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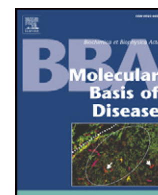

Review

Osteoclast heterogeneity: Lessons from osteopetrosis and inflammatory conditions

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ABSTRACT

The multinucleated osteoclast has a unique function: degradation of mineralized tissues. It is generally taken that all osteoclasts are alike, independent of the skeletal site where they exert their activity. Recent data, however, question this view as they show that osteoclasts at different bony sites appear to differ, for example in the machinery responsible for resorption. Support for the notion that there may be heterogeneity in osteoclasts is obtained from studies in which osteoclast activity is inhibited and from observations in osteopetrosis and inflammatory bone conditions. In this review we discuss the available evidence and propose the existence of bone-site-specific osteoclast heterogeneity.

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1. General introduction

Osteoclasts are defined as cells that can break down bone, the word being derived from the Greek *oston* bone and *klastos*, broken. In older literature a range of additional terms has been used to name cells that break down other types of mineralized matrices, for example: “chondroclasts” for cells that break down mineralized cartilage and “dentinoclasts” for cells that break down mineralized dentine. More recently, osteoclast has become the common term to denote any cell that has the capacity to break down mineralized matrices, as it is clear that in the growth plate the same cell can frequently be found to resorb both bone and cartilage simultaneously (Fig. 1) and cells taken from bone can easily resorb dentine as many laboratory studies indicate (Fig. 2). While undoubtedly osteoclasts can attack a wider range of matrices than their name suggests, recent studies have raised some new questions about the possible existence of different types of osteoclasts at different bone sites. In this review we discuss the available evidence, most of which comes from the study of osteopetrosis and from inflammatory bone conditions. It remains unclear whether heterogeneity in this cell type is the result of differential regulation in different bone sites. The implications of the possible presence of site-specific osteoclasts may be significant, especially when considering novel treatment options to inhibit resorption.

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2. Osteopetrosis and tooth eruption

As much evidence pointing to the possible existence of different subsets of osteoclasts comes from observations in osteopetrosis and especially from differences in osteoclast activities between the axial skeleton and the head region, as evidenced by (lack of) tooth eruption, we will first discuss osteopetrosis and pay some attention to the events that lead to tooth eruption.

2.1. Osteopetrosis

Osteopetrosis is defined as a disease characterized by a general increase in bone mass caused by inactivity, or absence of osteoclasts. While in osteopetrosis the bone mass is high, the bone itself is brittle and fractures are common. This differentiates the condition from other high bone mass disorders caused primarily by overactivity of osteoblasts, such as for example sclerosteosis or Van Buchem's disease [1] where bones are very strong.

Osteopetrosis is genetically heterogeneous with autosomal dominant and recessive forms. There is a wide spectrum of severity in the human condition with the most severe forms caused by autosomal recessive inheritance. Much has been learned in the past 25 years from the study of spontaneous mutations in animals that lead to osteopetrosis and more recently the genes causing such phenotypes have been identified and in most instances confirmed as causes of human osteopetrosis. In addition targeted gene deletion, or gene

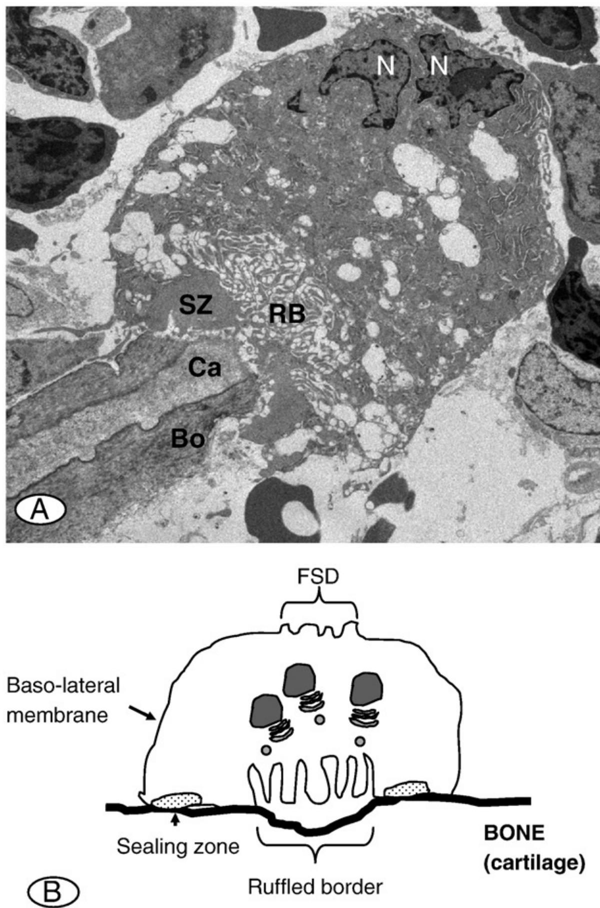

Fig. 1. (A) Electron micrograph of an osteoclast resorbing both calcified cartilage (Ca) and bone (Bo). Note the extensive ruffled border (RB), the site where the actual resorption occurs, and the sealing zone (SZ), the site where the osteoclast is attached to the bone/cartilage surface. N: nucleus. (B) Schematic drawing of an osteoclast in which the most important membrane domains are shown. The osteoclast is attached to the bone by the sealing zone (SZ), in the centre of this area resorption occurs underneath the ruffled border (RB), following resorption fragments are transported through the osteoclast to the functional secretory domain (FSD).

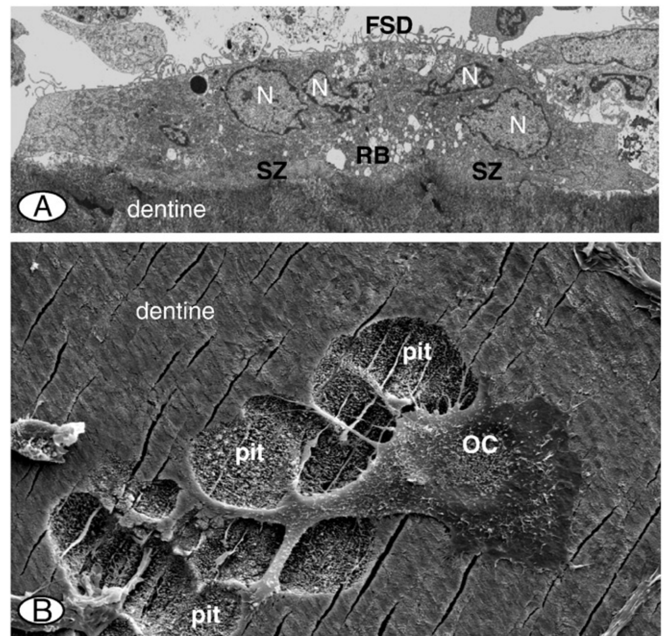

Fig. 2. (A) Electron micrograph (TEM) of an osteoclast seeded on a dentine slice. Resorption of the dentin occurs at the ruffled border (RB) and the cell is attached to the dentine by a sealing zone (SZ). Note the numerous small cellular extensions at the membrane opposite to the ruffled border, the functional secretory domain (FSD). N: nucleus. (B) Scanning electron micrograph of an osteoclast (OC) seeded on a slice of dentine. The osteoclast has resorbed part of the surface as shown by the presence of resorption lacunae, the pits.

overexpression in the mouse have, often quite unexpectedly, yielded additional osteopetrotic phenotypes and again pointed to some of the genes that are mutated in the human condition. Several reviews have recently summarized the remarkable progress made in this field and the reader is referred to those for detailed information [2,3]. Not only have these studies helped to understand osteopetrosis, they have also provided information about the essential genes necessary for osteoclast development and function. We now know that osteoclast development requires two growth factors, M-CSF, which stimulates the proliferation of osteoclast precursors, and RANKL, which promotes differentiation towards mature osteoclasts. Absence of either growth factor, or its receptors leads to severe osteopetrosis in rodents with lack of osteoclast formation [4–8]. Loss-of-function mutations in RANKL, or its receptor RANK on the osteoclast, have now also been identified in osteoclast-poor cases of human osteopetrosis [9,10]. More commonly however, osteopetrosis is caused by loss-of-function mutations in genes essential for osteoclast function. The genes identified so far in this subgroup are all involved in generation and transport of protons and the vesicles that contain them [11], to the ruffled border, the highly folded membrane domain facing the surface of the mineralized matrix the cell is resorbing (Fig. 3).

A common feature in these cases of osteopetrosis is that osteoclasts form, frequently in relatively high numbers (Fig. 4) and attach to the bone surface with a sealing zone, but that they do not form a ruffled border. The ruffled border is a unique membrane structure in osteoclasts and is the site where the cells lower the pH of the extracellular resorption compartment underneath by secreting protons. This activity results in the dissolution of the mineral (hydroxyapatite), thus exposing organic matrix components, such as type I collagen. A concomitant release of proteolytic enzymes, in particular the cysteine proteinase cathepsin K, results in breakdown of the organic matrix. The V-ATPase, the proton pump essential for the release of H^+ in the resorption area, is localized in the membrane of acidic vesicles in the osteoclasts and inserted into the ruffled border membrane when the osteoclast polarizes to start resorption. In the absence of functioning V-ATPase proton secretion and hence mineral dissolution are completely compromised [12]. To balance the charge of ions across the membrane, chloride is released concomitantly in the resorption lacuna through the use of a chloride channel, ClC-7 [13], which is also present in the membrane of acidic vesicles and inserted in the ruffled border membrane when resorption starts. Two recently identified proteins, Plekhm1 and Ostm1, appear to facilitate the transport of the acidic vesicles to the ruffled border, in case of Ostm1,

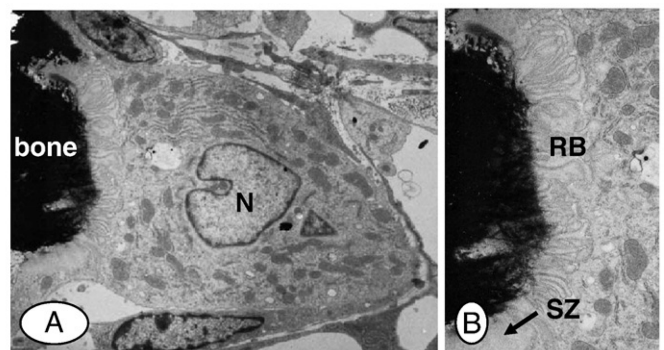

Fig. 3. (A) Electron micrograph of an osteoclast resorbing bone. The ruffled border (RB) is shown at a higher magnification in (B) SZ: sealing zone.

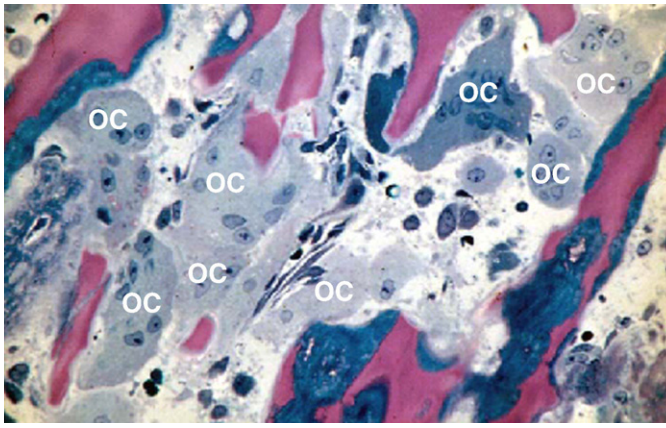

Fig. 4. Light micrograph of numerous osteoclasts (OC) present in the bone of an osteopetrosis patient. The osteoclasts are present but do not have the capacity to resorb the bone and/or calcified cartilage.

by acting as a subunit of the ClC-7 protein [14]. Their precise roles remain to be elucidated, but loss-of-function mutations in these proteins lead to osteopetrosis in rodents and in man [15,16]. The generation of H^+ in osteoclasts is dependent on the activity of the enzyme carbonic anhydrase II, which generates a proton and a bicarbonate ion from carbon dioxide and water. Mutations leading to reduced activity of this enzyme compromise osteoclast activity [17]. The bicarbonate is removed from the cytoplasm by exchange with chloride, facilitated by the anion exchanger AE2, which is active at the basolateral membrane of the osteoclast (Jansen et al., manuscript submitted).

In the case of malfunctioning of V-ATPase, ClC-7, CAII, Plekhm1, Ostm1, or AE2, the osteoclast is unable to form a ruffled border. This contrasts with the situation in which the proteolytic enzyme cathepsin K is inactive or not expressed. In such cases osteoclasts do form a – sometimes structurally abnormal – ruffled border and secrete acid, but they are unable to digest the organic bone matrix. A characteristic feature in bone biopsies of patients in which cathepsin K is not functioning normally, a disease called pycnodysostosis, is the huge amount of non-digested demineralized bone matrix adjacent to polarized resorbing osteoclasts [18,19]. In addition, the endosomal compartment of these cells contains high amounts of non-digested bone collagen fibrils; a phenomenon frequently seen in fibroblasts but only ever seen in osteoclasts from patients with pycnodysostosis [18].

2.2. Tooth eruption in osteopetrosis

Tooth eruption is critically dependent on osteoclast activity in the jaw to create a path for the developing tooth through the alveolar bone to the oral cavity. A characteristic feature of osteopetrosis is therefore the absence or severe delay in tooth eruption, something that, in rodent studies, is usually the first sign an animal is osteopetrotic (as reflected in strain names such as “toothless” or “incisor absent”). It is clear from histological and radiological studies that tooth germs are present in the jaw in all types of osteopetrosis and it has been shown that overcoming the defect causing osteoclast dysfunction may rescue tooth eruption (reviewed in [20]). However, as we will see further below, in some cases of osteopetrosis tooth eruption does not strictly correlate with osteoclast activity in other parts of the skeleton. A complication in the interpretation of some of these studies is the fact that only a small window of time exists for rescue of tooth eruption (see [20]).

The precise sequence of events leading to tooth eruption has been reviewed previously and many of the factors regulating bone remodeling are identified as important in this process [21]. The same factors are involved in the shedding of deciduous teeth and the replacement by permanent teeth, a process even more remarkable as it involves resorption of the root of the deciduous tooth by osteoclasts while also creating the eruption pathway for the underlying permanent replacement [22]. In human patients with osteopetrosis it is often during this second wave of tooth eruption when problems occur and supernumerary teeth may be seen, or alternatively lack of eruption of the permanent teeth.

Important in the context of this discussion is the fact that tooth eruption requires time-limited recruitment of osteoclast precursors, their local formation and subsequent activation. Not only diseases affecting osteoclast differentiation, or osteoclast function therefore can result in reduced or delayed tooth eruption, but also conditions in which osteoclast precursor recruitment is insufficient. This occurs in patients with cleidocranial dysplasia (CCD), who have a mutation in

Table 1

Mutation/gene defect	Disease	Skull/jaw bones	Long bones	
Sh3bp2$^{-/-}$	Cherubism	Affected (osteonecrosis)	Unaffected	[80]
Cathepsin K$^{-/-}$	Pycnody sostosis	Unaffected	Affected	[49,81]
Connexin 43$^{-/-}$		Affected	Unaffected	[82]
TRAF6$^{-/-}$		Unaffected	Affected	[83]
MMP-2$^{-/-}$		Affected	Unaffected	[84]
NFATc1$^{-/-}$		Unaffected	Affected	[85]
Ihh$^{-/-}$		Unaffected	Affected	[27,86]
HIFα$^{-/-}$		Unaffected	Affected	[29]
AE2$^{-/-}$		Unaffected	Affected	
Differences between osteoclasts				
TRACP expression		++++	++	[50,52]
MMPs used for resorption		++	−	[19,44,45]
CPs used for resorption		+	+++	Idem
Size of osteoclasts		++++	++	[87]
Experimental approach				
Sympathectomy		Affected	Unaffected	[36–38]
Pressure induced remodeling		Very sensitive	Less sensitive	[39]
Remodeling		Relatively fast	Relatively slow	[33]
PTH application		Unaffected	Affected	[35,88]
OPG overexpression		Unaffected osteoclastogenesis	Affected osteoclastogenesis	[26]
Bisphosphonate application	Paget's disease of bone	Limited effect on bone turnover markers	Normalization of bone turnover markers	[89]
PGE_2 application		Unaffected	Stimulated growth	[34]
Marrow cells		Fast proliferation	Slow proliferation	[90]
MMP-resistant bone collagen		Very thick	Unaffected	[44]

the gene for RUNX2, the critical osteoblast differentiation factor and often suffer from problems with tooth eruption and supernumerary teeth. While Runx2 has an important role in tooth formation, as it is expressed in odontoblasts and cementoblasts, the main reason for the problems in the CCD patients appears to be the reduced synthesis of RANKL and osteoclast precursor recruitment factors, such as M-CSF and MCP-1, by immature osteoblasts in the alveolar bone [23].

2.3. Bone-site-specific types of osteopetrosis

In addition to the systemic forms of osteopetrosis (i.e. where the osteoclast abnormalities are found throughout the body), a number of osteopetrotic phenotypes have been identified in transgenic or knockout mice in which osteopetrosis is bone-site specific (see Table 1). In these mouse models the sclerotic skeleton does not necessarily correlate with lack of tooth eruption, pointing to different osteoclast activities in the jaw, or even the whole head region, and the rest of the skeleton. For example, in RANKL$^{-/-}$ mice, that lack an essential osteoclast differentiation factor, a general osteopetrotic phenotype is seen. However, when Odgren et al. [24] performed rescue experiments with CD4-driven RANKL they noticed that the teeth did not erupt in the rescued mice. Their findings indicated continued osteoclastic underactivity in the jaw while at the same time osteopetrosis in the long bones was resolved, indicating normalization of osteoclast function. These findings suggested that osteoclasts at different bone sites respond differently to rescue by RANKL presented by CD4-positive immune cells. Similar findings have been reported for rescue of osteopetrosis in the op/op mouse using M-CSF. Interestingly, the op/op sclerotic phenotype resolves with age, but tooth eruption never spontaneously occurs. Systemic administration of M-CSF was able to rescue tooth eruption, but only if given before a critical time window [25]. While this indicates that timing of osteoclast recruitment is essential in the jaw, persistent lack of tooth eruption in the presence of normal osteoclastic bone remodeling elsewhere in the skeleton does suggest site-specific differences in the ability of a bone tissue to recruit and activate osteoclasts. Clearly, the jaw and in particular the dental follicle and roots of permanent teeth are a protected site where in general osteoclast activity is suppressed. The opposite situation to that seen in the op/op mouse or the RANKL$^{-/-}$ mouse, is found in mice over-expressing the RANK decoy molecule OPG. Here, tooth eruption occurs as normal indicating that the osteoclasts in the alveolar bone function normally. Osteoclasts in the periosteum of long bones are equally active, but those in trabecular bone were inactive [26].

The anion exchanger-2 (AE2) has several isoforms. Recently it was shown that mice deficient for three of its five isoforms have normal tooth eruption and normal resorption in skull bone, whereas long bones are severely osteopetrotic. In line with these observations, osteoclasts in the skull displayed a normal ruffled border, whereas this membrane structure was absent in long bone osteoclasts. Subsequently it was shown that the osteoclasts in the head region, but not those in the long bones, used a sodium transporter (SLC4a4) in addition to AE2 to transport chloride and/or bicarbonate across the membrane and could therefore compensate for the absence of AE2 (Jansen et al., FASEB J. in press). Together these observations clearly indicate that bone-site-specific differences in osteoclastic activity exist. In the following sections, we will discuss possible explanations for the presence of site-specific regulation of bone resorption and pay particular attention to the possibility that such differences may arise from differences in osteoclasts themselves.

3. Different bones, different cells and different responses?

How can bone-site-specific differences in osteoclastic activity be explained? Two possible explanations spring to mind. Firstly, it is possible that the composition of the various skeletal bones is different and therefore requires osteoclasts with different activity profiles. Alternatively, it is possible that osteoclasts at different skeletal sites are intrinsically different. In both scenarios the result is the presence of osteoclasts which are best equipped ("the fittest") for their role in their unique local microenvironment. To further discuss these two scenarios we first touch upon the two ways in which bone formation occurs during embryogenesis.

The bones of our skeleton are formed either by endochondral or by intramembranous ossification. Endochondral ossification, which is characterized by the initial formation of a cartilage model of the future bone, the bone *anlage*, which is gradually replaced by bone, is the process responsible for formation of the majority of the bones of the skeleton. Other bones (e.g. skull, jaws, and scapula) are formed by direct bone deposition in a condensed connective tissue, gradually replacing this connective tissue by bone. Long bones are considered to be formed by a combination of endochondral and intramembranous bone deposition. Part of the bone shaft (diaphysis) is formed by intramembranous ossification while he the rest is formed by endochondral ossification. Given the different ways of bone formation it may not be surprising that cells in different skeletal sites have different properties. We will first turn our attention to some of these as they relate to bone formation, i.e., osteoblasts and the matrix they produce, then to evidence for a different regulation of cells at different sites, before summarizing the evidence for intrinsic differences in osteoclasts at different sites.

3.1. Bone-site-specific differences in the formation of bone

Endochondral bone formation has been shown by Chung et al. [27] and Long et al. [28] to critically depend on Indian hedgehog (Ihh) expression and signaling. By contrast, lack of Ihh did not affect the presence or activity of osteoblasts at sites of intramembranous bone formation.

Hypoxia-inducible factor α (HIFα) similarly proves to be essential for osteoblastic activity during endochondral bone formation but not during intramembranous bone formation [29]. Angiogenesis proved essential for the remodeling of long bones whereas this was found less important for remodeling of intramembranous bone [29].

Together these data suggest considerable bone-site-specific differences in regulation of osteoblast activity. This, as we will see later on, results in differences in the matrices they produce. It seems that the situation in bone may be analogous to that in hyaline cartilage at different anatomical sites where significant differences in function and in composition are found, for example between cartilage in the growth plate and articular cartilage.

3.2. Bone-site-specific differences in matrix composition

A different mode of ossification may be expected to result in compositional differences of the matrix and its subsequent mineralization. This indeed appears to be the case. Not only the amount of collagen and non-collagenous proteins differs considerably between skull and long bone also the level of collagen cross-linking was shown to be greatly diverse [30]. These differences are considered to be important for the mechanical properties and mechanical demands made on the different bones. After all each bone has its own unique function in providing support and/or protection of specific parts of the body.

Each of the different types of bone appears to have its own unique osteoinductive components [31,32]. Extracts of long bone can induce formation of cartilage as an intermediate step in the process of formation of bone. Extracts of intramembranous bone do not seem to have this capacity [31].

In addition to differences in the composition, abundant data indicate differences in bone remodeling times. Remodeling of calvaria bone is much slower than the remodeling of long bones [33]. Whether

these differences are due to the compositional differences, to different osteoclastic activities, to differences in the proportion of cortical or trabecular bone, or to differences in the neuronal and/or hormonal modulation is still a matter of debate.

3.3. Bone-site-specific differences in hormonal responses

A series of elegant experiments has shown bone-site-specific responses to compounds such as prostaglandin E_2 (PGE_2) and parathyroid hormone (PTH). In 1998, Suponitzky and Weinreb [34] demonstrated that systemic *in vivo* application of PGE_2 to rats resulted in an increased bone formation in long bones without affecting calvaria bones. Also systemic injection of PTH had an anabolic effect on long bones but not on those of the skull [35]. Hens need to release large amounts of calcium from their skeleton during their egg-laying period and have a very high osteoclastic activity in specific bones, the medullary bones, whereas skull bones are spared. Systemic hormones regulate calcium release through regulating medullary osteoclast activity, thus indicating that not all osteoclasts respond similarly to circulating hormones. It remains to be seen, however, whether in this case the osteoclast or the osteoblast is the cell responsible for the bone-site-specific effect as osteoblasts are the cells expressing PTH receptors.

3.4. Bone-site-specific differences in neuronal response

Sympathectomy results in an increased resorption of intramembranous bone whereas it does not affect resorption of endochondral bone [36,37]. This resorption-stimulating effect was noted in bones of the middle ear and in mandibular bones, but not at other skeletal sites [38], leading the authors to suggest that blockage of sympathic nerves increases resorption whereas blockage of sensoric nerves decreases resorption. These data strongly suggest bone-site-specific differences in responses due to different neuropeptides to which the bone cells (osteoclasts and/or osteoblasts) respond [38].

3.5. Bone-site-specific differences in mechanically-induced responses

Different bone types have different turnover rates, which are largely dependent on the different mechanical strains experienced by the tissue. According to Chole [39] intramembranous bone is more sensitive to pressure-induced remodeling than endochondral bone. While the precise mechanism underlying mechanically-induced remodeling is not yet clear, we know that the osteocyte network is critically important in regulating bone formation and resorption. Osteocytes sense differences in strain and subsequently send signals (e.g. nitric oxide, PGE_2, sclerostin) to bone surface-associated cells, such as bone lining cells, osteoblasts and osteoclasts to stimulate or block their activity, thus modulating bone remodeling [40,41].

Given the differences in remodeling rate between the various bone types, it is of interest to note that osteocytes in bones of the skull differ from those in long bones. Vatsa et al. [42] recently showed that skull osteocytes were rounder than those of the long bones. Since a round cell appears to be more sensitive to mechanical signals than a flat cell [43], skull osteocytes may respond more easily to the lower mechanical strains in the skull. These observations could help to explain why differences exist in remodeling rates in different types of bone.

3.6. Bone-site-specific differences in osteoclasts

There is a substantial body of evidence to suggest considerable differences between osteoclasts at various bone sites. Already 10 years ago, in 1999, we showed that calvaria osteoclasts differ from those present in long bones with respect to the proteolytic enzymes used for bone matrix digestion [44]. Long bone osteoclasts synthesize primarily cysteine proteinases (especially cathepsin K), whereas calvaria osteoclasts additionally employ matrix metalloproteinases (MMPs). In a more detailed follow-up study we showed that the different osteoclast populations also synthesized and used additional cysteine proteinases [19], although the nature of these enzymes still needs to be established. In line with these findings is the study by Shorey et al. [45] who showed that osteoclasts, present in the intramembranous scapula, also use MMPs, in addition to cysteine proteinases, to digest organic bone matrix, thus generalizing the concept that intramembranous bones are degraded not only by cysteine proteinases but also by osteoclastic MMPs.

This is a departure from the general notion that cathepsin K is essential for the digestion of organic bone matrix by osteoclasts [46]. We have already mentioned the disease pycnodysostosis, caused by loss-of-function mutations in the cathepsin K gene [47] where osteoclasts are able to demineralize bone, but cannot degrade the organic matrix. Generation of cathepsin K deficient mice [48,49] confirmed the importance of cathepsin K in osteoclast-mediated bone degradation. However, interestingly, these studies also showed that absence of cathepsin K affected remodeling of calvaria bone much less than that of long bones, clearly pointing to differential use of cathepsin K by osteoclasts at different bone sites.

Another remarkable difference between osteoclast populations is the expression level of the enzyme tartrate resistant acid phosphatase (TRACP). Although this enzyme is highly expressed by multinucleated osteoclasts in all types of bone, expression levels are much higher (up to 25-fold) in calvaria osteoclasts compared to long bone osteoclasts [50]. The higher expression in calvaria osteoclasts seems to relate to the important role of TRACP in digestion of the non-collagenous protein osteopontin [51] as in TRACP-deficient mice higher levels of non-digested osteopontin are found adjacent to calvaria osteoclasts, compared to long bone osteoclasts [50]. In addition to differences in TRACP expression between different bone sites, osteoclasts also differed in TRACP expression within the same type of bone [52]. A higher level of TRACP expression was found in osteoclasts in the proximal epiphysis (resembling the “calvaria osteoclast phenotype”) compared with trabecular osteoclasts [52]. The findings that characterize the osteoclasts at different bone sites are summarized in Fig. 5.

Finally, several studies have shown heterogeneity in osteoclasts with respect to their size [53–55]. Several groups demonstrated that small osteoclasts showed less resorption [53,56] and adhered more strongly to prothrombin and thrombin than large osteoclasts.

4. How are bone-site-specific osteoclasts formed?

4.1. Different osteoclasts due to different osteoblasts?

The osteoblast or, more precisely, the bone lining cell, an osteoblast-lineage cell that does not produce osteoid, plays a crucial role in the generation of osteoclasts. It recruits osteoclast precursors to the resorption site by the release of chemokines [57]. Following this chemoattraction the osteoclast precursors attach to the bone lining cells via ICAM-1/VLA interaction [58] and this allows the receptor RANK expressed by the osteoclast precursors to bind its ligand RANKL expressed by the lining cell. This results in the priming of the precursor to start differentiation into an osteoclast expressing characteristic features such as TRACP activity, and receptors for calcitonin and vitronectin. Subsequently, the bone lining cells move out of the way thus exposing the bone surface to which the primed precursors migrate and where they complete maturation and fusion [59].

The ratio of RANKL with its decoy receptor OPG is essential for osteoclastogenesis. When Odgren et al. analyzed the effect of transgenically induced RANKL expression in RANKL deficient mice, they noted normalization of osteoclast formation in long bones but not in the jaw and concluded that “failure of bone resorption in the jaws is highly site-specific” [24]. Since RANKL is highly expressed by bone lining cells [60,61], this finding supports the notion that bone-site specificity exists with respect to expression of RANKL. In keeping with

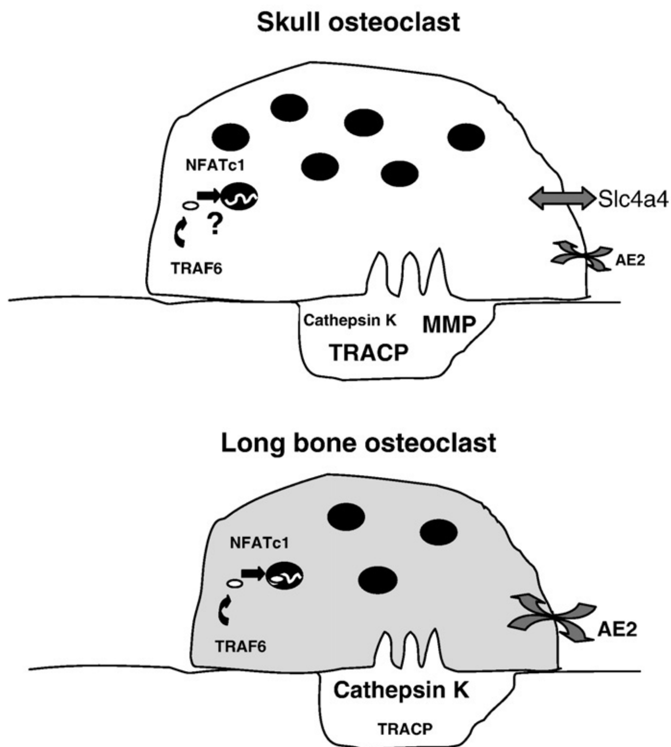

Fig. 5. Schematic presentation of known and putative differences between osteoclasts present in the skull and in long bone. Skull osteoclasts use MMPs and cysteine proteinases (among which cathepsin K) for resorption, whereas long bone osteoclasts use primarily cysteine proteinases. Skull osteoclasts are bigger and express a higher level of TRACP. The anion exchanger-2 (AE2) is essential for resorption by long bone osteoclasts, but less important for resorption by skull osteoclasts. The latter cells express the sodium transporter Slc4a4, which seems to compensate for the loss of AE2. Skull osteoclasts may use different signaling pathways than through TRAF6 and NFATc1, since skull bone is not affected in TRAF6 and NFATc1 null mutant mice.

this, overexpression of OPG has an inhibitory effect on osteoclastogenesis at the endosteal and trabecular bone sites, but not in periosteum or the jaw where osteoclasts are formed unhindered [26]. In line with a bone-site-specific response of bone lining cells/osteoblasts is the response to the hormone PTH. Several studies have shown that whereas the metaphysis is highly sensitive to this hormone the skull bone is hardly responsive (see [35]). Collectively, these data strongly suggest considerable differences between osteoblasts/bone lining cells in different bones. Whether such different populations of osteoblasts result in the formation of phenotypically different osteoclasts remains to be demonstrated.

4.2. Different osteoclasts due to differences in substrate?

The composition of matrix and mineral differs between bones at different sites of the skeleton (see above). Such compositional differences might alter the response and expression of proteins by the osteoclasts [62] and osteoclast precursors [63]. After all, it has been well established that extracellular matrix components affect cell behavior, including expression of proteolytic enzymes (e.g. [62,64]). In an attempt to study this in more detail we isolated osteoclasts from calvaria and long bones and compared their activities. The osteoclasts were seeded on their original and a different bone substrate and analyzed for the enzymes used in resorption. Calvaria osteoclasts seeded on their own substrate or on long bone slices used in both conditions MMPs and cysteine proteinases. Similarly, long bone osteoclasts seeded on either substrate used primarily cysteine proteinases. These findings indicate that the bone substrate itself does not affect the phenotype of mature osteoclasts, but does not exclude that there is an influence of substrate during osteoclast generation.

4.3. Different osteoclasts due to different precursors?

It is generally taken that osteoclast precursors reside in the circulation and that these cells leave the blood system upon attraction by chemokines locally produced by endothelial cells and/or bone lining cells [65,66]. Numerous findings, including the classic studies of Walker [67] who showed that osteopetrotic mice could be rescued by temporary parabiosis (connecting the blood stream) with a normal littermate, indicate that a mononuclear cell fraction present in the peripheral blood has the capacity to differentiate into osteoclasts. This feature, together with the availability of synthetic M-CSF and RANKL, has revolutionized *in vitro* osteoclast formation studies from easily accessible precursors in patients and volunteers. Despite this, convincing evidence that *in vivo* the osteoclasts present at the different bone sites always arise from blood-borne cells is not yet available. Alternative sources of osteoclast precursors are the spleen, liver (in very young animals) and the bone marrow. Spleen and bone marrow are regularly used in osteoclast culture experiments, especially in mouse studies.

Recently Richter et al. [68] showed that *oc/oc* mice can be cured by intraperitoneal injection of retrovirally transduced osteoclast precursors expressing normal osteoclast V-ATPase, demonstrating that precursors injected into the peritoneum find their way to the blood stream and subsequently into bone. However, this does still not answer the question whether precursors generally home to marrow spaces from where they are directed to the sites of resorption, or whether they use the blood stream to directly extravasate at sites of resorption. In addition, the question whether osteoclasts derived from blood-borne cells differ from those generated from marrow cells has not been investigated yet. Recent studies do show that various mononuclear cell fractions isolated from mouse bone marrow differ in their capacity to form osteoclasts and their requirement for cytokines [69,70]. It still has to be assessed, however, whether phenotypic differences, such as enzyme profiles, cell size, or others as mentioned above, exist between osteoclast populations derived from these different mononuclear cell fractions.

Strong evidence for the existence of different subsets of osteoclast precursors has recently come from studies in inflammatory conditions [71]. It was demonstrated that osteoclasts can form from mature dendritic cells through a process of transdifferentiation [71–75]. The dendritic cell-derived osteoclasts were indistinguishable from osteoclasts derived from blood monocytes using a wide range of phenotypic markers. *In vivo*, it is now considered that dendritic cells may transdifferentiate into osteoclasts due to interaction with cells producing high levels of RANKL, such as T cells, stromal cells and/or osteoblasts, conditions that may exist for example in the joint of patients with rheumatoid arthritis. In addition there is evidence for the contribution of B-cell-lineage cells to osteoclast formation [76]. Although this may only occur under special circumstances, for example under the influence of unusually high local levels of osteoclast-inducing factors and in the presence of high numbers of B cells and osteoclast precursors, such as in myeloma, it nevertheless indicates that different cell lineages may contribute to the formation of osteoclasts.

In line with the notion that under inflammatory conditions osteoclast precursors may differ is the study performed by Nose et al. [77], who found that comparable numbers of osteoclast precursors yielded higher numbers of osteoclasts if precursors were from patients with rheumatoid arthritis, compared with precursors from patients with osteoporosis.

5. Implications of osteoclast heterogeneity

The notion that there may be bone-site-specific differences in bone cells leading to local differences in bone remodeling has received relatively little attention in the literature so far. Yet, we have shown that there are abundant observations pointing to such differences in

osteoclasts. In addition we discussed the emerging evidence for site specificity in bone lining cells/osteoblasts and osteocytes. Focusing on osteoclasts, we would urge caution in the interpretation of studies performed with cells from one source of precursors and extrapolating results to all osteoclast populations. Bone phenotyping of whole animal models of bone disease is often restricted to analyses of long bones or vertebrae and, as we have shown above, this may not reveal differential effects of gene knockout, or mutation, which may specifically become apparent when comparing endochondral versus intramembranous bones. Differences within bone, such as those between trabecular and cortical bone will only be revealed upon careful histological and immuno-phenotypical analysis. One may question whether differences in osteoclasts are sufficiently important to warrant such additional analyses. We should consider however that novel osteoclast inhibitors are being developed, often using knowledge of essential osteoclast gene products gained in the study of osteopetrosis. One of these, a cathepsin K inhibitor, is in advanced stages of clinical development [78]. Based on the evidence presented above, we can be fairly sure that a cathepsin K inhibitor will primarily reduce osteoclast activity in long bones and vertebrae, but far less in the skull or jaw. Other inhibitors of osteoclast activity may have different site specificity. This is not necessarily a problem, but may in fact be seen as a new challenge and an opportunity. Studies are ongoing to understand the site-specific effects of bisphosphonates on the skeleton. When coupled with the notion of cellular site specificity, such information may lead to novel, rational drug treatments, even with drugs, or combinations of drugs, already licensed. In a similar way, the notion that anabolic PTH treatment does not benefit all bones (or bone sites) alike [79] clearly points to differences in the osteoblast compartment and urges caution with generalization of treatment effects observed in one site only to the whole skeleton. We expect there is a lot of additional data on bone-site specificity of treatment effects and cellular responses already collected, but not necessarily reported in the literature as this data may have been seen as "erroneous results". We would suggest that cellular heterogeneity in bone is more likely than not. Clearly this is an area that requires further careful study to fully exploit therapeutically the immense advances in knowledge of osteoclast biology gained from the study of rare osteoclast diseases such as osteopetrosis.

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