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Chapter 1.

General introduction

Bone cells and remodeling

Bone is important to give the body support, as an attachment site for muscles, and to maintain mineral homeostasis. Bone also surrounds the bone marrow that contains hematopoietic progenitors and mesenchymal stem cells. To keep its strength, bone is continuously renewed; a process called bone remodeling. Bone remodeling starts with bone resorption, i.e. degradation of bone, which is performed by osteoclasts [1]. Remaining demineralized bone fragments in the resorption pit are then ingested by bone-lining cells [2], in what is also referred to as the reversal phase, which couples bone resorption and bone formation [3]. Finally, osteoblasts deposit new matrix proteins and mineralize the newly formed osteoid [4]. In response to mechanical loading, bone embedded osteocytes regulate bone remodeling by influencing osteoclast and osteoblast activities [5,6]. In this chapter, the role of bone cells in maintaining bone homeostasis is explained. The main focus will be on osteoclasts and bisphosphonates; drugs that can be used to treat diseases that are characterized by excessive bone resorption, such as osteoporosis and bone cancer.

Osteoclasts

Osteoclasts are multinucleated cells formed by fusion of mononuclear precursors from the monocyte/macrophage lineage. These precursors are present in bone marrow and in blood, and migrate towards bone when resorption is needed. Upon contact with osteoblasts, osteoclast precursors are activated to fuse and to become osteoclasts [7]. Osteoblasts express two essential cytokines that stimulate osteoclast formation: macrophage-colony stimulating factor (M-CSF) [8] and receptor activator of nuclear factor κ B ligand (RANKL) [9,10]. M-CSF binds to its receptor c-Fms, which is present on the osteoclast precursors, thereby inducing RANK expression and priming the precursors to differentiate into an osteoclast [11]. RANK activation by RANKL is considered to be the main stimulus for fusion of osteoclast precursors.

Osteoclast heterogeneity

Osteoclasts from different bone sites have different characteristics, a phenomenon called osteoclast heterogeneity [12,13]. Osteoclast formation from different precursors was shown to occur at a different velocity [14,15]. *In vitro*, osteoclasts from long bone precursors are formed relatively early in time, whereas jaw osteoclasts are formed later. This can be explained by the finding that long-bone marrow contains more rapidly-differentiating myeloid blasts, whereas jaw marrow contains a relatively high number of monocytes that need more time to differentiate into osteoclasts [14,15]. Therefore, the study by de Souza Faloni et al. also shows that not only osteoclasts, but also the composition of the bone marrows are bone-site specific. Furthermore, the osteoclasts that were formed from these

bone marrows were shown to have a different morphology [16]. Moreover, the resorption machineries were shown to be bone-site specific. Osteoclasts derived from long bones, for instance, use primarily cathepsin K for the digestion of bone matrix, whereas calvarial osteoclasts use next to cathepsins also matrix metalloproteinases (MMPs) [17-19].

Bone resorption

During bone resorption (reviewed in [20]), the osteoclast first creates a sealing zone. As this zone contains mainly F-actin, it is also called the 'actin ring'. This ring also contains $\alpha_v\beta_3$ integrin, which tightly attaches to the bone. The area encircled by the actin ring is called the resorption lacuna, where a ruffled border is created, where chloride is secreted by the chloride channel ClC-7, and where protons are secreted by the proton pump vacuolar-type H⁺-ATPase (V-ATPase). Acidification leads to demineralization of the bone. Subsequently, matrix proteins are degraded by proteases such as cathepsin K. Degraded proteins are partly ingested by osteoclasts, which secrete them at the functional secretory domain at the basolateral membrane. However, some proteins remnants are left behind in the resorption pit and cleared by bone-lining cells [2].

Bone-lining cells and the reversal phase

As their name suggests, bone-lining cells are the cells lining the bone. They are osteoblast/fibroblast-like cells, and after attraction of osteoclast precursors, the bone-lining cells migrate away from each other to make space for the osteoclast precursors [21]. This phenomenon was also seen when periodontal ligament fibroblasts were cultured together with osteoclast precursors [22,23]. Osteoclasts can also transmigrate through a layer of osteoblasts, implying that osteoclasts can migrate underneath the bone-lining cells to the site of bone resorption [24]. After bone resorption, bone-lining cells clear the collagen fragments that have been left behind in the resorption pit [2]. Without this clearance of the resorption pit, new bone formation does not occur. Because this is called the reversal phase of bone remodeling, bone-lining cells are also called reversal cells.

Osteoblasts and bone formation

Osteoblasts are bone-forming cells derived from mesenchymal stem cells in the bone marrow. Runt-related transcription factor 2 (Runx2) was shown to be an essential transcription factor during osteoblast differentiation [25]. Mature osteoblasts synthesize new bone called osteoid by excreting proteins such as collagen type I, osteonectin, bone sialoprotein, and osteopontin. These and other matrix proteins might play a role in bone mineralization, which is also carried out by osteoblasts. Alkaline phosphatases play a role in this process by increasing the local phosphate concentration [26].

By creating a feedback loop towards osteoclasts through M-CSF and RANKL expression, osteoblasts are also regulating bone homeostasis. Interestingly, they also express osteoprotegerin (OPG), which inhibits RANK-mediated stimulation of osteoclastogenesis by competitively binding to its ligand, RANKL [27]. Other cell types such as periodontal ligament fibroblasts and osteocytes can also stimulate osteoclastogenesis [28-30].

Osteocytes

Osteocytes belong to the osteoblast lineage and have become deeply embedded in lacunae in the bone matrix. Thin cytoplasmic extensions protrude from the cell in canaliculi. This system forms a network which is surrounded by canalicular fluid. Through the canalicular network, osteocytes are connected to each other by gap junctions [31]. In response to mechanical loading, they regulate bone homeostasis [6,32]. As a result of loading, they stimulate osteoblasts to form new bone. On the other hand, when bone is unloaded, the osteocytes die and stimulate osteoclastogenesis, thereby inducing bone degradation [30]. Recently, it has become clear that osteocytes can, next to osteoblasts, also express RANKL and stimulate osteoclastogenesis during bone remodeling *in vivo* [33,34].

Bisphosphonates

In healthy bones, bone resorption and bone formation are in balance. When this balance is disturbed and directed towards bone resorption, too much bone is degraded, making it vulnerable to fracture. Such an imbalance occurs for instance in osteoporosis and cancers that have metastasized to the bone. Those diseases are commonly treated with bisphosphonates (BPs), which induce apoptosis and inhibit osteoclast activity. Due to their high affinity for calcium, BPs rapidly bind to bone after administration [35]. During bone resorption they are released into the resorption lacuna, where they become available for uptake by the osteoclast. BPs therefore mainly act on osteoclasts *in vivo*; *in vitro*, they can also be toxic to other cells such as osteoblasts [36], and reduce viability of macrophages [37,38] and periodontal ligament fibroblasts [39,40]. Yet, *in vivo* BPs were also shown to be taken up by monocytes [41], and they reduced the number of osteoclast precursors in human peripheral blood [42,43]. Interestingly, anti-apoptotic effects on osteocytes and osteoblasts with low concentrations of BPs were also reported [44], and were shown to be mediated by connexin 43 [45].

Two groups of BPs are known and characterized by the presence or absence of nitrogen (N), each using its own mechanism to inhibit osteoclast activity. BPs such as clodronate, which do not contain nitrogen (non-N-BPs), are converted into a non-

hydrolyzable form of ATP, leading to apoptosis [46,47]. Nitrogen-containing BPs act through a more complicated mechanism that is described below.

Nitrogen-containing bisphosphonates

Nitrogen-containing BPs (N-BPs) such as pamidronate, risedronate, and zoledronic acid, inhibit the enzyme farnesyl pyrophosphate synthase (FPPS), thereby preventing protein prenylation [48,49]. Prenylation is a post-translational protein modification, during which a lipid group is added to the protein. Prenylation is important for the homing of small GTPases to the cell membrane, where they play a role in cytoskeletal organization, migration, cell adhesion and cell survival [50]. In osteoclasts the formation of the actin ring is disturbed by N-BPs, leading to diminished osteoclast function.

As well as diminishing protein prenylation, inhibition of FPPS by N-BPs leads to accumulation of its substrate, isopentenyl diphosphate (IPP). This is converted into triphosphoric acid 1-adenosin-5-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) leading to apoptosis [51]. Thus, as well as diminishing osteoclast survival and activity by inhibiting small GTPase function, N-BPs can also induce apoptosis directly.

Osteonecrosis of the jaw

A rare, but serious side effect in patients receiving a high-dose BP is osteonecrosis of the jaw (ONJ). This means that necrotic bone has been exposed due to soft tissue damage, not being the result of radiotherapy (i.e. osteoradionecrosis) [52]. ONJ is most common after treatment with the N-BPs zoledronate and pamidronate, and can occur both in the mandible and in the maxilla [53]. Prevalence is highly dependent on the dose and is estimated between 0.1 and 10% for people that receive high doses for cancer treatment [53,54]. People that are treated for osteoporosis receive a relatively low dose and the incidence of ONJ is lower. Invasive dental treatment, e.g. a tooth extraction, seems to increase the chance to develop ONJ [55].

Several hypotheses have been proposed to explain the etiology of BP-related ONJ (reviewed in [54,56]). Infection, oversuppression of bone turnover, and toxicity of BPs to cells other than osteoclasts are the most commonly described. How these pathways may interact in the onset of ONJ is depicted in Figure 1, however, its exact mechanism is currently unknown. To mimic BP-induced ONJ, animal models were developed in which it was shown that BP administration resulted in necrotic jaw bone, however, either immunosuppressive agents were used in conjunction [57,58] or periodontitis was induced [59,60]. Thus, BP treatment alone was not enough to induce ONJ in these animal models

and its pathogenesis remains unclear. Also, it is not known why especially jaw bones are affected.

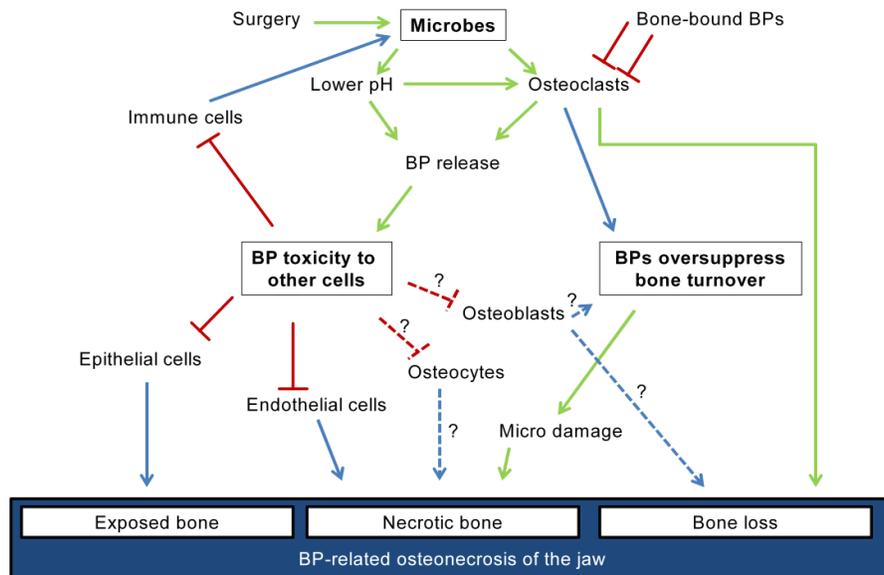


Figure 1. Current hypotheses for BP-related ONJ (squares). ONJ (blue square) consists of 3 events shown in the squares within the blue box. Green arrows represent positive effects, red blunted arrows show inhibitions. The blue arrows point out a result of the inhibitions indicated in red. The dashed arrows with question marks are speculative, since positive effects of BPs on osteoblasts and osteocytes have also been shown. It is not clear how the inhibition of BPs on osteoclasts on one hand, and a stimulation of osteoclasts by an infection on the other, can contribute to ONJ.

Hypothesis and thesis outline

We hypothesized that ONJ, the bone-site specific, negative effect of BPs, is related to the phenomenon of osteoclast heterogeneity. Therefore, we investigated whether BPs can have a different effect on long bone and jaw osteoclasts and their precursors.

In **chapter 2**, we investigated the internalization of BPs by long bone and jaw osteoclast precursors and the effect of BPs on long bone and jaw osteoclastogenesis and apoptosis. These *in vitro* studies with mouse cells were followed up by an *in vivo* study that is described in **chapter 3**. We subjected female C57BL/6J mice to weekly injections of zoledronic acid and investigated its effect on osteoclasts and bone formation markers in the jaw and long bones. This study revealed that BPs were able to induce osteoclast formation at the molar root. To investigate whether this stimulation may be induced by periodontal ligament fibroblasts that were treated with BPs we used human cells (**chapter 4**). In order to study the effect of BPs on osteoclast formation, time-lapse microscopy was shown to be a useful tool to study the fusion of long bone osteoclast precursors and multinucleated osteoclasts (**chapter 5**). Subsequently, we extended this in **chapter 6**, where we studied multiple steps of osteoclastogenesis, i.e. proliferation, migration, and fusion, from long bone and jaw bone marrow cells. We also analyzed the expression of genes involved in those processes and investigated the effect of BPs on migration. Finally, we discuss our major findings in **chapter 7** and propose a new hypothesis on how the effect of BPs on different osteoclasts may explain the pathogenesis of ONJ.

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Chapter 1

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