Mechanotransduction in bone—role of the lacuno-canalicular network

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ABSTRACT **The capacity of bone tissue to alter its mass and structure in response to mechanical demands has long been recognized but the cellular mechanisms involved remained poorly understood. Over the last several years significant progress has been made in this field, which we will try to summarize. These studies emphasize the role of osteocytes as the professional mechanosensory cells of bone, and the lacuno-canalicular porosity as the structure that mediates mechanosensing. Strain-derived flow of interstitial fluid through this porosity seems to mechanically activate the osteocytes, as well as ensuring transport of cell signaling molecules and nutrients and waste products. This concept allows an explanation of local bone gain and loss, as well as remodeling in response to fatigue damage, as processes supervised by mechanosensitive osteocytes.— Burger, E. H., Klein-Nulend, J. Mechanotransduction in bone—role of the lacuno-canalicular network.** *FASEB J.* **13 (Suppl.), S101–S112 (1999)**

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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 (1). 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 (2). The 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 (3, 4). 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 (5–7). Mechanical adaptation is a cellular process and needs a biological system that senses the applied mechanical loading. 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 (see ref. 8 for a recent review). However, the great 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 (9). Both osteocytes and lining cells derive from osteoblasts that have stopped producing bone matrix (10, 11) (**Fig. 1**).

Osteocytes are literally 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. 1, *A* and *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. 1*C*). Osteocytes remain in contact with the bone surface cells and with neighboring osteocytes via long slender cell processes that connect by means of gap junctions (12, 13). Differentiation of osteocytes from osteoblasts may facilitate the deposition of mineral in the newly formed collagen matrix (14). However, the matrix immediately around the 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. 1*C*). This three-dimensional network of interconnected
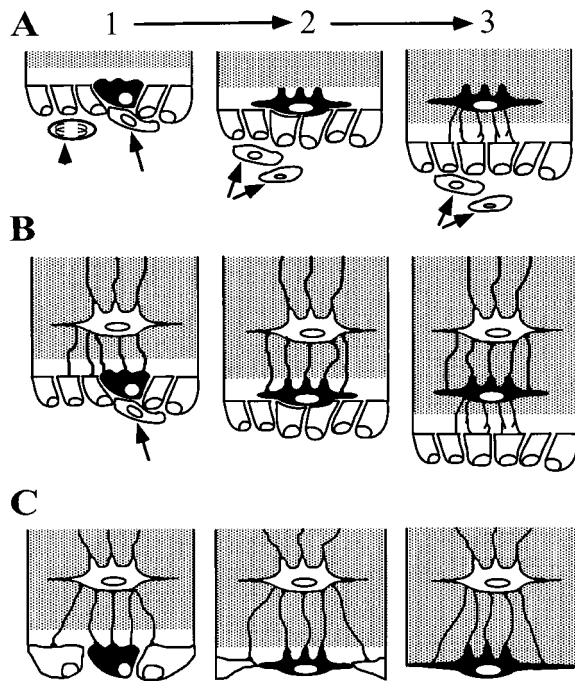
Figure 1. Schematic representation of the growth of bone tissue. *A* and *B*) a quiescing osteoblast (black cell, Fig. *A1, B1*) turns into an osteocyte (Fig. *A2, 3; B2, 3*) because its neighboring osteoblasts continue to produce osteoid, thereby embedding the quiescing osteoblast, or pre-osteocyte, in bone matrix. In *A*, during rapid growth, proliferation of progenitor cells (arrowhead) 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 until 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.

cells that is present throughout and around a piece of bone is a very attractive structure for the detection of local mechanical inadequacies (15–17). Because the cellular network neighbors 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 more easily 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.

We review the evidence that the combination of cellular network and lacuno-canalicular porosity performs the functions of mechanosensing and mechanotransduction in bone. We then propose a cell-based model to explain the role of osteocytes in regulating bone gain and loss in response to overuse and disuse, respectively, as well as bone remodeling

in response to fatigue damage. Finally, the consequence of this model for the behavior of bone under microgravity is discussed.

OSTEOCYTES AND THE LACUNO-CANALICULAR POROSITY IN MECHANOTRANSDUCTION

Because they are post-mitotic (18) 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 bone cell type. Nevertheless, information is increasing in conjunction with the recent interest in their putative role as mechanosensors. *In vivo*, osteocytes have been shown to express mRNA for β-actin, osteocalcin, connexin-43, insulin-like growth factor I (IGF-I)[2], c-fos, and c-jun, but not tumor necrosis factor α or tartrate-resistant acid phosphatase (19). *In vitro*, osteocyte cultures were found to reestablish their stellate morphology and again form a network via many slender cell processes and gap junctions (18, 20–22) (**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 (23). Evidence for parathyroid hormone receptors on their surface was found *in vitro* (18) as well as *in vivo* (24). Evidence for their role as mechanosensory cells in bone has been steadily growing over the last 10 years. Early strain-related changes in glucose-6-phosphate dehydrogenase activity were found in osteocytes after bone loading *in vivo* (25) and *in vitro* (26). At 1 h after 5 min of loading, transient expression of c-fos mRNA was induced in cortical osteocytes and lining cells of rat tail vertebrae *in vivo* (27). Osteocytic gene regulation by mechanical stress includes expression of IGF, although the reports are somewhat variable. Rapid induction of IGF-I was found by mechanical stimulation of rat caudal vertebrae (28) but another study using the same model found no effect (27). One *in vitro* study using rat bone cells found increased mRNA levels of IGF-II but not IGF-I (29), whereas other studies, also using rat bone cells, found elevated levels of IGF-I (30). IGF-I promotes bone formation (see ref. 31 for a recent review), and has been found to stimulate the differentiation of osteocytes from osteoblasts (32). Another mechanically regulated gene in bone is osteopontin, one of the major non-collagenous proteins in bone matrix. Mechanical loading increased OPN mRNA expres-

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[2] Abbreviations: IGF-1, insulin-like growth factor 1; PGE$_2$, prostaglandin E$_2$; PGI$_2$, prostaglandin I$_2$; NO, nitric oxide; TGF-β, transforming growth factor β.
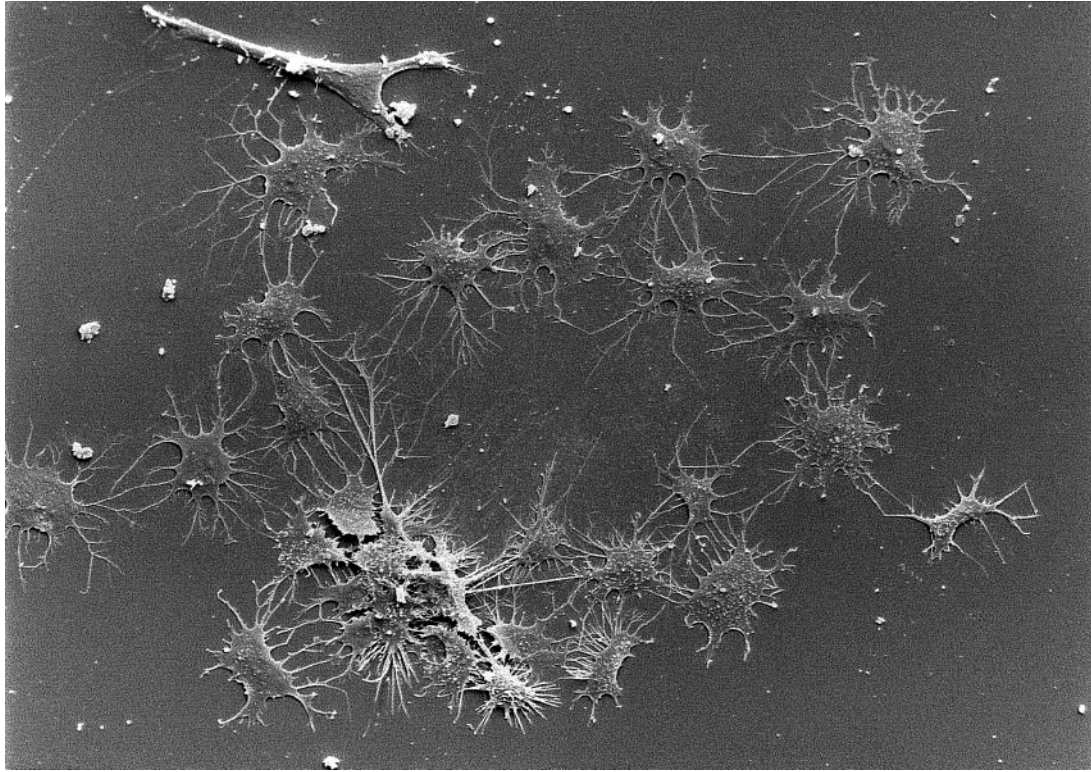
Figure 2. Scanning electron micrograph of a group of osteocytes, isolated from embryonic chicken calvariae, after 3 days of culture as monolayer. The cells have re-established a cellular network by moving away from each other and making thin, branching cell processes that connect with those of neighboring cells (see refs. 20 and 22 for more detail). 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. Bar = 100 μm. Micrograph kindly provided by Dr. P. J. Nijweide.

sion via the microfilament component of the cytoskeleton (33) and more in mature than in immature bone cells (34). Up-regulation of OPN mRNA levels was also found after bone loading *in vivo* (35). Thus, mechanical loading activates several cellular processes in osteocytes, including energy metabolism, gene activation, growth factor production, and matrix synthesis.

In a study on cell signaling after mechanical stimulation, monolayer cultures of osteocytes, isolated from embryonic chicken calvariae, responded to 1 h of pulsating fluid flow with a sustained release of prostaglandins E_2 and I_2 (PGE_2 and PGI_2, respectively) (36). Osteocytes were much more responsive than osteoblasts, and intermittent hydrostatic compression had less effect than fluid flow (36, 37). *In vivo*, prostaglandins are found to be essential for the transduction of mechanical stimuli into bone formation (38), whereas *in vitro* as well as *in vivo* exogenous prostaglandins, particularly PGE_2, stimulate osteoblastic cell proliferation (39) and bone formation (40). Klein-Nulend et al. (36) used fluid flow for mechanical stimulation 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 (41–43). According to this hypothesis, the prime mover 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 on the order of 0.2%) (44, 45). However, *in vitro*, strains on the order of 1–3% are needed to obtain a cellular response (46, 47). 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 toward 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) (48). 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 (42). 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 (42). It is interesting to note that 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 PGE$_2$ and PGI$_2$ after a 5-min application of flow (37, 49). Pulsatile fluid flow, at an average shear stress of 0.5 Pa, was found to be more effective than steady flow (0.4 Pa shear stress) in modulating gene expression in osteoblasts (50). Fluid flow rapidly increased intracellular calcium in bone cells (51), an effect that was inhibited using neomycin or gadolinium, suggesting calcium influx via stretch-activated channels (52) as well as release from intracellular stores (53). These studies confirmed the efficacy of fluid flow as a mechanical stimulus for bone cells, which was also concluded from *in vivo* studies (54, 55). Two independent studies subsequently found that fluid shear stress is more effective than mechanical stretching on bone cells (56, 57). In one study, unidirectional straining of the cell culture substratum in the range of 500–5,000 microstrain had no effect, whereas a fluid shear stress of 14.8 Pa rapidly induced both PGE$_2$ and NO production (56). In the other study, four-point bending was applied to a cell culture substratum of variable plate thickness. This study showed unequivocally that the rate of displacement, independent of the strain magnitude, correlated with the bone cell response (57). Because the rate of displacement is proportional to the fluid force, applied by the movement of fluid culture medium over the culture plate during bending, the data show that the fluid shear stress rather than the cell strain activated the cells (57). It is likely that this conclusion also applies to other models where cell monolayers are mechanically stimulated by stretching the culture substratum through a fluid culture medium (58).

Although flow of interstitial fluid through the canaliculi as a result of bone loading was already postulated in 1977 (59), experimental proof of this phenomenon was provided only quite recently (60, 61). 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 (61). Evidence supporting this conclusion was found in organ cultures of rat long bone diaphyses, where intermittent axial loading improved osteocyte viability and stimulated periosteal osteogenesis (62).

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 (63, 64). The latter are usually held responsible for the cellular responses in bone (42, 43, 54). However, a recent study found no effect of applying an external current, which either doubled or canceled the convective current density, on the calcium response of bone cells to fluid flow (65). These data argue 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, in line with an earlier experimental study (66). 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 now seems well established, the question of whether and how shear stress magnitude and/or pulse frequency are related to the type and magnitude of cellular responses remains open. High-frequency, low-magnitude stimuli (500 microstrain at 30 Hz) were sufficient to stimulate new bone formation in experimental animals, whereas low-frequency, high-magnitude stimuli (3,000 microstrain at 1 Hz) were insufficient (67). However, another study questioned the biological significance of high-frequency bone strains because of their small increase in active versus anesthetized animals (68). On the other hand, an *in vitro* study found differential electrophysiological responses in human bone cells to a cellular perturbation as small as 15 microstrain at different frequencies (69). More sophisticated methods, allowing independent variation of shear stress magnitude and pulse frequency, are needed to study dose-response aspects in detail (70).

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 (36, 37, 49, 58, 71). *In vivo*, the NO inhibitor L-NAME suppressed mechanically induced bone formation in rats (72). *In vitro*, fluid flow rapidly (within 1 h) induced the expression of prostaglandin G/H synthase II, or COX-2, in mouse bone cells (73), whereas *in vivo* in rats, specific inhibition of COX-2 but not COX-1, the constitutive form of the enzyme,

prevented the induction of bone formation (74). Because inhibition of NO release also prevented the enhanced release of PGE_2 after fluid flow (49), prostaglandin up-regulation seems to be dependent on NO up-regulation. The NO response was recently linked to the constitutive expression by bone cells of endothelial nitric oxide synthase, or ecNOS (75). Human bone cell cultures from several donors constitutively expressed ecNOS and showed a modest (twofold) up-regulation of ecNOS expression 1 h after a 1-h treatment with pulsatile fluid flow (75). ecNOS is the isoenzyme that was hitherto considered specific for endothelial cells (76). It is interesting to note that 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 (see ref. 76 for a recent review). 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 (77). Although the response to flow in bone cells is less well characterized, several similarities with the endothelial response have now been reported, including up-regulation of prostaglandins, release of NO by constitutively expressed ecNOS, regulation of ecNOS expression by shear stress, and induction of c-fos (76–79). 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, the 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.

THE LACUNO-CANALICULAR NETWORK AND BONE (RE-)MODELING—A HYPOTHESIS

As shown above, there is evidence supporting the concept that mechanical stimulation activates osteocytes to produce anabolic paracine factors (such as PGE_2 and IGF), which recruit new osteoblasts from periosteum and bone marrow stroma. To do so these factors must be brought to the bone surface, presumably in the same manner that waste products are removed, i.e., via the lacuno-canalicular porosity. Alternatively, an anabolic message is transported through the cellular network, via intracellular transport and gap junctions. A combination of inter- and extracellular signals is also possible. In any case, the integrity of the lacuno-canalicular network, both the cellular part and the porosity, are crucial for mech-

anotransduction because the porosity produces the ultimate mechanical signal for the cellular part. Using this concept it is possible to explain local bone gain as a result of local overuse, and local bone loss as a result of local disuse, as shown schematically in **Figure 3**. Physiologically "normal" bone use is needed to keep osteocytes viable by enhanced displacement of nutrients and waste, but also to provide them with a basal level of mechanical stimulation by fluid shear stress (Fig. 3A). Under these conditions no osteoblasts or osteoclasts are recruited. Overuse means overstimulation of the osteocytes by abnormally high fluid shear stress, resulting in recruitment of osteoblasts instead of lining cells to the bone surface (Fig. 3B). It is also possible that lining cells are activated to redifferentiate as osteoblasts (80). The extra bone produced by the osteoblasts restores the normal level of loading and therefore the "use" state of osteocyte stimulation (Fig. 3B). Disuse, on the other hand, reduces osteocyte shear stress stimulation as well as reducing transport of nutrients and waste products (Fig. 3C). The latter will reduce osteocyte viability and may even lead to osteocyte death. Osteocyte death could then be the signal for recruitment of osteoclasts. Indeed, a positive correlation between osteocyte apoptosis and osteoclastic attack has been described in growing bone (81). It is, however, also possible that well-stimulated osteocytes inhibit osteoclast recruitment, and that disuse leads via understimulation of osteocytes, to absence of osteoclast-inhibiting signals. Inhibition of bone resorption has been described in mechanically stimulated bone of experimental animals (82). Osteoclasts readily resorb slices of devitalized bone *in vitro* (83), but in mixed cultures of osteocytes and osteoclasts, osteocytes seemed to inhibit osteoclast activity (21). Recently an osteocyte-derived protein has been described that inhibits osteoclastic bone resorption (84). These findings suggest that active suppression of osteoclasts by osteocytes is feasible. In the concept of osteoclastic suppression by (well stimulated) osteocytes, osteocyte apoptosis and osteoclastic attack are two parallel results of lack of mechanical stimulation, rather than the former being the cause of the latter.

The theory of osteocytic suppression of osteoclasts is useful when trying to explain the process of bone remodeling (**Fig. 4**). In adult bone, osteoblastic and osteoclastic activity are largely confined to bone remodeling (85). Remodeling is a complicated process that starts by osteoclasts removing existing bone, by either digging a tunnel as in compact bone, or a more or less shallow groove along the surface of a trabecula. The tunnel or groove is subsequently refilled with new bone tissue by osteoblasts. Convincing evidence has accumulated over the last decade that remodeling serves to renew bone that was
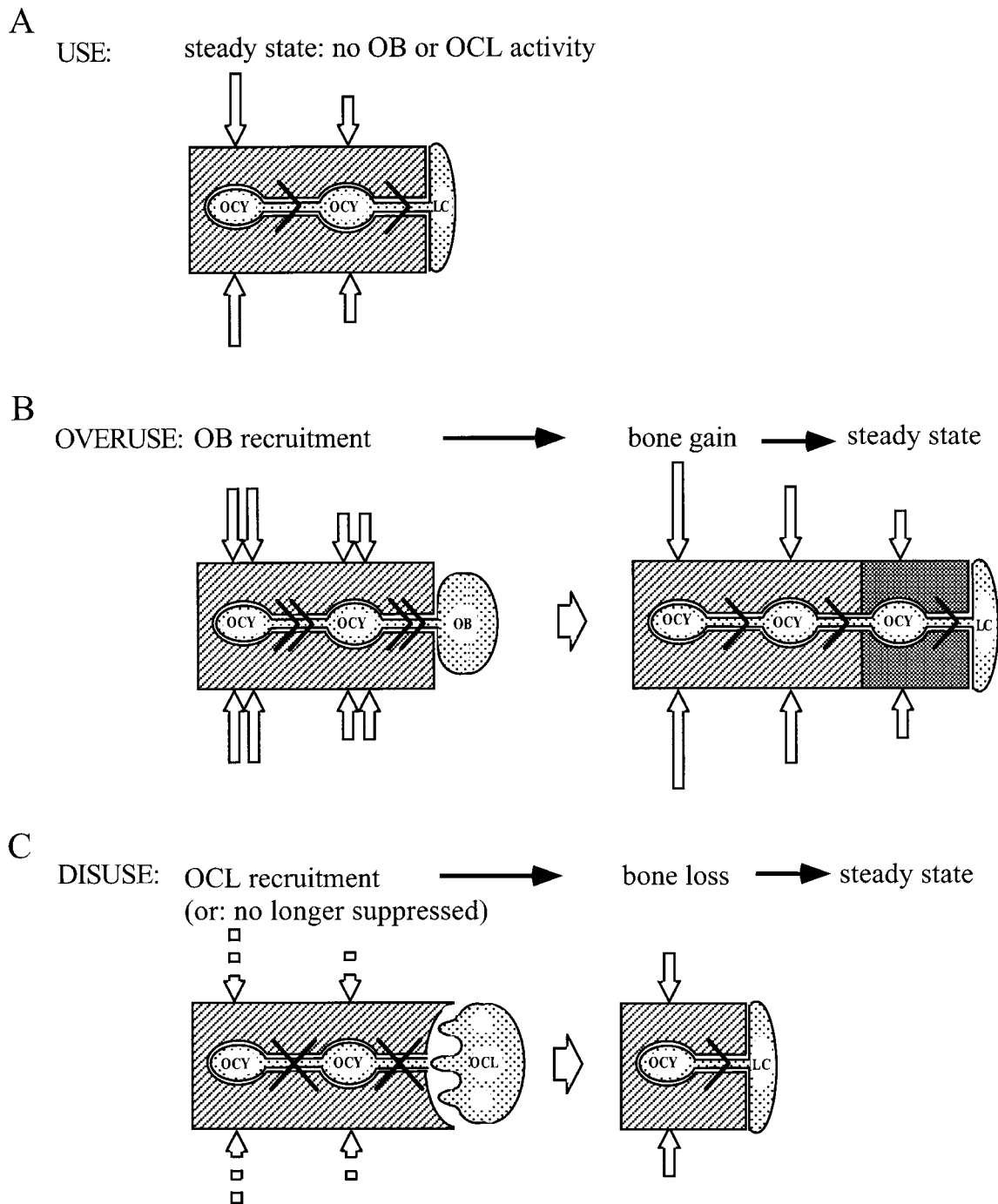
A

USE: steady state: no OB or OCL activity

B

OVERUSE: OB recruitment ⟶ bone gain ⟶ steady state

C

DISUSE: OCL recruitment ⟶ bone loss ⟶ steady state
(or: no longer suppressed)

Figure 3. Schematic representation of how the osteocyte network may regulate bone modeling. *A)* In the steady state, normal mechanical use ensures a basal level of fluid flow through the lacuno-canalicular porosity, indicated by an arrowhead through the canaliculi. This basal flow keeps the osteocytes viable and also ensures basal osteocyte activation and signaling, thereby suppressing osteoblastic activity as well as osteoclastic attack. *B)*During (local) overuse, the osteocytes are over-activated by enhanced fluid flow (indicated by double arrowheads), leading to release of osteoblast-recruiting signals. Subsequent osteoblastic bone formation reduces the overuse until normal mechanical use is re-established, thereby re-establishing the steady state of basal fluid flow. *C)* During (local) disuse, the osteocytes are inactivated by lack of fluid flow (indicated by crosses through canaliculi). Inactivation either leads to release of osteoclast-recruiting signals or to lack of osteoclast suppressing signals, or both. Subsequent osteoclastic bone resorption re-establishes normal mechanical use (or loading) and basal fluid flow. OCY, osteocyte; LC, lining cell; OB, osteoblast; OCL, osteoclast; hatched area, mineralized bone matrix; dark-gray area, newly formed bone matrix; white arrows represent direction and magnitude of loading.

impaired by fatigue microdamage (86–90). Fatigue microdamage results from repetitive loading in the normal physiological range and, when accumulating over time, leads to impairment of the mechanical properties of the bone matrix (see ref. 87 for a recent review). At the ultrastructural level, fatigue
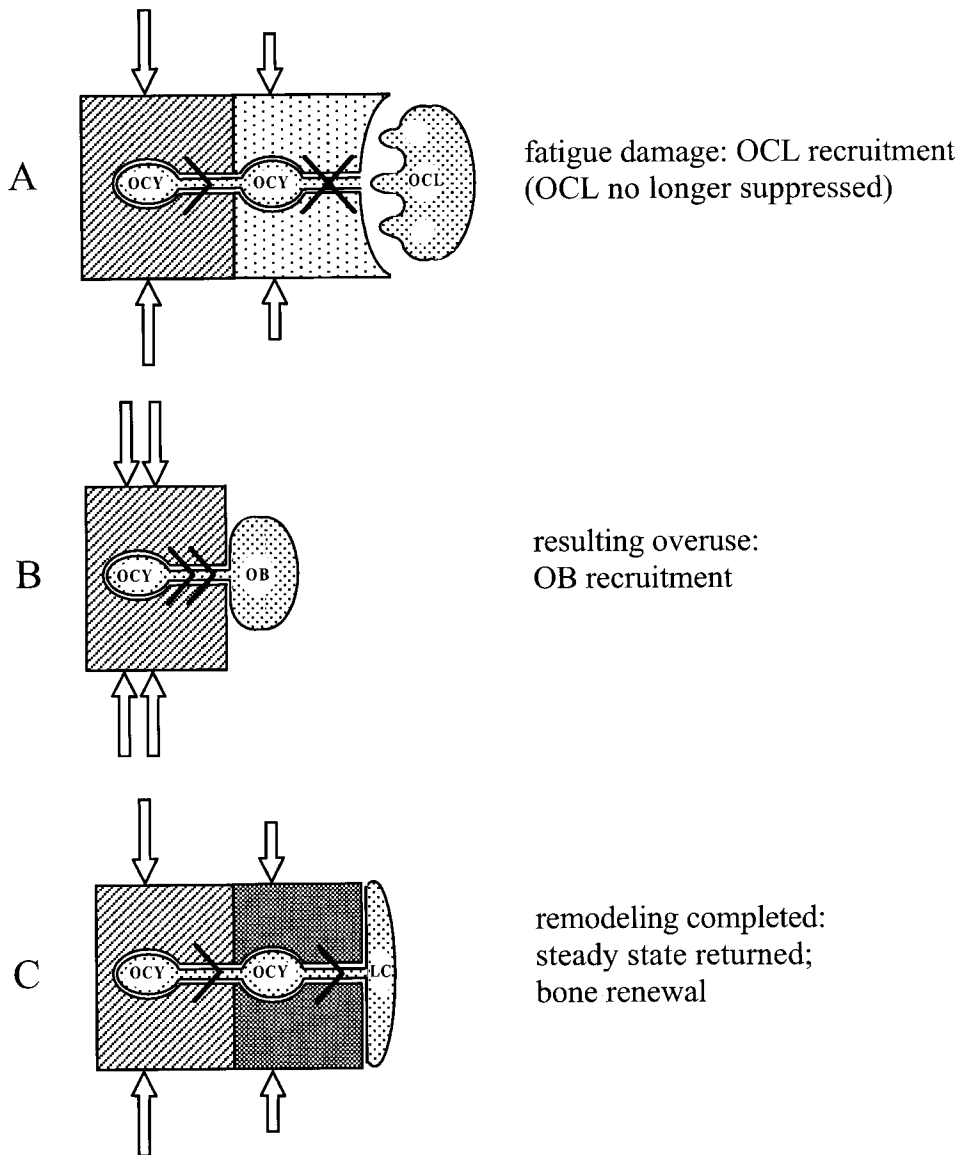
A fatigue damage: OCL recruitment (OCL no longer suppressed)

B resulting overuse: OB recruitment

C remodeling completed: steady state returned; bone renewal

Figure 4. Schematic representation of how fatigue damage may initiate bone remodeling. *A*) Accumulation of fatigue microdamage (stippled matrix) interferes with canalicular fluid flow and osteocyte signaling by disrupting canaliculi and severing osteocyte processes. Fatigue microdamage results in osteoclast recruitment, suggesting that osteocyte signaling suppresses osteoclast recruitment rather than activating it. Osteoclasts resorb damaged bone until undamaged bone is reached, when they are again suppressed. *B*) The local loss of bone after osteoclastic resorption leads to (local) overuse of the remaining undamaged bone. The resulting enhanced fluid flow through the lacuno-canalicular network leads to recruitment of osteoblasts. *C*) Subsequent osteoblastic bone formation reestablishes normal mechanical use and therefore the steady state of basal fluid flow in the renewed bone. Stippled matrix, matrix with fatigue microdamage; for other symbols see Figure 3.

leads to focal patches of ultrastructural damage of the fully mineralized matrix, shown as increased permeability of the matrix for stain (91). Ultrastructural microdamage precedes the appearance of very fine cracks observable only at greater than ×1000 magnification, and these eventually coalesce into microcracks that can be stained with basic fuchsin and are observable at low-magnification light microscopy (88). The cracks will run right through the mineralized matrix, independent of the lamellar and osteonal organization of the tissue (91). We may therefore assume that fatigue microdamage will interfere with the integrity of the osteocytic and lacuno-canalicular network by disrupting canaliculi and severing osteocyte processes. Finite element analysis predicts changes in strain at the microstructural, cellular level as a result of microdamage (92). Increased permeability between canalicular channel and mineralized matrix will decrease the fluid drag

forces in the channel (93). Fatigue damage may therefore create a situation resembling disuse at the level of the osteocyte cell body and disrupt the communication between osteocytes and bone surface. Accumulation of fatigue damage leads to bone remodeling (86), starting with osteoclast recruitment. Active recruitment of osteoclasts by osteocyte signaling is, however, difficult to reconcile with disrupted communication between damaged osteocytes and the bone surface. Rather, disrupted communication could abolish active suppression of osteoclasts by osteocytes, thereby allowing resorption to start.

Whatever the precise cellular mechanism of osteoclast recruitment, the canalicular fluid flow hypothesis predicts that fatigue microdamage will lead to bone resorption (Fig. 4*A*). Resorption of microdamaged bone will then lead to (local) overuse and stimulation of bone formation (Fig. 4*B*). Bone formation will continue until a new steady state of

normal loading (use) is reached, where osteocytes receive again the "normal use" mechanical stimuli (Fig. 4C). Hormonal regulation such as by parathyroid hormone, vitamin D, or sex hormones will modulate the general level of activity of the bone cells in a systemic manner, at the level of mechanotransduction or at the level of osteoclast/osteoblast recruitment and activity (94, 95). However, it is unlikely that hormones will interfere with the local accumulation of microdamage or with the principles of local mechanoregulated bone (re-)modeling (96).

Can the model as described above be experimentally tested? In an animal study, bending of bone proved a better stimulus for adaptive responses than vertical loading (54). Because bending also produces more canalicular fluid flow than vertical loading, this result supports the canalicular fluid flow hypothesis (54). A recent study in rats showed alteration of osteocyte and canalicular integrity around cortical resorption spaces activated by fatigue loading (96a). This study is the first to experimentally relate osteocyte integrity to initiation of bone remodeling. Other experimental verification may come from *in vitro* studies by submitting osteocytes to fluid shear stress and studying the production of osteoblast- and osteoclast-modulating factors. In the multitude of studies that have been performed on (chemical) regulation of osteoblasts and osteoclasts, several useful *in vitro* approaches have been developed, including bone organ cultures, cell cultures, and *in vitro* functional assays (97, 98). These should also be of value for testing theories of cellular mechanical regulation.

MICROGRAVITY AND BONE LOSS—A ROLE FOR THE LACUNO-CANALICULAR POROSITY?

Microgravity, as occurring during spaceflight, has negative effects on the skeleton, leading to bone loss (99–101). Histological studies in young rats suggest that the decreased bone formation rate during spaceflight may be due in part to reduced osteoblast activity (100, 102). Spaceflight decreased mRNA levels of osteocalcin, type I collagen, and transforming growth factor β (TGF-β) in rat bone periosteum (103, 104). In addition, osteoblastic cells in cell culture show a reduced growth and hormone responsiveness during spaceflight (105, 106). Finally, a decreased mineralization as well as increased mineral resorption were found in organ cultures of long bone rudiments exposed to spaceflight (107). These findings suggest that bone tissue is directly sensitive to spaceflight conditions.

The question remains as to how the lack of gravity is detected. Could microgravity act directly on the bone cells? Or more precisely, could osteocytes and osteoblasts read the gravitational field change directly? The effects of microgravity on bone cells in monolayer cell culture seem to suggest that such a detection system exists. However, a recent paper by Cowin shows that the adhesive forces acting on a living cell attached to a substratum are several orders of magnitude larger than the weight of the cell (108). Compared with the adhesive forces expressed by cells in monolayer culture (109), the gravitational forces on a cell at the earth's surface are minuscule, amounting to 10^{-3} to 10^{-4} of the adhesive force (108). This makes it unlikely that cells that are attached to their matrix, as in intact bone, or are attached to a tissue culture substratum, as in *in vitro* experiments, can sense directly the change in the gravity field of interest. Rather, the clear effects of microgravity that have been found in cultured bone cells (105, 106) may be the consequence of indirect effects of weightlessness. Microgravity strongly reduces convection in the fluid culture medium around cultured cells, thereby reducing the supply of nutrients and dispersion of waste molecules. This may have negative effects on the cultured cell's metabolism, which could explain the *in vitro* data. Unfortunately, experiments specifically addressing this point are lacking.

There is evidence that at the subcellular level, cytoskeletal macromolecule assembly may, under certain conditions, be sensitive to the gravitational field. *In vitro* studies of the self-ordering process of a cell-free preparation of tubulin in solution have shown that the morphology of the microtubule-related structures that form depends on the orientation of the sample with respect to gravity (110, 111). An important condition for this effect of gravity is that the microtubule preparation is chemically and structurally far from equilibrium (111). Such a situation might exist in a cell during the process of mitosis, when microtubular rearrangements are large. However, osteocytes are post-mitotic cells. Because they are also strongly attached to their surrounding matrix, even in monolayer culture (112), the current evidence for a direct effect of (micro)gravity on bone cells is inconclusive. Even if osteocytes or osteoblasts could sense gravitational change directly, it seems unlikely that such an ability could be related to the loss of bone mass in humans during spaceflight. Bone adaptation studies have shown that a constant, non-time-varying force applied to bone has the same effects as no force (113). Or, for the purpose of bone maintenance and adaptation, bone tissue only responds to time-varying forces, whereas the change in G force does not vary during spaceflight except for takeoff and landing.

On the other hand, spaceflight does produce a unique condition of skeletal unloading because of the near absence of gravity. Weightlessness not only

annihilates weight, but also reduces the amount of muscle contraction forces on the skeleton. Therefore, the negative effects of spaceflight on bone mass can be easily explained as resulting from disuse. Disuse leads to lack of canalicular fluid flow, osteoclastic bone resorption, and bone loss, as discussed above. The reported lack of recruitment of osteoblast progenitors during spaceflight in growing animals can also be explained by weightlessness-induced lack of canalicular flow, if we assume that the rate of osteoblast progenitor recruitment is (partly) determined by the osteocytes. Simply stated, the loss of gravity during spaceflight has a major effect on bones, but only a minuscule direct effect on bone cells. Spaceflight-related bone loss is therefore easily explained via the negative effects of microgravity on bone loading, which indirectly lead to bone loss as a result of disuse, but much more difficult as a direct effect of microgravity on bone cells.

Apart from the obvious disuse effects of microgravity, it may be worthwhile to consider the effect of spaceflight on fluid distribution in the human body. Under microgravity, important fluid redistribution occurs along the human body axis, because interstitial tissue fluid shifts from the legs and pelvis to the chest and head (114, 115). This redistribution seems to be due to a unique shift of tissue pressures, volumes, and lymphatic throughput values during spaceflight (116). There are also data suggesting that in humans, bone mineral loss during spaceflight is unevenly distributed along the body axis, being most pronounced in the legs and pelvis. In the head even a small but significant increase in bone mineral density has been observed (117, 118). Thus, changes in bone mineral density seem to correlate with changes in interstitial fluid pressure during spaceflight, reduced fluid pressure correlating with bone loss and enhanced fluid pressure with bone gain. In the model that we have just discussed (Fig. 3), reduced interstitial fluid pressure in the canaliculi would reduce canalicular fluid shear stress as a result of strain, whereas increased fluid pressure would increase canalicular fluid shear stress. This is compatible with bone loss in the former situation and bone gain in the latter, thereby providing indirect evidence for a relationship between fluid pressure and bone balance. Currently no direct studies linking these two issues are available but it may be worthwhile to further analyze their possible relationship. Manned spaceflight could then be a unique condition to test the canalicular fluid flow hypothesis as discussed in this review. **FJ**

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