J Bone Miner Metab (1999) 17:61–65

Journal of
Bone and
Mineral
Metabolism
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Establishment and characterization of an osteocyte-like cell line, MLO-Y4

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Abstract: Much attention has focused on osteoblast and osteoclast biology, but little research has been performed on the cell that composes 90% of all bone cells—the osteocyte. Osteocyte function has been difficult to study because these cells are embedded in mineralized tissue and are difficult to obtain in reasonable numbers and purity. Establishment of an osteocyte cell line makes it possible to study osteocyte function more readily and easily by the application of protein chemistry and molecular biology. We have established a cell line that appears to have the properties of primary osteocytes [1].

The cell line is designated MLO-Y4 (for murine longbone osteocyte) and was established from transgenic mice created using the osteocalcin promoter driving the large T antigen. This cell line, compared to osteoblasts, expresses long dendritic processes and produces small amounts of alkaline phosphatase and collagen type 1, large amounts of osteocalcin, and very large amounts of connexin 43, a gap junction protein, but similar amounts of osteopontin and CD44. Interestingly, this cell line does not express osteoblast-specific factor 2. This cell line also has functional gap junctions transmitting through the dendritic processes. On exposure to fluid flow, an increase in prostaglandin production occurs concomittant with a dramatic increase in functional gap junctions. Monoclonal antibodies have been generated using these cells, which appear to recognize osteocyte-specific antigens. This cell line and these antibodies should prove to be useful tools for examining osteocyte function.

Osteocytes

Osteocytes first attracted the attention of electron microscopists by their extensive networks within the mineralized bone matrix [2]. These networks connect the embedded osteocytes with the cells on the surface of bone. Osteocytes appear to be derived from the same precursors as osteoblasts and in fact appear to be terminally differentiated osteoblasts. During the process of osteocyte ontogeny, osteoblast precursors are recruited to sites of previous resorption; these cells then differentiate into matrix-producing cells that either become lining cells or pre-osteocytes embedded in the newly formed osteoid. A mature osteocyte is defined as a cell surrounded by mineralized bone.

The mature osteocyte is described as a stellate or star-shaped cell with a large number of slender cytoplasmic processes radiating in all directions but generally perpendicular to the bone surface. These cell processes pass through the bone in thin canals called canaliculi. Within each osteon, the osteocytes and bone lining cells appear to be coupled via gap junctions [3]. Osteocytes appear to be attached to the mineralized matrix via integrins such as beta 3 [4]. These properties support the hypothesis that osteocytes function as sensor cells in bone.

Mechanical strain

Clearly bone accommodates or responds to strain, as was first stated by Julius Wolff in 1892. To paraphrase Wolff's law, "the law of bone remodeling, alteration of internal and external architecture, occurs as a consequence of the stressing of bone." In general, athletes such as wrestlers and chronic exercisers such as tennis players have higher bone mineral density than matched, nonexercising controls. Astronauts subjected to long periods of space flight lose bone. The cells of bone suitable for sensing mechanical strain and translating these forces into biochemical signals include bone lining cells, osteoblasts, and osteocytes. It has been proposed that mechanical strain can be translated into electric

Received: Oct. 3, 1998 / Accepted: Oct. 15, 1998

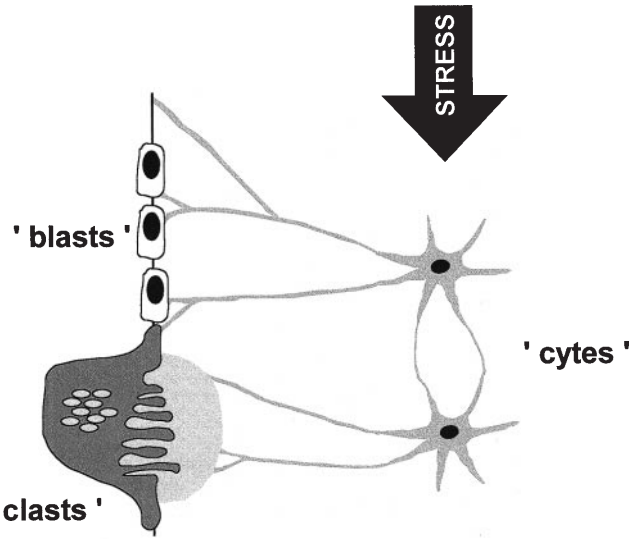

Fig. 1. The communication network between osteocytes and the cells responsible for bone formation, osteoblasts, and the cells responsible for bone resorption, osteoclasts. One popular hypothesis is that the osteocyte is the cell in the bone that is responsible for sensing the mechanical stress that is applied to bone. This mechanical strain on the osteocyte is then translated into signals to other bone cells. For example, a lack of stress would result in a signal to the osteoclast to resorb bone, as occurs in space flight and immobilization. An unknown magnitude of strain would be responsible for maintenance of bone, a higher magnitude for new bone formation as occurs in athletes, and an even higher magnitude for bone destruction as occurs in stress fractures

fields in these cells, which can induce an osteogenic stimulus [5].

Various models have been proposed to explain this responsiveness of bone to strain using animals and intact bone (for review, see [6]), but here the focus is cellular and biochemical. Experiments have been performed using intact bone subjected to strain and then examined for increased bone formation [7] or for biochemical markers [8]. Within a few minutes of loading, glucose 6-phosphate dehydrogenase, a marker of cell metabolism, is increased in osteocyes and lining cells. The increase in this marker appears to be mediated by prostaglandins as this effect is blocked by indomethacin [9]. By 2 h, c-*fos* mRNA is evident and by 4 h, transforming growth factor-β (TGF-β) and insulin-like growth factor-I (IGF-I) mRNA is increased in osteocytes [10].

It was also found that mechanical forces applied to bone cause fluid flow through the canaliculi surrounding the osteocyte, causing deformations in the extracellular matrix and the cell membrane [11,12]. Recently, it has been proposed that integrins are the "mechanosensors" or "mechanoreceptors" or "mechanotranducers" in bone cells [13]. Movement of integrins could be responsible for opening of calcium channels and changes in membrane potential resulting in intracellular signaling (Fig. 1).

Mechanical loading also appears to affect the number and distribution of apoptotic osteocytes in cortical bone [14]. Osteocytes appear to undergo apoptosis during both growth and remodeling. Nobel and coworkers found that loading was associated with a reduction in numbers of apoptotic osteocytes. Osteocytes are capable of living for decades. Humans in their seventies have been reported to contain live osteocytes in ossicles [15]. Therefore, mechanical loading may be important in maintaining osteocyte viability.

Gap junctions

Gap junctions are transmembrane channels that connect the cytoplasm of two adjacent cells. These channels permit molecules with a molecular weight less than 1 kDa such as small metabolites, ions, and intracellular signaling molecules (i.e., calcium, cAMP, inositol triphosphate) to pass through [16]. These channels have been demonstrated to be important in modulating cell and tissue function in many organs, i.e., heart, liver, peripheral nerve, ovary, ear, and eye lens [17–21]. Gap junction channels are formed by members of a family of sequentially and structurally related proteins known as connexins (Cx). So far, 13 connexins have been identified and cloned from various tissues and cells [22]. Phosphorylation and dephosphorylation are responsible for the change in connexin structure that is responsible for the opening and closing of the channels (for review, see [23]).

The morphological proof of the existence of gap junction structures has been obtained for osteoblasts and osteocytes [24]. Connexins are expressed in bone tissues and by established osteoblastic cell lines. Cx-43 is expressed in cultured osteoblasts from newborn rat calvaria [25], primary osteocytes in vivo [23], and in MC3T3-E1 cells [26]. Both Cx-43 and Cx-46 are expressed in the ROS 17/2.8 cell line [27]. Cx-45 is expressed in UMR-102 osteoblastic cells [28]. In addition, in our newly characterized osteocyte-like MLO-Y4 cells, Cx-43 is the major connexin identified [1]. Gap junction channels in osteoblasts are known to be regulated by several factors such as hormones [29] and mechanical loading [30]. Cyclic stretch has recently been shown to enhance the phosphorylation of Cx-43 and gap junctional communication in osteoblastic cells [30].

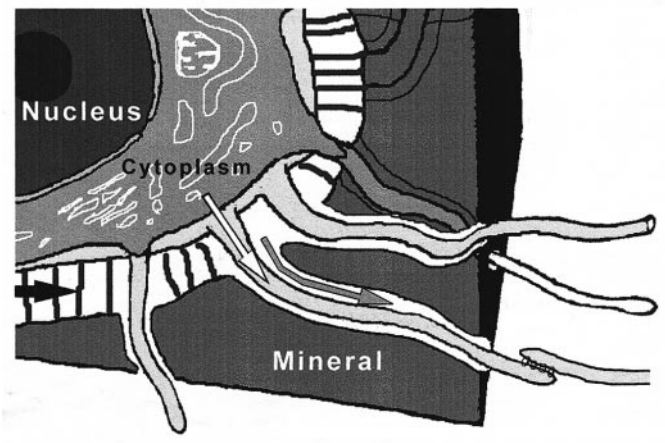

Fig. 2. The effects of fluid flow or strain on osteocyte intracellular communication (*white arrow*) or extracellular communication (*gray arrow*). The osteocyte is surrounded by a nonmineralized, proteoglycan, fluid-filled annulus. Structures can be observed connecting the osteocyte membrane with the mineralized bone tissue (*black arrow*). Canaliculi extend along the dendritic processes, which penetrate the mineralized bone matrix and physically connect with other osteocytes or cells along the bone surface. Strain applied to the mineralized component of bone is thought to perturb the fluid in the canaliculi and to perturb structures attaching the mineralized matrix to the osteocyte cell membrane, resulting in changes in the cell membrane. These perturbations of the cell membrane are thought to generate intracellular and extracellular signals. The intracellular signals are thought to move from cell to cell through channels called gap junctions, whereas the extracellular signals are thought to move through the fluid in the canaliculi

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The cell line is designated MLO-Y4 (for murine longbone osteocyte) and was established from transgenic mice created using the osteocalcin promoter driving the large T antigen. This cell line produces long dendritic processes, small amounts of alkaline phosphatase, large amounts of osteocalcin, and small amounts of collagen type 1 and expresses very large amounts of Cx-43 but amounts of osteopontin and CD44 similar to osteoblasts. Interestingly, this cell line does not express osteoblast-specific factor 2, a bone adhesion molecule described by Takeshita and coworkers [31]. OSF2 has been renamed periostin because of its location in the periosteum. These cells should prove to be useful tools for examining osteocyte function.

MLO-Y4 cells possess functional gap junctions

Dye-transfer coupling assays were performed, an approach commonly used to study cell couplings through gap junctions. MLO-Y4 cells on culture dishes were analyzed by the "scrape loading" method. In this method, cells were scratched with a fine needle in the presence of two types of fluorescent dyes: lucifer yellow (LY), which can penetrate through gap junction channels and rodamine (RD) (MW, 10 kDa), which is too big to pass through the channels, thus serving as a tracer for the original dye-receiving cells. Our experimental results show that MLO-Y4 cells allow LY to transfer to the nondisrupted neighboring cells. If the cells are plated at low density, the dye is transferred only through the long processes. Treatment by octanol, a gap junction channel blocker, inhibited the dye-transfer process and LY was not delivered into the neighboring cells. Together, these results support the existence of gap junction-mediated communication in the MLO-Y4 cells.

MLO-Y4 cells produce prostaglandins in response to fluid flow

Next, the effects of fluid flow on the production of arachidonic acid metabolites, the prostaglandins PGE_2 and PGF_1, were examined. MLO-Y4 cells respond to fluid flow with the release of PGE_2 and PGF_1. Both MLO-Y4 cells and MC3T3-E1 cells were subjected to fluid flow at 4.5 dynes/cm^2. Media were collected at the indicated times and PGE_2 and PGF_1 were quantitated. No significant effect was observed with the MC3T3 cells whereas the MLO-Y4 cells responded with an increase in PGE_2 and PGF_1 in a time-dependent manner under low fluid flow conditions.

Generation of an osteocyte-specific antibody

As the definition of an osteocyte is a bone cell surrounded by mineralized bone, it may be questioned whether the MLO-Y4 cell line represents the osteocyte phenotype. Therefore, we have generated a monoclonal antibody that appears to be specific for osteocytes. Rats were injected with MLO-Y4 cells; the spleens were removed and fused with mouse myeloma NSF-1 cells. The pooled fusion clones were screened using ELISAs of fixed MC3T3-E1 cells and fixed MLO-Y4 cells and by western blots of MLO-Y4 lysates. One clone, 9C11,

reacted with a 40-kDa band in MLO-Y4 cell lysates by western blotting. This clone was then tested against MC3T3-E1 and OCT-1 lysates and shown to be highly specific for MLO-Y4 cells.

The clone 9C11 was then tested against a series of cell and bone lysates from mouse long bone. Long bones were removed from 6-week-old mice, the marrow flushed, and the bone cut into pieces and then serially digested with collagenase. The residual bone pieces were boiled in SDS sample buffer for 5 min. No reactivity was observed with cell lysates from digestions 1 through 7, but the lysate of the remaining bone pieces revealed a strong band at 40 kDa. These observations have been repeated three times with similar results. No cross-reactivity exists with other tissues such as brain, liver, testis, lung, and heart. This further suggests that the antibody is osteocyte- and not osteoblast specific.

The 40-kDa band as detected by clone 9C11 is expressed in murine calvarial osteoblasts and OCT-1 cells. OCT-1 cells and murine calvarial osteoblasts differentiate in culture to form bonelike nodules that mineralize in the presence of ascorbic acid and beta-glycerolphosphate [32]. The antigen detected by 9C11 antibody is expressed by these cells only after 4 weeks of culture. These observations suggest that primary osteoblasts and the osteoblast cell line OCT-1 can differentiate in culture to become osteocytes after the period of nodule formation and during mineralization of the nodules.

In summary, we have shown that MLO-Y4 cells have the properties of osteocytes and should prove to be useful tools to examine osteocyte function. These cells have functional gap junctions and respond to fluid flow with an increase in gap junctions and production of prostaglandins. The availability of an experimentally highly manipulatable cell model system for osteocytes will advance investigation of osteocyte biology and function.

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