【国外研修報告】

The Role of Osteocytes in the Initiation of Targeted Remodeling

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ターゲティッドリモデリング開始における骨細胞の役割

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Abstract:Microdamage in bone contributes to fractures and acts as a stimulus for bone remodeling. Osteocytes are the most abundant cells in bone, and their death by microdamage has been suggested to be the major event leading in the initiation of osteoclastic bone resorption. Even though there is increasing evidence that osteocyte density, microcracks and targeted remodeling are related, there still exist several questions. For example, how osteoclasts are targeted to the specific site of microdamage for repair. It has been proposed that apoptotic osteocytes could secrete a specific signal to target osteoclasts. The other question is the nature of this signal. To elucidate the role of microdamage-induced osteocyte cell death in the initiation of targeted remodelling, this paper discusses the potential use of a newly developed *in vitro* model, in which osteocytes can be three-dimensionally cultured and locally damaged. Furthermore, the method enables one to study the osteocyte-derived soluble interactions with bone marrow cells. It was demonstrated that damaged osteocytes locally affect osteoclast precursors by secreting osteoclastogenic factors, and thus can have a role in the initiation of resorption in bone remodelling. This strongly supports the idea that damage to osteocyte cellular network has the potential to stimulate osteoclastic proliferation and therefore the activation of Basic Multicellular Units (BMUs).

Keywords: Osteocyte, Osteoclast, Microdamage, Targeted Remodeling, In Vitro Model

1. Introduction

Osteocytes are the most abundant cells in bone and there are approximately ten times as many osteocytes as osteoblasts in adult human bone [1]. Osteocytes represent the final differentiation phase of osteoblasts that become embedded within the mineralized bone matrix. For a long time, osteocytes were considered as the "quiescent" cells of bone, serving a mainly structural function. However, the abundance and special location of this cell type has puzzled scientists for a long time. The knowledge of the role of osteocytes in mechanosensing and in the consequent regulation of bone remodeling is constantly increasing [2-4]. It has also been reported that osteocyte death or apoptosis induced by fatigue microdamage coincide with osteoclastic bone resorption, meaning that the initiation of bone remodeling would be targeted to microdamage [5-6]. Furthermore, it has been suggested that the rupturing of osteocyte cell processes due to microcracks could actually contribute to the regulation of bone adaptation [7].

The mechanism of targeted remodeling could efficiently repair the damaged bone, which is essential for maintaining the functional capacity of load-bearing bone. Considering the cell-to-cell networks inside bone, osteocytes are the key instrument to detect local environment surrounding them, and to control the bone resorption for removing the damaged matrix. Even though osteocytes are located within the hard mineralized matrix, they remain in contact with each other and the cells on bone surface. Osteocytes can spread their processes to osteoblasts, and also to some extent directly to the cells on the vascular facing surface [8]. This suggests the possibility of communication without any interference of the osteoblasts or bone lining cells. Functional gap junctions have been described between osteocytes and osteoblasts [9]. Since gap junctions have, furthermore, been described in osteoclasts [10], it is possible that direct cell-to-cell interactions can occur between osteocytes and osteoclasts. Indeed, MLO-Y4 osteocyte-like cells have been shown to support osteoclast formation and activation from spleen and bone marrow cells *in vitro* via the RANKL expressed in their cellular processes [11].

Osteocytes can also control bone remodeling via secreted proteins. Since osteocytes share the same origin with osteoblasts, it is clear that they share, at least partially, the expression patterns of growth factors. Due

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to their location within the bone matrix, the expression and especially the secretion of specific factors by osteocytes is far more difficult to study. Nevertheless, the osteocytic expression of some growth factors, such as TGF-β [12] has been demonstrated *in situ*. It has been previously demonstrated that osteocytes can control osteoclast function by secreting TGF-β *in vitro* and that estrogen can modulate this effect [13]. Furthermore, osteocytic expression of the major regulators of osteoclastogenesis, OPG and RANKL, has recently been confirmed [14-15]. This suggests that osteocytes are able to regulate the osteoclastic bone resorption.

Despite of many indications that osteocytes have the capacity to regulate bone resorption, there are no previous studies showing that the change of osteocyte activity could locally influence on the differentiation of bone-resorbing cells. In our studies, we have been aiming at studying the soluble interactions between osteocytes and osteoclasts and have demonstrated that MLO-Y4 osteocyte-like cell line represents a good model to study the soluble interactions between osteocytes and osteoclasts. More recently, the aim has been to develop a relevant *in vitro* model to demonstrate the role of damaged osteocytes in the induction of osteoclastic cell differentiation. This paper discusses osteocyte cell death and survival, as well as microdamage and targeted remodelling, and the novel culture system, which can be used to study these events *in vitro*.

2. Osteocyte apoptosis

The life span of osteocytes is probably mostly determined by bone turnover, when osteoclasts resorb bone and "release" osteocytes. In addition to engulfment by osteoclasts, osteocytes may also die by apoptotic or necrotic processes, or just senescence. Osteocytes may live for decades and an average half-life of 25 years has been estimated [16]. The importance of apoptosis, or programmed cell death, in normal tissue development is increasingly recognized. It is a form of individual cell suicide originally defined by morphological changes in nuclear chromatin and cytoplasm [17]. When apoptosis is induced, cells undergo contraction, lose attachment to their neighbors, and break up into fragments, apoptotic bodies, which get phagocytosed quickly by neighboring cells. Osteocyte apoptosis is getting more attention, since it is supposed to be related to decreased mechanotransduction, which possibly leads to development of osteoporosis. Apoptotic osteocytes with condensed chromatin and degraded DNA have been identified in tissue sections [5] and the osteocytic expression of death receptor Fas/CD95 has been demonstrated both *in vivo* [18] and *in vitro* [19].

2.2. Osteocyte viability and mechanical loading

It was demonstrated in the mid-1990s that mechanical loading increases osteocyte viability *in vitro* [20]. Pulsating fluid shear stress of physiological magnitude reduces osteocyte apoptosis, and static control conditions induces higher levels of apoptosis in osteocytes than in osteoblasts and periosteal fibroblasts [21], indicating that mechanical loading leading to fluid flow is important in osteocyte survival. Physiologically, mechanical loading contributes to solute transport through the lacuna-canalicular system in bone, and enhances oxygen and nutrient exchange and diffusion to osteocytes. Because most osteocytes reside remote from blood supply, their metabolic needs are fulfilled by both passive diffusion and active transport induced by loading. Indeed, skeletal unloading has been reported to induce osteocyte hypoxia *in vivo* [22]. Although many cell types survive hypoxia, prolonged hypoxia and consequent reoxygenation-induced injury may induce cell death. It has been suggested that during hypoxia, osteocytes undergo apoptosis and recruit osteoclasts to resorb bone. It was recently demonstrated that disuse-induced hypoxia upregulated the expression of both hypoxia-dependent transcription factor HIF-1α and osteopontin in osteocytes [23-24]. Since osteopontin induces osteoclast chemotaxis and attachment to bone, it has been speculated that osteopontin expression in hypoxia-induced osteocyte would mediate the disuse-induced osteoclastic bone resorption.

2.3 The ageing osteocyte and accumulation of microdamage

Osteocyte death has long been associated with ageing [25]. Even though the general view is that osteocytes can live for decades, it has been demonstrated that already 40% of osteocytes in ear ossicles are dead within the second year of life [26], emphasizing the importance of mechanical loading for osteocyte survival. Later it was confirmed that osteocyte density indeed declines with age, and that the age of bone, rather than the age of an individual, would be more important in determining the fate of osteocytes [27]. It has been proposed that the age-related loss of osteocytes is associated with impaired bone remodeling e.g. during the removal of microdamage. Microdamage occurs in bone as a result of repetitive events of cyclic loading. Small cracks accumulate in the mineralized matrix leading to reduced strength of bones but unlike most materials, bone is capable of removing the damaged area by "targeted remodeling". It has been postulated that only 10-20% of remodeling in adult human bone would be targeted, and the rest would be non-targeted, background remodeling [28]. A significant increase in new remodeling has been reported after the generation of microdamage [29], suggesting that targeted remodeling involves the active removal of damaged matrix by

osteoclasts. Recent studies have indeed shown that osteoclasts time-dependently migrate into the site of microdamage [6, 30].

Interestingly, decline in osteocyte lacunar density is associated with the accumulation of microcracks with age [31] and osteocyte lacunae have been considered as preferential sites for microdamage initiation [32]. Indeed, it was recently demonstrated that the interstitial bone, in which osteocyte number is low, would be the preferential site for microdamage accumulation [33]. It appears that microdamage and osteocyte deficiency occur at the same anatomical sites but it is difficult to say which comes first.

Even though there is increasing evidence that osteocyte density, microcracks and targeted remodeling are related, there still exist several questions. For example, how osteoclasts are targeted to the specific site of microdamage for repair. It has been implied that osteocytes and their apoptosis are related to this function. Microdamage in bone appears be associated with osteocyte death by apoptosis, at least in the rat ulnar model of fatigue fracture [6, 30]. Osteocyte apoptosis was observed to precede osteoclast invasion in the damaged regions [30] and increased osteocyte apoptosis was associated with the upregulation of the proapoptotic Bax protein [34]. There exists the possibility that apoptotic osteocytes secrete a signal to target osteoclasts to perform remodeling at a damaged site. Thus, the other question that remains to be answered is the nature of this signal. It is possible that during and after microfracture, apoptotic osteocytes upregulate a stimulatory signal or repress an inhibitory signal for resorption. Both possibilities exist, since there are reports concerning osteocyte-derived stimulators [11] as well as inhibitors of resorption [35-36]. It is clear that the possible molecular links between damage-induced apoptosis and targeted bone remodeling need to be studied further, since so far none have been confirmed *in vivo*.

3. *In vitro* model to study osteocytes as the regulators of bone resorption

To address some of the questions discussed above, we have set up an *in vitro* model to study the role of osteocytes and microdamage in the regulation of osteoclastic activity, and to identify possible paracrine factors involved. Our hypothesis was that osteocytes would regulate of osteoclast function, and that this regulation could be differential depending on the survival status of osteocytes.

3.1 Mechanical damage of gel-embedded osteocytes

Osteocyte-like MLO-Y4 cells were three-dimensionally (3D) cultured within the collagen matrix [37]. After 5 days of culture, it was observed that MLO-Y4 cells were 3D distributed in the gel, had elongated cell processes and formed cell-to-cell contacts to each other (Fig.1). A scratching device was developed to induce local mechanical damage to the cells and their processes. Scratching pick indeed was settled inside collagen gel so that only the very local osteocytes and their processes are suffered from the mechanical damage. This was demonstrated by cell viability assays, in which dead cells and destruction of the cell-to-cell networks were observed along the way that the embedded pick passed through. In contrast, the number of dead cells at the peripheral area was as small as that in the non-damaged control culture, in which only a small number of dead cells were observed.

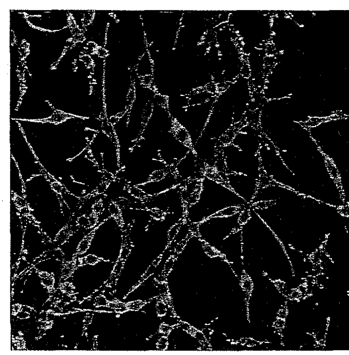

Fig.1 MLO-Y4 cells cultured in collagen matrix. Cells were stained with phalloidin-TRITC and then observed by a laser confocal microscopy. Bar: 50μm.

3.2 Damaged osteocytes induce bone marrow cell differentiation by secreting RANKL and M-CSF

The scratching device was used to clarify how the mechanically damaged osteocytes are involved in local bone marrow cell differentiation. Mouse bone marrow cells were co-cultured on the damaged MLO-Y4 cells for a week. The mechanical scratching of osteocytes induced the formation of tartrate resistant acid phosphatase (TRACP)-positive osteoclast precursors on top of the gel along the damaged region. No TRACP-positive cells were formed at the peripheral regions, which suggested that mechanically damaged osteocytes could locally stimulate osteoclast precursor cell differentiation.

Furthermore, osteocytes were again cultured inside collagen gel and after 3 days, the whole gel was cut into small pieces to gain a higher ratio of damaged cells. After 24-hour recovery, conditioned medium was collected and added to bone marrow cell cultures. A significant increase in the TRACP activity of mouse bone marrow cell lysates was observed after 7 days of culture. The conditioned medium collected from gel-embedded fibroblast culture with and without mechanical scratching had no effects on TRACP-positive cell differentiation or TRACP activity, suggesting an

osteocyte-specific effect.

TRACP antibody and Hoechst staining was performed to confirm the phenotype of the differentiated bone marrow cells. The presence of conditioned medium from the damaged MLO-Y4 cells significantly increased the formation of TRACP-positive cells, most of which were mononuclear. In addition, some multinucleated cells could be observed in cultures with the damaged and intact MLO-Y4 conditioned medium.

To identify the soluble factors secreted from damaged osteocytes, ELISA assays were performed. The enhanced secretion of osteoclastogenic factors, RANKL and M-CSF was observed when mechanical scratching was applied on MLO-Y4 cells. On the other hand, there was no substantial M-CSF and RANKL secretion from the gel-embedded fibroblast culture. These findings indicated that soluble factors secreted from damaged osteocytes could locally induce and activate the initial phase of osteoclastic cell formation.

3.3 RANKL and OPG secretion by cyclically stretched osteocytes

RANKL is one of the major regulators of osteoclastogenesis and might be a factor for announcing where a microcrack is. As shown in the previous section, RANKL secreted from microdamaged osteocytes would be a promising signalling pathway that might activate osteoclastic activity and consequently the formation of BMUs. In order to confirm this, MLO-Y4 cells were seeded in a 3D collagen gel inside silicone wells (Fig.2) and cultured for two days to form a cellular network. Small incisions of 3, 10 and 30 mm were made in the collagen gel containing the cells to disrupt the cellular network. The silicone wells were consequently cyclically stretched at 5000με using an original linear loading device (Fig.3). Conditioned medium was collected and analysed using an OPG and RANKL ELISA kit. Figure 4 shows that RANKL concentrations in the conditioned medium continued to increase over a twelve hour period. The results indicate that the amount of RANKL is not related to the length of the induced defect (3, 10 or 30mm). When the cell cultures were simultaneously dynamically loaded for 24 hours using 5000με at 1Hz, levels of RANKL were approximately 1.5 higher than those at static conditions (Fig. 5). However, no OPG could be detected. This might imply that OPG, the decoy receptor for RANKL, has bound together and therefore blocking it for detection or is hardly expressed by osteocytes. Although more work is required to understand the biochemical signalling within bone, it gives insight in how a possible mechanism for crack detection might work.

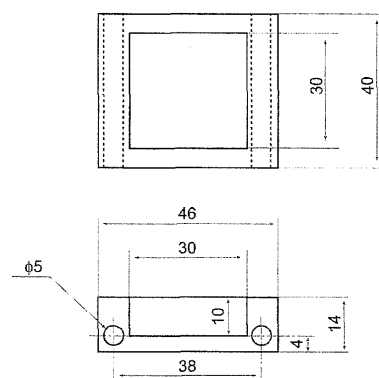

Fig.2 Silicone well designed for applying mechanical stretching to the gel-embedded MLO-Y4 culture.

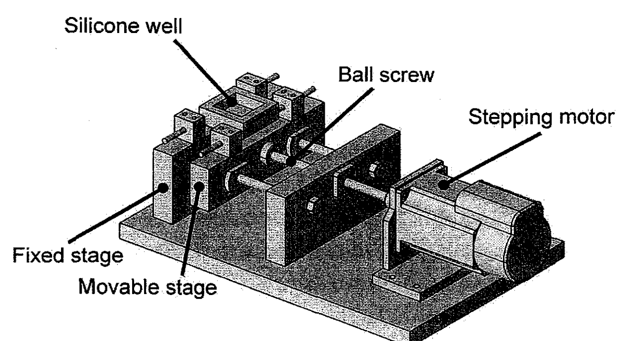

Fig.3 Linear loading device. The gel-embedded culture could be subjected to quantitative loading regime controlled by a PIC and stepping motor.

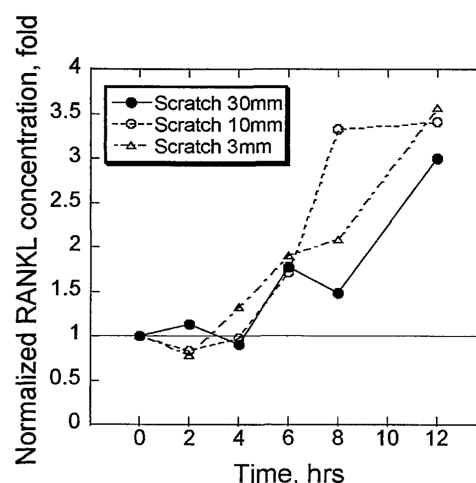

Fig.4 Periodic RANKL secretion from MLO-Y4 gel culture with small incisions of 3, 10, 30 mm.

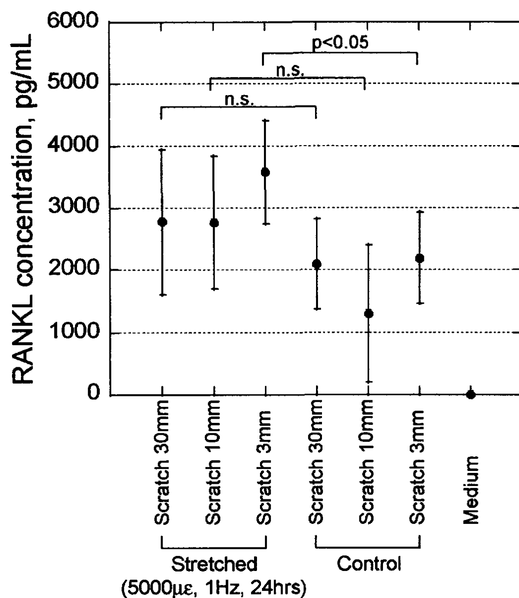

Fig.5 Comparison of RANKL concentration after 24-hour culture of MLO-Y4 with and without mechanical stretching.

4. Discussion

Our results demonstrate that the MLO-Y4 osteocyte-like cell line represents a good model to study the interactions between osteocytes and osteoclasts. It has been earlier reported that MLO-Y4 cells can inhibit the bone resorption of mature osteoclasts via soluble factors, and that this effect was mediated by TGF-β and estrogen [13]. TGF-β is a controversial growth factor but high concentrations of TGF-β have been reported to inhibit the function of isolated osteoclasts, suggesting that viable osteocytes indeed can secrete large amounts of this cytokine.

It has been suggested that apoptotic osteocytes could act as stimulators of bone resorption, e.g. in the initiation phase of targeted remodeling after microdamage. Repetitive, cyclic loading events in bone lead to microdamage, which needs to be repaired by removal of damaged matrix and rebuilding new material. The existence of targeted remodeling has been argued for some time but there now exists evidence to support this hypothesis [6, 29, 30]. It has been suggested that since both osteocyte apoptosis and microdamage associate with disuse [21], ageing [27] and estrogen withdrawal [38], conditions all of which lead to enhanced bone resorption, osteocytes would be major candidate cells to send a "targeting" signal to osteoclasts. However, so far there have been no *in vitro* models to study the accumulation of microcracks with respect to the viability of osteocytes.

To approach this question, we have reported the development of a novel *in vitro* model, in which osteocytes can be mechanically and very locally damaged [37]. Osteocytes were 3D cultured inside the collagen gel, which mimics the *in vivo* environment, even though it lacks the mineralized matrix. Osteocytes were able to form cell-to-cell contacts inside the gel and they remained viable, indicating that the matrix allowed sufficient transport of nutrients and oxygen from the culture medium. When osteocytes were mechanically damaged and bone marrow cells were cultured on top of the gel, there was a local induction of TRACP-positive cell formation along the scratching path, suggesting the presence of an osteocyte-derived "targeting" signal. The soluble nature of the phenomenon was confirmed by adding conditioned medium from mechanically damaged MLO-Y4 cells to bone marrow cells, which promoted their TRACP activity. Furthermore, this effect was demonstrated to be due to soluble M-CSF and RANKL in the MLO-Y4 conditioned medium. It was reported earlier that MLO-Y4 cells express RANKL on their surface and also secrete large amounts of M-CSF, and thus stimulate osteoclast formation and function [11]. Interestingly, it has been earlier suggested that the membrane-bound form of M-CSF could be more efficient than the soluble form in promoting osteoclast formation *in vitro* [39]. Our data suggested that when MLO-Y4 cells are mechanically damaged, the membrane-bound forms of M-CSF and RANKL are released, and could thus act as a "targeting" signal to bone marrow cells to differentiate toward the osteoclast phenotype.

5. Conclusions

We hypothesize that osteocytes express high amounts of TGF-β in normal condition and thus repress bone resorption. When bone grows old, levels of estrogen and mechanical loading decrease and microcracks accumulate, and part of the osteocytes die by necrosis or apoptosis. The expression levels of TGF-β decrease and the expression of osteoclast-stimulatory factors, such as RANKL and M-CSF increases, and bone resorption is enhanced, leading to net bone loss. In order to gain a better understanding of the processes leading to osteoporosis, the basic cellular mechanisms need to be understood. There is growing evidence that osteocytes have an important role in the regulation of bone resorption and bone formation. Our recent results strongly suggest that soluble factors secreted from damaged osteocytes can locally induce and activate the initial phase of osteoclastic cell formation. There appears to be a relationship between the damaged osteocytes and the local initiation of resorptive stage in bone remodeling, which strongly supports the existence of the targeted remodeling.

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