Mechanotransduction in Bone — Role of the Osteocyte Network

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The capacity of bone tissue to adapt to changing mechanical demands is well documented, but how the cells of bone perform this task remains poorly understood. Over the last decade, significant progress has been made in understanding how bone cells may sence and transduce mechanical signals derived from bone loading. These studies emphasize the role of osteocytes as the "professional" mechanosensory cells of bone, and the lacuno-canalicular network as the structure that mediates mechanosensing. The regulatory process of mechanical adaptation produces flow of interstitial fluid in the bone lacunar-canalicular network along the surface of the osteocytes, which is likely the physiological signal for bone cell adaptive responses in vivo. As a result, the maintenance of a mechanically efficient architecture is likely to depend on a balance between the intensity and spatial distribution of the mechanical stimulus and the responsiveness of the bone cells. At the tissue level, strain distributions occur during the bone remodeling process, that show a relationship to the activity of osteoblasts and osteoclasts. This suggests that the subsequent activation of osteoclasts and osteoblasts during remodeling, is a strain-regulated phenomenon. Here, we present a theory that explains the alignment of secondary osteons as well as the constancy of their diameter as a product of osteoclast attraction and rejection by osteocytes under opposite local strains, leading to reduced and enhanced canalicular flow, respectively. Nitric oxide is likely a key molecule in this process, as its absence would lead to osteoclast attraction by causing osteocyte apoptosis, while its production by well-strained osteocytes leads to osteoclast withdrawal.

Key words: *bone remodeling, osteocyte, mechanotransduction, fluid shear stress, nitric oxide.*

1. Bone Growth and Remodeling

Anatomically, two types of bones can be distinguished in the skeleton: flat bones (skull bones, scapula, mandible, ilium) and long bones (tibia, femur, humerus, etc.). These two types are derived by two distinct types of development, intramembranous and endochondral, respectively, although the development and growth of long bones actually involve both types of processes.

The adult skeleton is in a dynamic state, being continually broken down and reformed by the coordinated actions of osteoclasts and osteoblasts on trabecular (also called cancellous) bone surfaces and in haversian systems. This turnover or remodeling of bone occurs in focal and discrete packets throughout the skeleton. The concept of bone remodeling by Bone Multicellular Units or BMUs is well established, but how the resorbing osteoclasts find their way through the pre-existing bone matrix remains unexplained. The alignment of secondary osteons and trabecular hemi-osteons along the dominant loading direction, suggests that remodeling is guided by mechanical strain.

1.1. The Skeletal System

The skeleton is multifunctional in that it provides the rigid framework and support that gives shape to the body, serves to protect delicate internal organs, endows the body with the capability of movement, acts as the primary storage site for mineral salts, and functions in haematopoiesis. The vertebrate skeleton is composed of two main subdivisions: axial and appendicular components. The axial skeleton encompasses the skull, spine, sternum, and ribs, whereas the appendicular skeleton defines the bones of the extremities. The skull, in turn, is best regarded as consisting of two units: the chondrocranium whose elements first develop in cartilage, and the cranial vault and most of the upper facial skeleton, which arise from the direct conversion of undifferentiated mesenchymal cells into bone.

Bone formation arising from a cartilagenous template is referred to as endochondral bone ossification. This is a complex, multistep process requiring the sequential formation and degradation of cartilagenous structures that serve as templates for the developing bones. Formation of calcified scaffold, however, occurs not only during skeletogenesis, but is also an integral part of postnatal growth, bone modeling, and fracture repair. Intramembranous

bone differs from the endochondral component in that it is formed in the ab-
sence of a cartilagenous blastema. Rather, it arises directly from mesenchymal
cells condensating at ossification centers and being transformed directly into
osteoblasts.

The organization and morphology of the developing skeleton are estab-
lished through a series of inductive interactions. The functional elements
in these inductive and morphogenetic processes are not individual cells but
rather interacting populations that elaborate an extensive extracellular ma-
trix, which in turn feeds back onto these populations controlling their differ-
entiation potential.

1.2. Intramembranous Bone Formation

Intramembranous bone formation is achieved by the direct transforma-
tion of mesenchymal cells into osteoblasts, the skeletal cells involved in bone
formation. It is the process responsible for the development of the flat bones
of the cranial vault, some facial bones, and parts of the mandible and clavi-
cle. The addition of bone within the periosteum on the outer surface of long
bones is also described to arise from intramembranous bone formation.

1.3. Endochondral Ossification

The axial and appendicular skeleton develops from cartilagenous blaste-
ma, the growth of which arises in a variety of ways. Cartilage is unique among
skeletal tissues in that it has the capacity to grow interstitially, i.e. by di-
vision of its chondrocytes. This property is what allows cartilage to grow
very rapidly. Moreover, cartilage utilizes apposition of cells on its surface,
matrix deposition, and enlargement of cartilage cells as additional means
of achieving maximal growth. Appositional growth is the principal function
of the periochondrium, which envelopes the epiphyses and the cartilagenous
diaphysis, serving as the primary source of chondroblasts. With time, these
cells differentiate to chondrocytes that secrete type II collagen, aggrecan, and
a variety of matrix molecules that constitute the extracellular matrix of the
hyaline cartilage. As development proceeds, capillaries invade the perichon-
drium surrounding the future diaphysis and transform it into the periosteum,
while osteoblastic cells differentiate, mature, and secrete type I collagen and
other bone-specific molecules, including alkaline phosphatase. This will ulti-
mately mineralize by intramembranous ossification and give rise to the bony
collar, the cortical bone.

A predetermined program of chondrocyte differentiation then ensues in the central diaphysis, just underneath the bone collar, leading to chondrocyte hypertrophy, synthesis of type X collagen, and calcification of the cartilage matrix, likely in response to signals emanating from periosteal osteoblasts. In turn, matrix mineralization is followed by vascular invasion from vessels originating in the periosteal collar that allows for the migration of osteoblast precursor cells into the cartilagenous blastema (primary ossification center). These cells transform into mature osteoblasts and initiate new bone formation on the degraded matrix scaffolding. The primary growth plates are then established and serve as a continual source of cartilage conversion to bone and linear growth of the long bone during development and postnatally. In late fetal life and early childhood, secondary centers of ossification appear within the cartilagenous epiphyses by a mechanism very similar to that used in the formation of the primary center. Cartilage is retained at the joint surface, giving rise to articular cartilage, and at the growth plate, extending the full width of the bone and separating epiphysis from diaphysis. Cessation of growth occurs at the end of puberty, when growth plates are replaced by bone.

1.4. Bone Remodeling

Once growth and modeling of the skeleton have been completed, the bones continually alter their internal architecture by remodeling, which is the localized removal of old bone and replacement with newly formed bone. The process is complex, requiring interactive cellular activity, and is regulated by a variety of biochemical and mechanical factors. It is likely that the major reason for remodeling is to enable the bones to adapt to mechanical stresses. Remodeling also allows the bone to repair microdamage and thus maintain its strength. Finally, remodeling is an important component of mineral metabolism.

Bone as a material compares poorly with other engineering materials. At repetitive loading equal to 100 miles of running, fatigue damage will occur. However, unlike the other materials, bone can repair itself by directing remodeling to the damaged site. In some situations, the rate of repair cannot keep up with the rate of damage, and the material fractures. In addition to remodeling which repairs cracks and fatigue damage, there is remodeling acting to continually renew bone. This prevents accumulation of older, densely mineralized bone, which is more brittle.

The remodeling events in bone are slow. It takes 4 or 5 years for an area on the bone surface to complete one bone remodeling cycle. Although the dynamics of bone physiology are slower than other organs, many of the same principles apply. Bone senses and then responds to external forces or stimuli, and adjusts itself to different environments.

A fundamental property of bone remodeling is that it occurs in discrete locations and involves a group of different kind of cells. This secondary level of organization was named the basic multicellular unit by Frost [1]. The BMU is not a permanent structure. It forms in response to signal or stimulus, performs its function, and disbands, leaving a few residual lining cells and osteocytes. Each BMU undergoes its functions in the same sequence: origination and organization of the BMU, activation of osteoclasts, resorption of old bone, recruitment of osteoblasts, formation of new bone matrix, and mineralization.

1.5. Remodeling at the BMU Level

At any given time, approximately 20% of the cancellous bone surface is undergoing remodeling, and at any one surface location, remodeling will occur on average every 2 years. The skeleton contains millions of BMUs, all at different stages. What initiates the organization of a new BMU? This question has not yet been answered, but evidence shows that mechanical stress can be sensed by osteocytes that can signal lining cells to form a new BMU at either cortical or cancellous surfaces. The osteocytes excrete paracrine factors when subjected to mechanical stimuli. Following fatigue loading, osteocyte apoptosis is seen in association with microdamage as well as resorption [2].

Origination is the first step in organizing a BMU, and thus it must involve gathering of the initial cells that will form the new BMU. Precursor cells must proliferate and be available. Many hormones and cytokines exert most of their influence at this step.

The life span of a BMU is not well defined. Cortical BMUs can wander for months, usually in a straight line. Estimates are from 2-8 months. The BMU front travels at a rate of about $10 \, \mu m/day$. In cortical bone, the BMU progresses into solid bone, and the signal comes from existing BMU cells. Replacement osteoclasts must come from the capillaries that are formed within the BMU. As the BMU progresses, new osteoclasts are required at shifting locations. Most research on osteoclast recruitment has focussed on

differentiation and proliferation, and not on localization to the precise site of resorption.

In a BMU, the resorption phase lasts about 8 days of rapid resorption followed by 34 days of slower resorption. During resorption, bone-derived growth factors are released, and collagen is digested. After the maximum eroded depth has been achieved, there is a reversal phase, that lasts approximately 9 days. During this phase, osteoblasts converge at the bottom of the cavity and start to form osteoid. After 15 days, the osteoid begins to mineralize. The time to fill in the cavity at any given point of the surface is 124-168 days.

Each BMU is associated with a capillary. In cortical bone, the capillary grows along the excavated tunnel. On trabecular surfaces, small capillaries are frequently seen adjacent to osteoblasts.

2. In Search of the Third Cell

The question is how the different strains around cutting and closing cone in a BMU are sensed by osteoclasts and osteoblasts. Many recent studies show evidence that mechanosensing in bone is primarily a task for the *osteocytes*, the mature, long-lived, terminal differentiation stage of osteoblasts that lie buried in the mineralized bone matrix. Comparison of the responsiveness of osteocytes, osteoblasts and periosteal pre-osteoblasts to mechanical strain in vitro, showed that the osteocytes were most responsive, more than osteoblasts and these more than pre-osteoblasts. Thus, in the course of differentiation from immature pre-osteoblast via osteoblast to osteocyte, bone cells increase their sensitivity to mechanical strain, which is suggestive of a specific role for osteocytes in mechanosensing.

2.1. Mechanotransduction

Mechanotransduction is the process by which mechanical energy is converted into electrical and/or biochemical signals. In principle, all eukaryotic cells are probably mechanosensitive and physical forces, including gravity, tension, compression, and shear, influence growth and remodeling in all living tissues at the cellular level [3]. In vertebrates, bone is the tissue best suited to cope with large loading forces because of its hard extracellular matrix. This matrix can be considered a toughened composite material with collagen and calcium phosphate mineral as the structural elements [4]. The
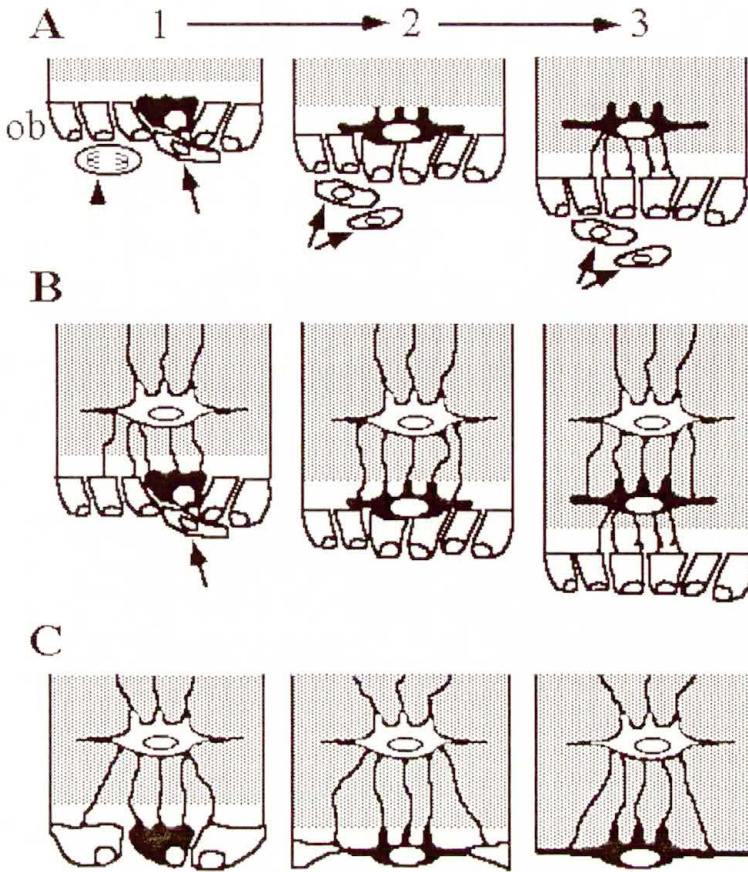
FIGURE 1. Schematic representation of the growth of bone tissue. In A and B, a quiescing osteoblast (black cell, Figs. A1, B1) turnes into an osteocyte (Figs. A2,3; B2,3), because it's neighbouring osteoblasts continue to produce osteoid, thereby embedding the quiescing osteoblast, or pre-osteocyte, in bone matrix. In A, during rapid growth, proliferation of progenitor cells (arrow head) ensures a plentiful supply of postmitotic pre-osteoblasts (arrows) which may take the place of pre-osteocytes. In B, growth starts to diminish, because supply of proliferating progenitors has stopped, and only postmitotic pre-osteoblasts (arrow) remain. In C, no pre-osteoblasts are left. All remaining osteoblasts stop producing osteoid, and the mineralization process continues up till the last layer of flattening osteoblasts (Fig. C2), which become lining cells (Fig. C3). Note that in all three cases the kinetics of bone tissue growth are regulated by the rate of osteoblast progenitor cell recruitment, apart from osteoblast lifetime and osteoblast synthetic activity.

notion that bone and bones not only develop as structures designed specifically for (future) mechanical tasks, but that they can adapt during the life of an individual toward more effective mechanical performance, stems from the last century [5, 6]. Although functional adaptation is a general phenomenon and not specific for bone tissue, it remains intriguing that such a hard and seemingly inert material as bone can be gradually altered during life, and in such a "sensible" manner. Mechanical adaptation ensures efficient load bearing: the daily loads are carried by a surprisingly thin structure. In trabecular as well as in compact bone the three-dimensional organization of the elements (plates and struts in the former, osteons in the latter) depends on the direction of the principal mechanical stresses during daily loading and movement. Mechanical adaptation is a cellular process and needs a biological system that senses the applied mechanical loading [7]. The loading information must then be communicated to effector cells that can make new bone or destroy old bone. Osteoblasts are the cells that produce new bone by synthesizing collagen and making it calcify; osteoclasts are the cells that can degrade bone matrix by subsequent demineralization and collagen degradation. However, the majority of the cells of bone tissue, some 95% in the adult skeleton, are osteocytes, lying within the bone matrix, and bone lining cells, lying on the surface. Both osteocytes and lining cells derive from osteoblasts that have stopped producing bone matrix (Fig. 1).

2.2. Osteocytes

Osteocytes are litterally buried in bone matrix. They form as long as new osteoblasts are recruited, to take the place of the buried osteoblast, now osteocyte, on the actively forming bone surface (Fig. 1A, B). When the recruitment of new osteoblasts stops, the last remaining osteoblasts flatten out and cover the now inactive bone surface as lining cells (Fig. 1C). Osteocytes remain in contact with the bone surface cells and with neighbouring osteocytes via long slender cell processes that are connected by means of gap junctions. Differentiation of osteocytes from osteoblasts may facilitate the deposition of mineral in the newly formed collagen matrix. However the matrix immediately around osteocyte cell body and processes does not calcify, and thus a three-dimensional network of lacunae and canaliculi is formed containing non-mineralized, osteoid-like matrix and the osteocyte cells. The cell network is connected, again via gap junctions, with the bone lining cells on the bone surface (Fig. 1C). This three-dimensional network of interconnected

cells that is present throughout and around a piece of bone, is a very attractive structure for the detection of local mechanical inadequacies [8, 9]. Since the cellular network neighbours on the bone marrow stroma as well as on the periosteum, recruitment of new osteoblasts and osteoclasts by the network is also easily foreseen. In addition, the non-mineralized matrix of lacunae and canaliculi is much easier penetrated by water and (small) molecules than the mineralized matrix. Therefore this network may also be considered a complex structure of pores and channels, the lacuno-canalicular porosity.

2.3. Osteocytes and the Lacuno-Canicular Porosity in Mechanotransduction

Because they are post-mitotic and embedded in hard matrix, osteocytes are difficult to study. This and their general appearance of inactive cells as to protein synthesis, has made them the least studied cell type of bone. Nevertheless knowledge is increasing, in conjunction with the interest during the last couple of years in their putative role as mechanosensors. In vitro, osteocyte cultures were found to re-establish their stellate morphology and again form a network via many slender cell processes and gap junctions [10–13] (Fig. 2).

The osteocyte cultures produced small amounts of collagen and fibronectin (much less than osteoblasts), but were more active than osteoblasts in producing osteocalcin, osteonectin, and osteopontin [14]. Evidence for parathyroid hormone receptors on their surface was found [10]. Evidence for their role as mechanosensory cell in bone has been steadily growing over the last ten years. Early strain-related changes in glucose-6-phosphate dehydrogenase activity were found in osteocytes following bone loading [15]. Loading resulted in transient expression of c-fos mRNA in cortical osteocytes and lining cells of rat tail vertebrae. Osteocytic gene regulation by mechanical stress includes expression of IGF, although the reports are somewhat variable. IGF-I promotes bone formation, and stimulates the differentiation of osteocytes from osteoblasts. Another mechanically regulated gene in bone is osteopontin, one of the major non-collagenous proteins in bone matrix. Mechanical loading increased OPN mRNA expression via the microfilament component of the cytoskeleton, and more in mature than in immature bone cells [16]. Thus, mechanical loading activates several cellular processes in osteocytes, including energy metabolism, gene activation, growth factor production, and matrix synthesis.
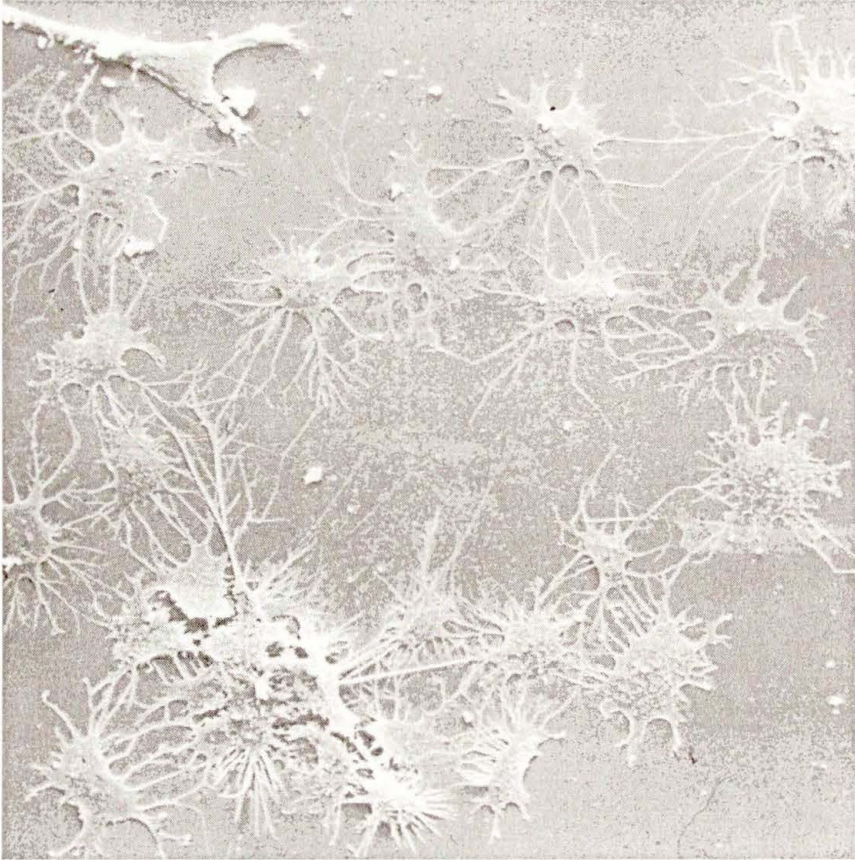
FIGURE 2. Scanning electron micrograph of a group of osteocytes, isolated from embryonic chicken calvariae, after 3 days of culture as monolayer. The cells have re-established a cellular network, by moving away from each other and making thin, branching cell processes that connect with those of neighbouring cells. The non-random distribution of the cell processes, and their straightness, suggest that the processes have a means to sense each other's presence. The cell in the upper left corner is a contaminating osteoblast. Micrograph kindly provided by Dr. P.J. Nijweide.

2.4. Osteocytes and Fluid Flow—the Canalicular Fluid Flow Hypothesis

In a study on cell signalling after mechanical stimulation, monolayer cultures of osteocytes, isolated from embryonic chicken calvariae, responded to one hour pulsating fluid flow with a sustained release of prostaglandins E_2 and I_2 [17]. Osteocytes were much more responsive than osteoblasts, and

intermittent hydrostatic compression had less effect than fluid flow [17, 18]. Prostaglandins are essential for the transduction of mechanical stimuli into bone formation, while prostaglandins - particularly PGE_2 - stimulate osteoblastic cell proliferation and bone formation. Klein-Nulend et al. [17] used fluid flow for mechanical stimulation [19] of the osteocytes, to test the hypothesis, developed by Cowin and associates, that in intact bone the osteocytes are mechanically activated by flow of interstitial fluid through the lacuno-canalicular porosity [20–22]. According to this hypothesis, the prime driving force for bone adaptation is the strain-driven motion of interstitial fluid through the canaliculi and along the osteocyte processes, which is sensed and transduced by osteocytes. Because bone matrix is so stiff, the deformation – or strain – imposed by physiological loads is only very small (maximally of the order of 0.2%). However in vitro, strains of the order of 1-3% are needed to obtain a cellular response. The Canalicular Fluid Flow hypothesis proposes that, rather than the bulk strains resulting from loading the whole bone, a local force derived from that strain (or rather, strain rate), activates the osteocytes. When bone is loaded, interstitial fluid is squeezed through the thin layer of non-mineralized matrix surrounding cell bodies and cell processes towards the Haversian or Volkmann channels, thereby producing fluid shear stress at the osteocyte cell membrane. In trabecular bone the lacuno-canalicular network drains on the bone marrow sinusoids. Haversian channels, Volkmann channels, and sinusoids themselves will not generate meaningful amounts of shear stress during physiological loading, because they are much too wide (Haversian channels are roughly 3,000 times wider in cross section than canaliculi) [23]. However the combination of canalicular diameter and the diameter of the osteocyte process, produces an annular porosity that is well suited to generate appreciable fluid shear stress during physiological bone loading [22]. Assuming that these stresses perturb the osteocyte surface, in particular the osteocyte processes in canaliculi, a magnitude of 8-30 dynes/cm^2 (or 0.8-3 Pa) fluid shear stress was predicted during physiological loading [22]. Interestingly, pulsating fluid flow with a mean stress of 0.5 Pa and 5 Hz pulses of ± 0.02 Pa, provoked an immediate response in osteocytes, measured as a two times increased release of nitric oxide (NO) and a five times increased release of prostaglandins PGE_2 and PGI_2, after 5 minutes application of flow [18, 24]. Pulsatile fluid flow was also found to be more effective than steady flow in modulating the bone cell's response [25]. These studies confirmed the efficacy of fluid flow as a mechanical stimulus

for bone cells. Fluid shear stress has been shown to be more effective than mechanical stretching on bone cells [26].

Although flow of interstitial fluid through the canaliculi as a result of bone loading was already postulated in 1977 [27], experimental proof of this phenomenon was provided only a couple of years ago [28]. Using low and high molecular weight tracers, the diffusive transport as well as the convective transport resulting from load-induced fluid flow was studied in intact bones. These studies found that diffusion alone was not efficient for transport, in the canaliculi, of larger molecules such as microperoxidase, and that load-induced fluid displacements are necessary for the maintenance of metabolic activity in osteocytes as well as activation or suppression of modeling processes.

Flow of fluid over the cell surface subjects the cell to two types of stimuli, fluid-induced drag forces (or fluid shear stress) and streaming electrical potentials. The latter are usually held responsible for the cellular responses in bone [21, 22]. However, there are data that argues that the fluid-induced shear stress, the direct mechanical perturbance of the cell(membrane), is the stimulus that conveys the mechanical message to the bone cell. A combination of shear stress and streaming potentials for complete cell activation is also possible, and needs further study.

Although the case for canalicular fluid flow in mechanotransduction seems now well established, the question was if and how shear stress magnitude
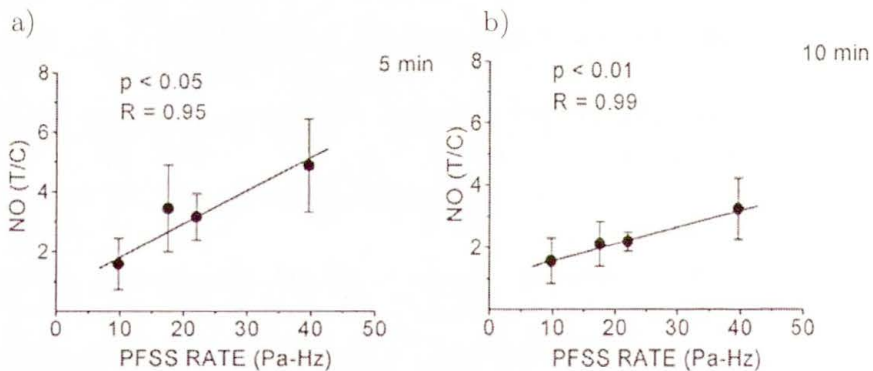

FIGURE 3. Nitric oxide production by bone cells is linearly proportional to the rate of fluid shear stress. The steepest slope was found at 5 min. ($0.11\,Pa\,Hz^{-1}$), indicating that the highest bone cell response to fluid shear stress rate occurs rapidly. At 10 min., NO levels were lower than those found at 5 min. Pa-Hz, Pa times Hz; PFSS—pulsating fluid shear stress. Values are mean treatment-over-control ratios (T/C)±SEM.

and/or pulse frequency are related to the type and magnitude of cellular responses. Bacabac et al. [25] found that the rate (determined by frequency and magnitude) of mechanical loading determines the bone cell's response. It was shown that the fluid shear stress rate is an important parameter for bone cell activation (Fig. 3).

2.5. Nitric Oxide and Prostaglandins as Mediators of Loading-Induced Adaptive Bone Responses

The importance of NO and prostaglandins as mediators of loading-induced adaptive bone responses has been substantiated by a number of studies. Transient rapid increase of NO release was found in several in vitro systems, including osteocyte monolayer cultures and bone organ cultures [17, 18, 24]. In vivo, the NO inhibitor L-NAME suppresses mechanically induced bone formation in rats. In vitro, fluid flow rapidly (within 1 hour) induced the expression of prostaglandin G/H synthase II, or COX-2, in mouse bone cells [30], while in vivo in rats, specific inhibition of COX-2 but not COX-1—the constitutive form of the enzyme—prevents the induction of bone formation. As inhibition of NO release also prevented the enhanced release of PGE_2 after fluid flow [24], prostaglandin upregulation seems to be dependent of NO upregulation. The NO response could recently be linked to the constitutive expression by bone cells of endothelial nitric oxide synthase, or ecNOS [31]. Human bone cell cultures from several donors constitutively expressed ecNOS, and showed a modest (two-fold) upregulation of ecNOS expression 1 hour after a 1 hour treatment with pulsatile fluid flow [24]. ecNOS is the isoenzyme that was hitherto considered specific for endothelial cells. Interestingly, in endothelium ecNOS expression is related to the sensitivity of endothelial cells to blood fluid shear stress which is part of the mechanism whereby blood vessels can adapt their diameter to changes in blood pressure. The response to fluid shear stress in endothelial cells has been extensively characterized, and includes activation of a number of kinases and multiple transcription factors followed by induction of gene expression. Although the response to flow in bone cells is less well characterized, several similarities with the endothelial response have now been reported, including upregulation of prostaglandins, release of NO by constitutively expressed ecNOS, regulation of ecNOS expression by shear stress, and induction of c-fos. The similarities of these early responses suggest that both cell types possess a similar

sensor system for fluid shear stress. Sensitivity for fluid shear stress appears to be a differentiated trait of the osteocytic phenotype, same as in endothelial cells. As such, this finding is an argument in favor of fluid flow as the mediator of mechanotransduction in bone - as postulated by the Canalicular Fluid Flow hypothesis.

3. Histomechanics of Bone Remodelling

The alignment of secondary osteons and trabecular hemi-osteons along the dominant loading direction in bone suggests that remodeling is guided by mechanical strain. This means that bone adaptation (Wolff's Law) takes place throughout life at each remodeling cycle. We propose that alignment during remodeling occurs as a result of different canalicular flow patterns around cutting cone and reversal zone during loading. Local strain and flow patterns around a remodeling osteon comply with local bone resorption and formation: "coupling" can be explained by changing mechanical conditions around the cutting versus closing cone.

3.1. Bone as a Natural Composite

Bone is a natural composite with a rich hierarchical structure. At the highest level, cortical bone is distinguished from trabecular bone by differences in structure and density, but at lower levels all bone can be considered as a continuum (Fig. 4).

In humans, after 1 year of age, bone is renewed bit-by-bit and replaced by units of secondary bone. This remodeling process continues throughout life. The structural unit of secondary cortical bone is the osteon, essentially a thick-walled cylinder of concentric lamellae containing blood vessels and nerves. On the other hand, trabeculae consist of lamellar bone segments called trabecular packets; these may be seen as "hemi-osteons" and in fact are formed in a similar way. All secondary bone is aligned to the dominant loading direction, which is illustrated in Fig. 5.

In addition, its density (mass) increases with the magnitude and frequency of daily loads. Load-related alignment and density of bone are in fact the foundations of the law of bone transformation [6]. Computer simulations confirmed the postulate that bone apparently adapts its form according to rules of mathematical design. However, the cellular mechanism of mechanical adaptation is still unexplained.
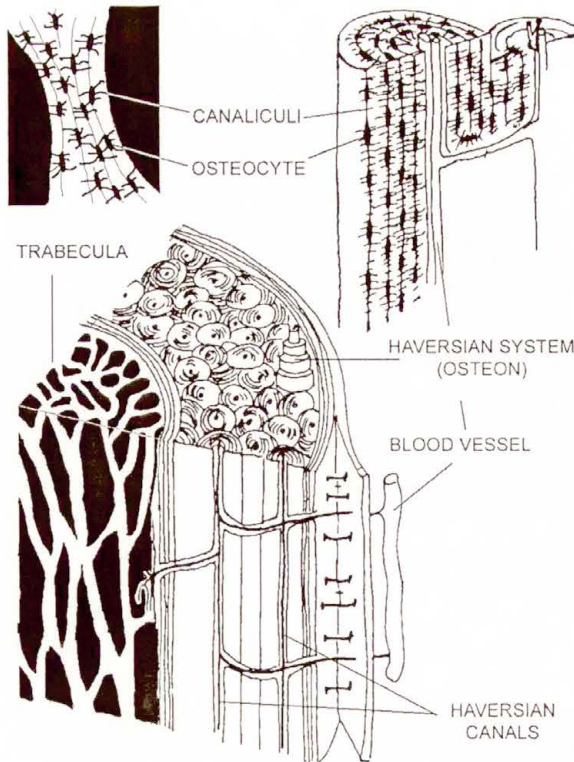
FIGURE 4. The hierarchical structure of secondary bone. Both trabeculae and osteons, shown on top in an exploded cross-sectional view, have a lamellar structure (thin lines) aligned to the longitudinal (loading) direction. Osteocytes are flattened out parallel to the bone surface, and their protrusions run perpendicular to that through the bone matrix. The bone marrow around the trabeculae is black.

Bone remodeling involves groups of different cells, which collaborate in basic multicellular units (BMUs). In cortical bone, BMUs proceed by tunneling, during which osteoclasts excavate a canal that is refilled partly by osteoblasts (Fig. 6). On the other hand, trabeculae are renewed by the excavation and refilling of trenches along the surface. Osteoclasts and osteoblasts are cell types that originate from different lineages (stromal and haematopoietic stem cells, respectively), and their molecular and functional characteristics have been studied extensively. However, it is unclear how the concerted action of osteoclasts and osteoblasts is orchestrated. Smit et al. [32] discussed possible mechanisms, explaining the typical dimensions of (hemi-)osteons and their alignment to the loading direction.
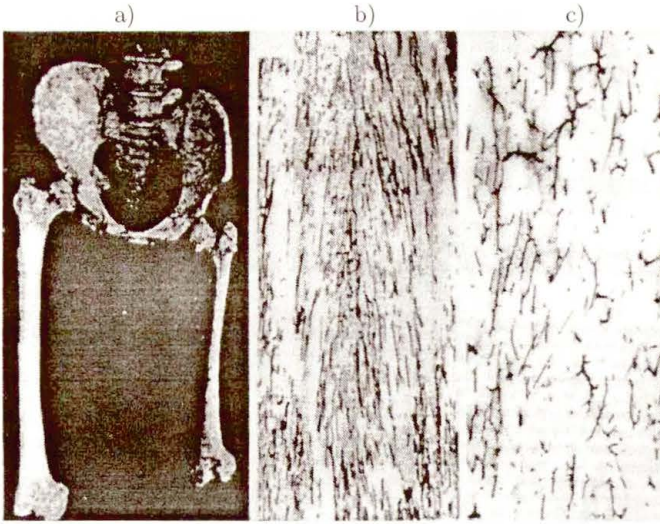
FIGURE 5. Mechanical load and osteonal alignment in cortical bone. a) case of an individual with left-sided hypotrophy due to poliomyelitis. b) Osteonal organization in the normally loaded (large) femur shown by a technique with black Indian ink that stains the Haversian canals. c) Osteonal organization in the marginally loaded (small) femur. Anisotropy and number of osteons are small compared with the contralateral normal femur, indicating that the remodeling activity was strongly reduced and not well oriented [33].
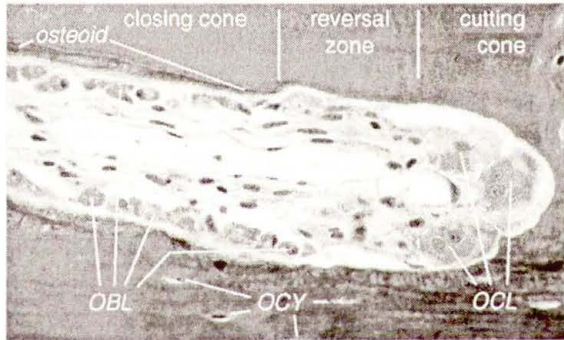

FIGURE 6. The cellular activity during bone remodeling. At the tip (cutting cone) multinucleated osteoclasts (OCLs) excavate the mineralized bone tissue. At some distance, after the resting zone, osteoblasts (OBLs) appear at the surface to refill the tunnel with osteoid that subsequently is mineralized. Osteocytes (OCYs) are former osteoblasts that were entombed within the bone matrix but remained connected to the bone surface by numerous long slender protrusions (not visible). Typical outer diameter of an osteon in human cortical bone is approximately $200\,\mu$m.

The key issue in load-directed bone remodeling is that sensors must exist to detect mechanical strains and to direct subsequent cellular activities. Osteoclasts or osteoblasts cannot be the sensors themselves because they do not receive a mechanical signal before they attach to the bone surface, and attachment occurs only after recruitment of the progenitor cells from the bone marrow. Better candidates are therefore the osteocytes, the cells that reside inside the bone matrix and thus have a good position for mechanosensing. With their long slender protrusions they form a three-dimensional network that reaches to the bone surface, which allows them to signal the effector cells. In a theoretical study on the nutrition of osteocytes, Piekarski and Munro [27] suggested that on mechanical loading of bone, extracellular fluid flows through the the lacuno-canalicular porosity toward the bone surface and back into the bone on unloading. Streaming potentials that have been measured in bone specimens confirm that ions actually are transported through the bone matrix. Knothe-Tate et al. [28] found direct experimental evidence that molecules of relevant size are transported throught the lacuno-canalicular porosity by this mechanism.

3.2. Effects of Fluid Flow

Fluid flow may give rise to at least three biophysical effects. First, an enhanced mass transport will occur, ensuring that osteocytes receive sufficient nutrients for survival within the bone matrix far away from the blood vessels, and that their waste products are washed away to the bone surface. Second, because of the charged bone matrix and the ionic composition of the extracellular fluid, an electrokinetic effect occurs in the form of streaming potentials. Streaming potentials might modulate the movement of ions such as calcium across the cell membrane and, subsequently, cell behavior. Third, a fluid shear stress is generated on the cell membrane, which is a well-known stimulus for cells. Bone cells, in particular osteocytes, are extremely sensitive to fluid shear stress, responding by rapid release of, among others, nitric oxide (NO), cyclic adenosine monophosphatase (cAMP), and prostaglandins (PGs), and by expression of cyclo-oxygenase-2 (COX-2). It is difficult to estimate the actual role of each of the three biophysical effects in mechanotransduction because in the in vivo situation they occur simultaneously. Nevertheless, all three are potentially powerful modulators of cell behavior, and, thus, strain-induced fluid flow appears to be a good mediator of mechanical information.

4. Cell Biology and Histomechanics

Bone adaptation (Wolff's Law) takes place throughout life at each re-modeling cycle. Alignment during remodeling occurs as a result of different canalicular flow patterns around cutting cone and reversal zone resulting from loading. Low canalicular flow around the tip of the cutting cone, reduces NO production by local osteocytes thereby causing their apoptosis. Osteocyte apoptosis attracts osteoclasts, leading to further excavation of bone in the direction of loading. At the transition between cutting cone and reversal zone, enhanced canalicular flow stimulates osteocytes to release NO, which induces osteoclast retraction and detachment from the bone surface. Together this leads to a treadmill of attaching and detaching osteoclasts in respectively the tip and the periphery of the cutting cone, and the digging of a tunnel or trench in the direction of loading.

4.1. Bone Strains and (Hemi)Osteons

It has become generally accepted that bone tissue renews itself throughout life by means of basic multicellular units (BMUs), groups of osteoclasts and osteoblasts that act in a coordinated fashion to first resorb existing bone tissue and subsequently refill the gap with new bone tissue. The new bone, organized as osteon in compact bone and hemi-osteon in trabecular bone, is aligned along the dominant loading direction, suggesting local strain gradient is a regulating factor [33, 34]. However, our understanding of the cellular mechanisms producing such a mechanically meaningful structure remains poor.

BMUs literally move through existing bone tissue during the process of bone renewal. Osteoclasts appear first, and dig a tunnel through compact bone or a trench along the surface of trabecular bone. The tunnel or trench is subsequently filled with bone tissue by osteoblasts that appear to follow the osteoclasts. The question of how osteoclasts and osteoblasts are able to collaborate to produce such (hemi-)osteons that run along the direction of dominant strain of the particular piece of bone, remains a mystery. A few theories have been put forward. The first theory holds that osteoblasts are able to instruct osteoclasts when and where to resorb bone matrix, and there-fore determine both the catabolic and anabolic phases of bone remodeling. This theory is based on experimental data that hormonal regulation of os-teoclast activity is directed by osteoblastic cells, which express the hormone receptors and produce signaling molecules that regulate osteoclast activity.

A second theory, that of "coupling", holds that after resorption is finished the surface of the remaining bone attracts osteoblasts, possibly by releasing growth factors from the matrix. A modification of the coupling concept proposes that osteoclast and osteoblast precursors are subsequently recruited by the endothelium of the capillary of a progressing BMU. These concepts are able to explain the sequential appearance of osteoclasts and osteoblasts in a progressing BMU. However, for an explanation of the *geometry* of bone renewal, they are completely inadequate because they make no link with local strains.

In order to understand how strain can determine the geometry of remodeling, it is important to realize when and where alignment is produced. In the remodeling process, a team of osteoclasts digs the tunnel or groove. Through that activity, the osteoclasts determine not only the direction of the the future (hemi-) osteon but also its diameter. Osteoblastic activity determines to what extent the defect is refilled. This is important, as in the long run it determines bone *mass*. *Orientation* however is determined by osteoclastic activity, more precisely by the direction in which the team of resorbing osteoclasts moves. So how can we explain that osteoclasts "know" their way while they are "eating" themselves through the bone tissue? As the orientation of (hemi-) osteons follows the direction of the prevalent local stress, bone loading must somehow direct the activity of the osteoclasts. Sensitivity for mechanical strain is likely a characteristic of all eukaryotic cells, so osteoclasts might in principle determine their own direction based on the mechanical information they receive. Direct mechanical information from deforming matrix to moving cell requires a close contact between cell and strained matrix, and resorbing osteoclasts do make such close contact in the sealing zone around the ruffled border. However, *how* would that strain direct cell movement? Only one theory has been put forward that deals with this issue. Martin et al. [35] have proposed that osteoclasts are activated by *tensile* stress while osteoblasts are activated by *compressive* stress. In a piece of bone that is loaded under compression, increased tension appears at the tip of the cutting cone during remodeling and increased compression around its base. The increased tension at the tip could direct the movement of the osteoclasts, thereby guiding them along the direction of loading. However in a piece of bone that is loaded in tension, as at the tensile side of bending bones, the stress fields are *inverted*, and compressive stress appears at the tip of the cutting cone. Tensile versus compressive stress thus can not explain

the behaviour of osteoclasts or osteoblasts, and therefore the theory must be rejected [34].

It was recently proposed that local tissue deformations around the cutting and closing cone of a progressing BMU determine osteoclast and osteoblast activity [34]. Using equivalent strain as a scalar measure for the deformation of the bone tissue around a remodeling (hemi-) osteon, opposite strain fields were found around the cutting and closing cone, both under compressive and tensile axial loading. Decreased strain was found in front of the cutting cone, just where resorption continues to proceed. Elevated strain however was found behind the cutting cone, where osteoblasts are active [34]. This observation suggests that the subsequent activation of osteoclasts and osteoblasts in a BMU could be regulated by very local strains of different magnitude in the surrounding bone. However, the question remains how the different strains around cutting and closing cone are sensed by osteoclasts and osteoblasts. Many recent studies show evidence that mechanosensing in bone is primarily a task for the *osteocytes*, the mature, long-lived, terminal differentiation stage of osteoblasts that lie buried in the mineralized bone matrix (see for a review [7]). Comparison of the responsiveness of osteocytes, osteoblasts and periosteal pre-osteoblasts to mechanical strain in vitro, showed that the osteocytes were most responsive; next came osteoblasts, and subsequently the pre-osteoblasts [17, 18, 24, 36]. Thus, in the course of differentiation from immature pre-osteoblast via osteoblast to osteocyte, bone cells increase their sensitivity to mechanical strain, which is suggestive of a specific role for osteocytes in mechanosensing. The manner whereby osteocytes sense the strains of the mineralized matrix has been considered in light of the very small strains in bone during daily loading, as compared to muscle tissue for example [21, 22]. This has led to the concept of strain-derived canalicular fluid flow, or its derivative, fluid drag force, as the physical mediator of mechano-sensing by osteocytes in bone tissue [22, 37]. Several studies support this concept [17, 18, 24, 36]. Together they suggest that the osteocyte network with its accompanying lacuno-canalicular porosity is the site of mechanosensing in bone tissue. Mechanotransduction then includes the translation, by osteocytes, of canalicular flow into cell signals that can recruit osteoclasts and osteoblasts. Therefore, if local strain differences around the cutting- and closing cone of a BMU should regulate the activity of osteoclasts and osteoblasts, these strain gradients must produce local canalicular flow differences that can be related to the recruitment of these two cell types.

4.2. Relation of Volumetric Strain in the Bone around a BMU Cutting Cone to Canalicular Fluid Flow

At maximal loading, a typical volumetric strain pattern appears in the wall of the tunnel, around the BMU (Fig. 7). The volumetric strain results in a flow of canalicular fluid that is different at the tip of the cutting cone and at its base (Fig. 8). At the tip of the cutting cone however, fluid is pressed into the canaliculi as a result of local volumetric expansion (Fig. 8). Influx occurs only in a shallow layer some $10 \, \mu$m deep, after which the flow changes into an efflux (Fig. 9). So, just below the surface of the cutting cone, where influx and efflux meet, the net canalicular fluid flow is about zero at maximal loading of the bone.
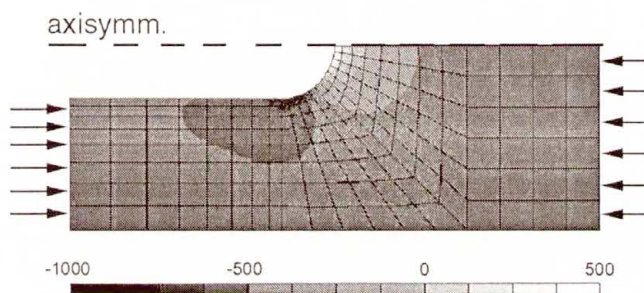

FIGURE 7. Volumetric strain within the bone matrix around the progressing end of an osteonic BMU at maximum loading during the walking cycle. The direction of loading is indicated by arrows. A superficial area of volumetric expansion appears at the tip of the cutting cone. At the base of the (hemi-)cone an area of high volumetric compression appears. Values are microstrains.
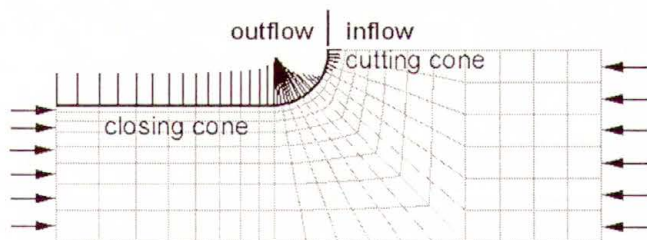

FIGURE 8. Fluid flow pattern in a remodeling osteon at maximum load during a walking cycle. Volumetric expansion leads to influx of canalicular fluid at the tip of the cone. At the base of the cone, high volumetric compression produces high efflux of canalicular fluid.
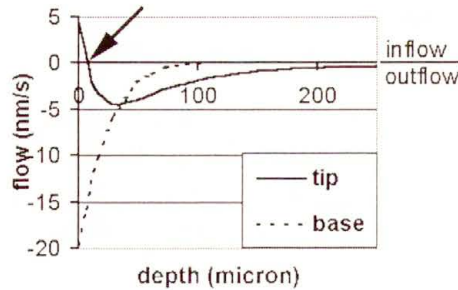
FIGURE 9. Fluid flow pattern within the bone tissue at maximum load during
the walking cycle. At the tip of the cutting cone (continuous line), the inflow
(resulting from volumetric expansion of the superficial bone layer) changes into an
outflow because of volumetric compression of the deeper bone layer. The reversal
(indicated by arrow) occurs at a depth of about 10 micrometer. At this depth,
canalicular fluid flow will be zero. At the base of the cutting cone (dashed line),
high volumetric compression leads to high fluid flow in the canaliculi, which runs
towards the cone void and is maximal near the bone surface.

4.3. Proposal of a Mechanism Explaining the Behaviour of the Team of Osteoclasts in the Cutting Cone [38]

The different canalicular flow patterns around the tip and the base of
a cutting cone during loading indicate that the osteocytes in these two loca-
tions receive different mechanical information. First of all, the flow pattern
was reversed between the two sites. Both at maximal loading and unloading,
the flow in the bony wall of the tip was opposite to the flow in the wall of the
base. More important however is the magnitude of flow at these two locations.
At the tip of the cutting cone, a small superficial zone, some 10 micrometer
deep, of volumetric expansion was followed by volumetric compression which
reached it's maximum at 30 micrometer depth. The combination produced
opposite flow directions in the canaliculi, a superficial *in*flux of fluid from the
cutting cone void that reverted to an *out*flux of canalicular fluid from deeper
bone toward the cutting cone void. As a result, the net fluid flow is close to
zero at a depth of some 10 micrometer in the wall of the cutting cone tip,
where the opposite flows meet. At the base of the cutting cone, the flow pat-
tern was unidirectional under both loading and unloading. At loading, fluid
was pressed out of the bone, reaching maximal canalicular flow at the border
of bone and cutting cone void. At unloading this strain pattern was reversed,
but because strain rate was slower, flow magnitude was considerably lower.

These volumetric strain patterns suggest very different canalicular fluid shear stresses during cyclic loading such as walking, for the local osteocytes around a remodeling BMU. At the tip of the cutting cone, the phase of maximum loading produces a near stand still of canalicular fluid at some 10 micrometers deep, and the phase of unloading a flow of low magnitude. The amount of shear stress experienced by the most superficial osteocytes around the tip, will therefore be extremely low. At the base of the cutting cone the situation is quite different. Maximal loading here results in an outflow of fluid that produces maximal shear stress in the canaliculi of the most superficial osteocytes. Further on, along the reversal zone, this pattern continues, with highest shear stress around the most superficial osteocytes.

What will be the effect of these wholly different stress conditions on the osteocytes? We have shown that in cell culture experiments, osteocytes produce high levels of nitric oxide (NO) in response to fluid shear stress [24]. NO production results from the activity of endothelial cell nitric oxide synthase or ecNOS, a plasma membrane bound enzyme originally believed to be specific for endothelium but later also found in osteocytes and osteoblasts [31]. Interestingly, ecNOS is the NO producing enzyme isoform specifically involved in the cellular response to fluid shear stress, as occurs in the endothelium of blood vessels in response to blood flow. The finding that osteocytes also express ecNOS, and that the bone cell enzyme is also activated by fluid shear stress, suggests that there may be common functions for ecNOS in endothelium and osteocytes. In endothelial cells, NO production by ecNOS in response to fluid flow plays a major role in preventing apoptosis of the endothelial cells. In areas of reduced blood flow, as at the periphery of blood vessel bifurcations where atherosclerotic plaque accumulates, endothelial cell death occurs. This endothelial cell apoptosis is believed to be caused by insufficient NO production as a result of insufficient fluid shear stress over the cells in that area of the vessel wall. NO production in response to adequate shear stress protects the endothelial cells against apoptosis. We propose that such a mechanism also operates in bone, and that osteocytes are also protected against apoptosis by a basal amount of NO production under normal canalicular shear stress. At the tip of the cutting cone of a BMU therefore, osteocytes enter apoptosis as a result of insufficient NO production due to insufficient fluid flow in their canaliculi.

In endothelium, apoptosis of endothelial cells attracts monocyte/macrophages that phagocytose the dying cells. We propose that in bone, the apop-

totic osteocytes at the tip of the cutting cone attract osteoclasts, that resorb the bone matrix as well as phagocytosing the dying osteocyte (Fig. 10). Evidence that osteoclastic attack is directed towards apoptotic osteocytes has been reported [2, 39]. Similar to macrophages, osteoclasts are attracted to apoptotic cells that expose phosphatidylserine on their outer cell surface at an early stage of apoptosis [40]. In the case of osteoclasts, the apoptotic cells that attract them are osteocytes and hypertrophic chondrocytes of the growth plate [40]. Exposure of phosphatidyl-serine on osteocytic cell "fingers" in canaliculi abutting on the wall of the cutting cone might therefore be the signal that urges the osteoclasts to continue resorption in that direction. As osteoclasts further excavate the cutting cone, the zone of low fluid flow and osteocyte apoptosis also moves further under the influence of the strain fields so as to steer osteoclastic resorption in the correct direction.

The mechanism discussed above can explain why osteoclasts resorb in the direction of loading, which is the first key issue that must be solved for a cellular explanation of bone adaptation or "Wolff's Law". A second question is why osteoclasts *stop* resorbing, at the base of the cutting cone. Here too, NO production in response to canalicular flow may play an important role. At
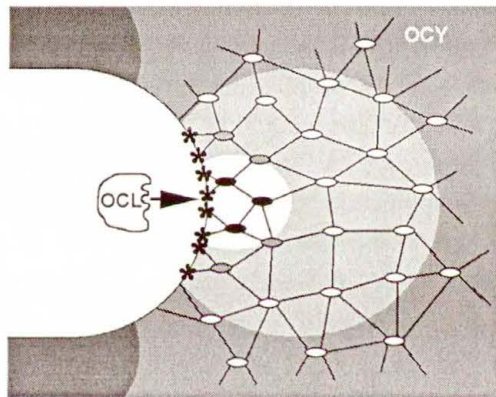

FIGURE 10. Cartoon of the cutting cone tip, showing the relation between apoptotic osteocytes and a progressing osteoclast. Osteocyte apoptosis (indicated as black lacunae) is caused by canalicular stasis, which directly results from the volumetric strain pattern caused by cyclic loading in the normal direction. As osteoclasts are attracted towards apoptotic osteocytes because of changes on the apoptotic cell surface (indicated by asterisks), the direction of the osteoclastic attack follows the direction of loading. OCY, osteocyte network; OCL, osteoclast.

the base of the cutting cone and further down the reversal zone, osteocytes receive enhanced fluid shear stress during loading. NO production will therefore be even higher than normal. Particularly the superficial osteocytes will produce much NO, because shear stress is highest close to the surface. The high NO level will prevent futher osteocyte apoptosis, but may also have another effect, that of promoting the retraction and detachment of osteoclasts from the bone surface. In cell culture experiments, NO has been shown to rapidly reduce the osteoclast spread area followed by retraction of the cells from the tissue culture support. A similar response may be expected at the base of the cutting cone where osteocytes produce high levels of NO. NO has a short half life, of only a few minutes, and must therefore always act locally when functioning as a paracrine cell modulator. This perfectly suites a role as a very local inhibitor of further osteoclastic attack (Fig. 11).

These two mechanisms, attraction of osteoclasts to the cutting cone tip and induction of osteoclast detachment from the cutting cone base, together explain the mechanically meaningful behaviour of osteoclasts during remod-
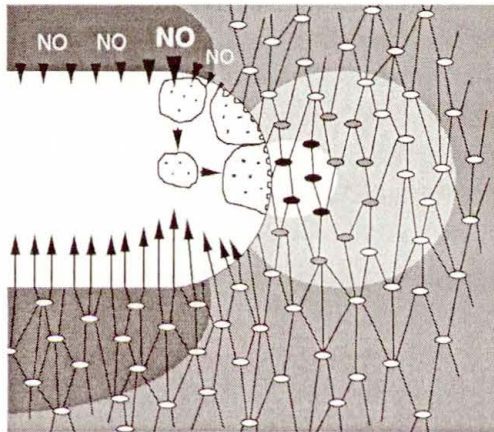

FIGURE 11. Cartoon of postulated events in the cutting cone of a progressing BMU. Osteoclasts are attracted by apoptotic osteocytes in the cutting cone tip, but forced to withdraw again from the bone surface at the cutting cone base, as a result of high amounts of NO produced by well stressed osteocytes. As NO production remains high further down the reversal zone, osteoclasts remain within the cutting cone and may even re-enter the resorption cycle, leading to a "treadmill" of active and in-active osteoclasts that together dig the resorption tunnel or trench. Vertical arrows indicate direction and magnitude of canalicular fluid flow; vertical arrow heads indicate release of NO by well stressed osteocytes.

eling. Osteoclasts are attracted to the tip by understressed, apoptotic osteocytes to resorb bone tissue in the direction of loading, while they are forced to detach and stop resorbing at the base of the cutting cone by well stressed osteocytes. In both instances NO production by osteocytes is a key issue, although it is likely that other auto- and paracrine signaling molecules such as prostaglandins are also involved [18, 36]. NO causes the detachment of osteoclasts, but does not kill them. In cell culture experiments, removal of the NO from the culture medium led to recovery of the osteoclasts and re-attachment without evidence of cytotoxicity. This suggests yet another point, namely the possibility of a treadmill of osteoclasts in the cutting cone. Osteoclasts could subsequently attach at the tip of the cutting cone, detach at its base, and reattach again at the tip. As NO production remains high all along the reversal surface and the detached osteoclasts can only reattach at surfaces not protected by NO, they can only reattach at the tip. This suggests a treadmill of the giant cells in a progressing BMU (Fig. 10).

The concept of a group of osteoclasts that digs a tunnel in response to strain-dependent osteocyte signals is attractive, because it explains the alignment of secondary osteons to the dominant loading direction, and also why the diameter of these osteons remains within certain limits. In healthy bone, the size of osteons is fairly constant, meaning that osteoclasts stop resorbing when a certain gap size has been reached. Our proposal links osteonic size directly to the magnitude of local strain, as strain induces osteocytes to inhibit further osteoclastic resorption. Local strain magnitude at the cutting cone base is of course finally determined by the amount of strain of the whole bone. As maximal bulk strain remains within fairly narrow limits during normal daily movement, within an individual, a species and even between species, it is to be expected that the osteonic diameter determined by that strain will also be constant. However if bone is not strained sufficiently, our model predicts that osteocytes are not sufficiently activated to inhibit further osteoclastic attack, leading to perforation of trabeculae and trabecularization of cortical bone, as observed after stress shielding.

Can this proposal be experimentally tested? It is largely based on the assumption that basal production of NO under adequate fluid flow promotes the survival of osteocytes by preventing apoptosis. Current tissue culture techniques did allow to apply fluid flow of well defined characteristics to cultures of freshly isolated ostecytes or bone chip-derived bone cell cultures (Klein-Nulend et al., [17, 31]). One way to test our proposal was to study

if flow applied to these cell cultures indeed modulates cell apoptosis. Our recent data show that osteocyte survival is indeed quite sensitive to fluid shear stress, and that absence of shear stress leads to their programmed cell death [41].

Summarizing, we present a theory that explains the alignment of secondary osteons and hemi-osteons, as well as the constancy of their diameter, as product of osteoclast attraction and rejection by osteocytes under opposite local strains, leading to respectively reduced and enhanced canalicular flow. NO is likely a key molecule in this process, as it's absence would lead to osteoclast attraction by causing osteocyte apoptosis, while its production, by well strained osteocytes, leads to osteoclast withdrawal.

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