General discussion
GENERAL DISCUSSION
To shed some light on mechanisms involved in osteoclast functioning and their heterogeneity, in this thesis we analyzed (1) the process of fusion and fission of osteoclasts, (2) the role of anion exchanger 2 and lysosome-membrane protein 2 in osteoclastogenesis and resorption (3) the effect of the inflammatory cytokine IL-1β on osteoclast precursors from different bone sites, and (4) the response of these precursors to bisphosphonates.

In Chapter 2 it is shown, by the use of life cell (time lapse) imaging that fusion occurs not only between mononuclear cells, between mononuclear and multinucleated cells, but, surprisingly, also between multinucleated cells. The most exciting phenomenon was, however, the fission of multinucleated cells. The fission of multinucleated osteoclasts started with the formation of cytoplasmic compartments that were linked to each other by very thin cytoplasmic extensions. Each of these compartments contained a number of nuclei, giving rise to so-called multi-compartmented osteoclasts [1]. The compartments moved away from each other resulting in the elongation and thinning of the interconnecting cytoplasmic extensions. Finally, these connections broke, thus separating the multinucleated osteoclast in several multinucleated osteoclasts. This latter process was frequently associated by a small mononuclear cell that proved to be of hematopoietic origin. Just prior to splitting the extension into two parts this small round cell was moving along the extension and when it stopped, at that site, the extension was clipped. How this was accomplished is not known and needs further investigation. We propose that due to selective separation and compartmentalization of nuclei, slightly different osteoclasts can be formed, which are better equipped for their site specific functions. Youn et al. [2] demonstrated that not all nuclei present in an osteoclast are transcriptionally active. This supports the idea that separation of specific nuclei could play a role in the appearance of osteoclast heterogeneity. Andersson et al. [3] showed in patients with myeloma that osteoclasts may contain nuclei of myeloma cell origin, thus possibly contributing to an altered phenotype of the osteoclast. These nuclei are likely incorporated into the osteoclast by fusion and or fission [3, 4], which also suggests that fusion and fission can play a role in the appearance of osteoclast heterogeneity. In addition, fission activity may play a role in the removal of apoptotic nuclei. In this way the osteoclast may prolong its lifetime by getting rid of inadequately functioning parts of the cell. Thus, the combination of fusion and fission may perpetuate the lifetime of osteoclasts.
Novel information regarding osteoclast heterogeneity was obtained by analyzing mice lacking proteins that are putatively involved in osteoclast activity. The osteoclasts present in mice deficient for 3 of the 5 isoforms of \textit{Ae2} (\textit{SLC4a2}) showed bone site specific differences in the use of membrane-bound ion exchangers that are involved in the modulation of the intracellular pH level. During bone resorption osteoclasts acidify the extracellular space where bone degradation occurs, i.e. the space adjacent to the ruffled border membrane. This acidification of the extracellular space will result in the rise of the intracellular pH. To maintain the intracellular pH at physiological levels different membrane-bound ion pumps and exchangers are present [5, 6]. Without these regulators, osteoclasts are unable to resorb bone and the bones become osteopetrotic [7-9]. The long bones of the \textit{Ae2\textsuperscript{-/-}} mice were indeed osteopetrotic; they had a smaller marrow space and a thicker bone shaft. Surprisingly, however, the teeth of these mice showed a normal eruption pattern. This finding indicated that the osteoclasts present in the jaw and probably also in the skull were active. Our findings presented in \textbf{Chapter 3} show that skull osteoclasts make use also of another ion transporter: \textit{SLC4a4}. We demonstrated that the anion exchange activity of long bone osteoclasts depend solely on \textit{Ae2}, whereas osteoclasts present in the skull have next to \textit{Ae2} also the other transporter, \textit{SLC4a4}, by which they can modulate the intracellular pH level. The data presented in this study provide convincing evidence for the occurrence of bone site specific differences between osteoclasts.

Furthermore mice deficient in lysosome-associated membrane protein-2 (LAMP-2) provided new insights. This protein is highly expressed in the lysosomal membrane [10-12]. Lysosome fusion occurs at the ruffled border of osteoclasts and LAMP-2 is also present in the ruffled border membrane. Several functions for LAMP-2 are described in the literature, but a possible function in the ruffled border was still unknown [10, 11, 13-19]. Electron microscopic evaluation of osteoclasts of the LAMP-2 deficient mice showed that they were present and active (\textbf{Chapter 4}), thus suggesting that LAMP-2 is not essential for osteoclast functioning. In an attempt to obtain more information, osteoclast precursors of LAMP-2 deficient mice were cultured with the osteoclastogenic cytokines M-CSF and RANKL. In these cultures multinucleated osteoclasts were formed normally, thus indicating that also the formation of osteoclasts did not depend on the presence of LAMP-2. It is known that osteoclast formation \textit{in vivo} depends largely on a close interaction of the osteoclast precursors with cells of the osteoblast lineage. Therefore, we
analyzed osteoclast formation induced by osteoblasts. Under this condition we found a complete absence of osteoclast formation when LAMP-2 was absent in the osteoblasts, regardless of their bone origin. This occurred irrespective of the origin of the precursors (LAMP-2 deficient or wild type). These findings indicate that expression of LAMP-2 by osteoblasts is essential for their osteoclastogenesis-inducing capacity. In search for an explanation, the expression of RANK and RANKL was investigated in co-cultures of LAMP-2 deficient and wild-type mice. Analysis of mRNA expression of RANK and RANKL showed no difference. Also RANKL protein was present in the LAMP-2 deficient osteoblast. Yet, the expression of RANKL at the cell membrane was severely hampered in the LAMP-2 deficient osteoblasts. Thus the essential interaction between RANK on the precursor membrane and RANKL on the osteoblast membrane was probably severely diminished. In line with a decreased interaction was the lower expression of downstream genes that depend on RANK-RANKL interaction, DC-STAMP and v-ATPase [20, 21]. How LAMP-2 is involved in the membrane expression of RANKL is not known, but recently it became clear that LAMPs are involved in processes such as recycling of the mannose-6-phosphate receptor. This receptor is involved in the transport of many lysosomal enzymes [10, 13, 22, 23, 24]. We propose that LAMP-2 is somehow involved in the transport of RANKL to the plasma membrane; in its absence RANKL accumulates in lysosomal vacuoles [25].

Osteoclasts are lysosome-rich cells and it is assumed that the formation of the ruffled border depends on lysosomal function. Therefore, it is quite remarkable that the osteoclasts and their functioning seemed unaffected when a protein highly expressed in the lysosomal membrane, LAMP-2 is deficient. Such a seemingly independence of important lysosomal proteins in osteoclast functioning has been noted before. Also in the absence of an important lysosomal transport protein, mannose-6-phosphate, an osteoclast phenotype was lacking [26, 27]. Mannose-6-phosphate (M6P) is involved in the sorting of the lysosomal proteins. In M6P deficient patients and mice, skeletal abnormalities were present, even though the resorbing activity of the osteoclasts was found to be unaffected. In these mice, comparable to our findings with LAMP-2 deficiency, the osteoblasts proved to be affected. Probably due to the accumulation of undegraded molecules, the osteoblasts were less differentiated, resulting in an altered signaling to the osteoclasts [26, 27].
An explanation why the osteoblasts were affected and the osteoclasts not, might come from the different functions lysosomes have. Lysosomes are involved in processes such as secretion, plasma membrane repair, signaling and energy metabolism [28]. Not all these processes are similarly active in all cell types. For instance in osteoclasts one of the main functions of the lysosomes is the secretion of different enzymes, e.g. proteases, in the resorption lacuna [29]. It might be possible that cells contain different subclasses of lysosomes, each class with its own specialized function.

Very recently it became clear that lysosomes regulate their own biogenesis through the subcellular localization of a transcription factor, called transcription factor EB (TFEB) [29]. Binding of RANK to RANKL triggers the phosphorylation and therefore stabilization of TFEB, resulting in enhancing the level of lysosomal enzymes, acidification, the number of lysosomes and the fusion of lysosomes to the plasma membrane and thereby osteoclast activity [28, 29]. Unphosphorylated TFEB translocates to the nucleus. In the nucleus TFEB induces the expression of lysosomal genes and genes involved in autophagy. If indeed different lysosomes exist with specialized functions, the phosphorylation state of TFEB might play a role in their adaptation [29].

Bone degradation by osteoclasts is enhanced at inflammatory sites such as those occurring in periodontitis or rheumatoid arthritis. An explanation for this localized increased osteoclast activity could be related to differences in osteoclast sensitivity to inflammatory mediators such as interleukin-1β (IL-1β); a cytokine highly expressed at such sites [30, 31]. Chapter 5 describes osteoclasts generated in the presence of IL-1β. Addition of IL-1β to osteoclast precursors resulted in larger osteoclasts with a higher resorptive activity. This phenomenon was found irrespective of the origin (calvaria or long bone) of the osteoclast precursors. However, site specific differences were found in the mRNA expression of the decoy receptor IL-1R2. A dose dependent upregulation of IL-1R2 mRNA was found in the calvaria cultures; long bone cultures did not show such a response.

Two receptors for IL-1β are known: IL-1R1 and IL-1R2 [32]. Following binding of IL-1β to IL-1R1, signal transduction will occur, which eventually results in an enhanced production of inflammatory cytokines [33]. However, IL-1β has a higher affinity for the decoy receptor IL-1R2 [34]. Binding to the IL-1R2 receptor does not result in transduction of a signal and therefore no release will occur of additional
inflammatory cytokines [35, 36]. Binding of IL-1β to IL-1R2 will lower the concentration of IL-1β and therefore result in lower levels of the cytokine that bind to IL-1R1 [33]. Thus IL-1R2 might control the activity of IL-1β [34]. This control step appears to be present more profoundly in osteoclast precursors of the calvaria. This suggests that calvaria cells are more capable to suppress inflammatory cytokine upregulation. Then how to explain the similarity in response of the different osteoclast precursors in the formation of larger osteoclasts and increased activity of these osteoclasts? The expression of IL-1R2 was very low during the first culture days, even though IL-1β was present. This IL-1β can bind to the available IL-1R1 and induce larger osteoclast with a higher activity. At a later time point IL-1R2 expression is upregulated. In turn IL-1R2 may capture an excess of IL-1β and further inhibit inflammatory signaling. At that time point the formation of larger osteoclasts with a higher resorptive activity was already accomplished.

Several studies have shown that bisphosphonates (BPs) can induce osteonecrosis of the jaw [37-40]. Why osteonecrosis is observed particularly in the jaw is not clear [41]. In Chapter 6 it is shown that jaw marrow cells exhibit a higher uptake of BPs than marrow cells isolated from long bone. This higher uptake, however, did not result in differences in osteoclast formation. This could be explained by the finding that gene expression of the anti-apoptotic genes Bcl-2 and Bcl-xL was higher in jaw cells than in long bone cells, suggesting that the jaw osteoclast precursors were more resistant to BP-induced apoptosis. An explanation for the occurrence of necrosis in the jaw, might be that most of the osteonecrosis incidents appear when bisphosphonate treatment coincided with an injury such as tooth extraction [42]. This injury probably influences the local concentration of inflammatory cytokines, such as IL-1β. As shown in Chapter 5 IL-1β accelerates osteoclast activity. Also shown in Chapter 5 was that not all osteoclasts were affected equally by IL-1β. It might be possible that IL-1β selectively accelerates a subtype of osteoclasts present in the jaw and that this subtype react differently to IL-1β compared to the osteoclasts normally present in the jaw. This might suggest that the combined presence of inflammatory cytokines and bisphosphonate eventually results in osteonecrosis.
Chapter 7

CONCLUSIONS
The data in this thesis shed some new light on differences between osteoclast subtypes. Besides the already known differences described in recent reviews [8, 43] the studies presented in this thesis demonstrate differences in use of membrane-bound pH regulators, sensitivity to IL-1β, and bisphosphonates. In addition, we showed pathways used by osteoclasts or their precursors to regulate their number and activity.

Osteoclasts are very shapable cells. They are able to regulate their number of nuclei and cell-body size by fusion and fission. The trigger to do this might lie in a need of local specialization of osteoclasts, resulting in a local occurrence of osteoclast diversity.

In the present study, it was observed that as opposed to long bone osteoclasts, head region osteoclasts express additional mechanisms that enable them to suitably react to the cranio-facial physiological environment. The calvaria cells showed: (1) an upregulation of IL1R2, probably to suppress an IL-1β-induced inflammatory cytokine upregulation, (2) expression of SLC4a4 to modulate intracellular pH, and jaw bone cells showed (3) a lower sensitivity to bisphosphonate-induced apoptosis.
REFERENCES


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