow induced by intramyocardial pressure (Imp) has been demonstrated to mediate bone modeling in the presence of mechanical strain. To further evaluate the potential mechanism of this fluid flow effect on altering the nutrient supply, we hypothesize that fluid flow generated by Imp oscillation will generate nutrient vasculature adaptation which may trigger bone remodeling. The objective is to investigate the relationship between Imp induced bone fluid flow and nutrient arterial adaptation.

Methods
Under anesthesia, the left ulnae of 29 adult, one year old male turkeys were operated. A 3-mm in diameter hole was drilled and tapped near the proximal end of each ulna, allowing the insertion of special designed fluid loading device. Following the same procedure as the left, a sham device was placed into the contralateral right ulna as a sham control. Repetitive Imp was applied via an oscillation system for 10 minutes per day, 5 days per week. The loading protocol is shown in table 1.

After euthanization, the nutrient arteries were dissected, embedded in paraffin wax, sectioned to â¼8μm and stained with H&E for histomorphometry analysis. The average arterial wall area and thickness were measured using Osteomeasure™. Smooth muscle cell apoptosis was analyzed using TUNEL (Roche, USA). The elastin and collagen fibers were examined using Van Gieson stain (EMS, USA). The nonparametric Wilcoxon test was used to evaluate significance (p<0.05) within and between groups.

Results
Nutrient arteries subjected to Imp stimulation demonstrated changes in wall area (+27% for 3Hz, 3-weeks; +50% for 30Hz, 3-weeks; -6% for 3Hz, 4-weeks; +18% for 30Hz, 4-weeks) and in wall thickness (+20%, +17%, 4%, and 9%, respectively). When comparing between groups, significant changes in arterial wall area and thickness were found between loading at 3-weeks and 4-weeks, as indicated in figure 1. Preliminary analysis of the cross-sectional images did not show any apoptotic smooth muscle cells, yet the distribution of the connective fibers were slightly different between the loaded vessel and its sham control. However, additional analyses are necessary in order to quantify the results.

Discussion
The histomorphometry results strongly suggested that Imp induced bone fluid flow affect the adaptive response of the nutrient artery. Three to four weeks of Imp fluid loading may be a critical time point for arterial wall adaptation. In addition, our results suggest that Imp stimulation leads to nutrient artery hypertrophy, by thickening of the arterial wall and potentially altering the distribution of its connective tissues. This study implies that bone fluid flow induced via Imp may further influence the blood supply to bone and ultimately trigger pathological remodeling in bone tissue.

Table 1. In vivo loading treatment

<table>
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<th>Loading Conditions</th>
<th>n</th>
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<tr>
<td>30Hz, 3 weeks</td>
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<td>3</td>
<td>4</td>
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<tr>
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</table>

Fig. 1. Histomorphometry analysis of the nutrient arteries subjected to 3Hz or 30Hz Imp stimulation, 10 minutes per day for 3-week or 4-week. (A) Arterial wall area. (B) Arterial wall thickness. Mean ± SE. * indicates p<0.05 & # indicates p<0.01.
Introduction

Bone poroelasticity theory developed by Biot, predicts load induced bone fluid flow as a result of the pressure gradient. When the applied load is rapid compared to the fluid relaxation time and the fluid has no time to drain, it is locked within the solid phase which results in an increase in stiffness. Cortical bone ultrasound velocity (SOS) increases with increasing stiffness of the bone so may be used to measure changes in stiffness.

Methods

Ultrasound velocity (SOS) measurements (“Sunlight, Equs”), Tel-Aviv, Israel) were taken before and during applied hydrostatic pressure at a midshaft dorsal site on the third metacarpal (MC3) bones of the right forelimb (RD) or the left forelimb (LD).

In vitro intramullary pressure was induced by saline fluid injected through the nutrient foramen of both third metacarpal bones in six yearlings, (RD and LD) under constant high pressure (~400mmHg). Drain condition was achieved when the fluid visibly flowed from the cortical bone cut surface.

In vivo changes in hydrostatic pressure were induced when one forelimb was elevated while the opposite forelimb was measured in 3 adult horses. Standard SOS measures were taken with both forelimbs on the ground and then a treatment measure taken while the opposite forelimb was raised. These horses were measured at rest and then following a dynamic loading of treadmill exercise for two minutes at a speed of 10kph.

Results

In vitro SOS increased by 2% (RD) and 2.6 % (LD) in the undrained condition, but these changes were not significantly different from the standard. There was a reduction in SOS in vivo in the drained condition which was significantly different from the standard after exercise. There was a high correlation (r² = 0.98) between in vivo RD dynamic load drain results and in vitro RD drain results.

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>SOS m/s</th>
<th>Δ SOS m/s</th>
<th>P</th>
<th>t-value</th>
<th>SD</th>
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<td>951.4</td>
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<td>25.2</td>
<td>0.11</td>
<td>25.9</td>
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</tbody>
</table>

Discussion

This study suggests that increased hydrostatic pressure generates pore consolidation which increases SOS as Biot’s poroelasticity theory predicted. There was a reduction in SOS when fluid was seen to drain from the cut surface, which might be due to pressure released (drained).

The undrained condition showed a greater increase in SOS magnitude due to increased hydrostatic pressure compared with the drained condition effect on SOS magnitude. The SOS response of the equine third metacarpus to hydrostatic pressure as well as the low fluid flow effect indicates that fluid flow may not have an important role in MC3 dorsal site and that the bone at this site is relatively stiff.

Responses to fluid flow during early osteogenic differentiation of mesenchymal stem cells

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Introduction

Human mesenchymal stem cells (hMSCs) are multipotent cells capable of self-renewal and differentiating into cells of mesenchyme origin, such as bone, cartilage, adipose, tendon, ligament and skin. Exogenous mechanical stresses can regulate an increasing amount of cellular components and mechanosensitive markers of multiple cell types including hMSCs.

Methods

Human MSCs were cultured in either basal or ‘osteogenic’ medium (hMSC-Obs) for 3 days (group A) or 7 days (groups B and C). Both hMSCs and hMSC-Obs were subjected to fluid flow at 9 dyne/cm² for 24 hrs in a parallel plate flow chamber with flow loop. Following flow, groups A and B were returned to culture in basal or osteogenic medium, respectively, for 3 additional days, and then assayed for alkaline phosphatase (AP) activity and DNA content. Group C was assayed for cellular and released AP activity and DNA content immediately after flow treatment.

Results

Figure 1. Alkaline phosphatase activity per cellular DNA. (A) Fluid flow-induced shear stress reduced sustained cellular AP/DNA at two time points but had no effect on immediate AP/DNA. (B) Fluid flow-induced shear stress reduced released AP/DNA at 8 days but had no effect on cellular or total AP/DNA. Black boxes are hMSC – no flow, white boxes are hMSC – flow, striped boxes are hMSC-Ob – no flow, dotted boxes are hMSC-Ob – flow. Values are mean ±SEM of independent experiments: *, p<0.05; **, p<0.01; ***, p<0.005 ([A] 7 days, n=4; 8 days, hMSC n=6; hMSC-Ob n=7; 11 days, n=5; [B] Cellular AP, n=6; released AP, n=4; cellular plus released AP, n=4).

Discussion

This regime may inhibit early osteogenic differentiation of hMSCs, and help to understand development of early osteogenic differentiation.
Understanding osteopenia in the context of interstitial fluid flow

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Decades of studies on bone adaptation have helped to clarify the critical role of interstitial fluid flows in mediating growth and resorption responses in bone tissue. Surprisingly, little of this knowledge base has been translated to clinical medicine, which would permit improvements to be made in the prevention and treatment of bone adaptation problems such as osteopenia and osteoporosis. Consequently, these clinical conditions are primarily treated through the use of drugs such as bisphosphonates which block bone resorption, but which can produce significant long term complications.

To advance such translational efforts, our work over the past five years has been focused on clinical studies of bone adaptation, with an emphasis on relating changes in fluid flow through bone to changes in bone density. Fluid flow in bone is driven, in part, by mechanical strain induced pressure gradients. However, for the majority of the day and night, bone fluid flows are driven predominantly by blood pressure and hydrostatic pressure gradients.

A series of recent clinical studies from our lab, which have addressed the role of these chronic interstitial flows in mediating bone adaptation, will be reviewed. This review will include investigations on the effect of upright posture on bone density; the effect of postural sway on bone density; age related changes in postural muscle dynamics; the role of skeletal muscle pump activity on lower limb fluid flows; the role of blood pressure; the role of muscle stimulation; and the differential effects of blood flow and interstitial flow in mediating bone growth.

These studies demonstrate that consideration of fluid flow rates and patterns in bone provides a more clear and mechanistic understanding of bone adaptation than possible through the traditional focus on mechanical stress and strain distributions. Moreover, such a perspective has set the stage for the development of non-pharmacologic clinical interventions which could be utilized to prevent bone loss or even enhance bone formation in the elderly, those in extended bed rest, and as well, those with a distinctly sedentary lifestyle.
The First International Bone Fluid Flow Workshop was held at The City College of New York on September 8th, 1997. Steve Cowin, CCNY, organized it.

The Second International Bone Fluid Flow Workshop with the objective of summarizing the state of research on bone fluid flow and its role in the bone tissue mechanosensory system was held on September 20th, 2000 also at the City College. Susannah Fritton, CCNY, organized it. It was so highly successful that the workshops became annual events.

The Third International Bone Fluid Flow Workshop was held just before the American Society for Bone and Mineral Research annual meeting in Phoenix, AZ on October 11, 2001. John Frangos, UCSD, organized it. The attendance was poor because the meeting occurred just one month after September 11, 2001.

The Fourth International Bone Fluid Flow Workshop was held in Amsterdam on May 6 and 7, 2002. J. Klein-Nulend, Department of Oral Cell Biology at the Free University, chaired it. It was highly successful.

The Fifth International Bone Fluid Flow Workshop was held in Cleveland, Ohio on September 17-18, 2003. Melissa Knothe Tate of the Lerner Research Institute at the Cleveland Clinic Foundation chaired it. It was the most attended workshop to date and it was highly successful.

The Sixth International Annual Bone Fluid Flow Workshop—Frontier Research on Bone Growth, Modeling/Remodeling, and Adaptation was held on September 30th and October 1st 2004 in Seattle, WA. The Chairman for the Workshop was Professor Yi-Xian Qin of the Departments of Biomedical Engineering and Orthopaedics at the State University of New York at Stony Brook. It was highly successful.

The Seventh International Annual Bone Fluid Flow Workshop—Translational Bone Fluid Flow will be held on September 20th and 21st, 2005 in New York City. The chairman for the Workshop is Steve Cowin, CCNY.
Seventh International Bone Fluid Flow Workshop: Translational Bone Fluid Flow
September 20–21, 2005
New York City