PERSPECTIVES

The Pathogenesis of Osteoclast Diseases: Some Knowns, but Still Many Unknowns

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Abstract

Genetic diseases of osteoclasts include those in which osteoclast function is compromised, such as in osteopetrosis, and diseases of osteoclast overactivity, such as the pagetic disorders. The genetic basis of these conditions is now largely known and this review gives an update on the most recent findings and functional studies supporting the role of genes in disease pathogenesis. Loss-of-function mutations in the genes *TCIRG1, CLCN7, OSTM1* and *PLEKHM1* are found in autosomal recessive osteopetrosis, and current evidence suggests they are mechanistically linked by their involvement in the trafficking of acidic vesicles in osteoclasts. The genetic defects in osteoclast-poor osteopetrosis remain to be found. Autosomal dominant osteopetrosis type II is also caused by loss-of-function mutations in *CLCN7*. Juvenile Paget's disease is caused by loss-of-function mutations in the gene for osteoprotegerin (OPG), *TNFRSF11B,* and is an endocrine disorder which can be treated by reconstituting OPG levels. The other pagetic diseases appear to be more osteoclast-autonomous. Classic Paget's disease of bone (PDB) is associated with mutations in *SQSTM1*, the complex syndrome known as inclusion body myositis with PDB and frontotemporal dementia (IBMPFD) with mutations in *VCP,* and the severe pagetic disorders early-onset PDB, familial expansile osteolysis and expansile skeletal hyperphosphatasia are all associated with mutations in *TNFRSF11A*, the gene for RANK. The common pathway affected by these genes is less easily deduced, but most likely involves perturbation of the proteasomal degradation pathway. We suggest that these pagetic disorders have many similarities with "inclusion body diseases" of the brain and skeletal muscle. Further understanding of pagetic disorders may require animal models that faithfully represent the pathology seen in patients, since cellular models show only part of the complex disease pathology. *BoneKEy-Osteovision.* 2007 February;4(2):61-77.
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The genetic basis of inherited bone diseases has been the focus of attention in many laboratories over the past 10 years or so, since advances in DNA sequencing allowed for more systematic analysis of putative causative genes. There was great optimism that, by knowing the mutated genes, the pathogenesis of the disorder would become immediately obvious, and present novel "rational" therapeutic avenues. Without question, this genetic approach has uncovered important, previously unrecognized players in bone physiology. For many disease genes, however, a full understanding of pathogenetic mechanisms remains unknown. Here, we discuss the current state of our knowledge of osteoclast diseases. We focus on the two extremes of osteoclast dysfunction: osteopetrosis, a series of rare disorders characterized by a lack of osteoclast activity, and Paget's disease, a more common disorder caused by osteoclast overactivity.

Genes Causing Osteopetrosis in Man

Osteopetrosis can be considered the quintessential osteoclast disease. Osteoclasts fail to resorb, or insufficiently resorb bone, and hence bone modeling and remodeling is disturbed to varying degrees, resulting in too much bone and the persistence of mineralized cartilage. A number of naturally occurring rodent models

of osteopetrosis exist and it is from careful studies of these that the lineage of the osteoclast was first established and bone marrow transplantation (BMT) demonstrated as a possible cure for the condition (1). However, despite much effort in functional analysis of the osteoclast defect in animal models, it was not until the era of modern genetics that the majority of causative genes in animal models were discovered and the first genes causing osteopetrosis in man found. Although it was clear from clinical assessment that osteopetrosis is heterogeneous in transmission (both autosomal dominant (ADO) and autosomal recessive (ARO) forms exist) and in severity, it is only now becoming evident that this is related to the presence of different genetic mutations and possibly to other genetic or environmental factors.

Figure 1 shows the genes currently linked to osteopetrosis in man (reviewed in (2;3)). It is well established that this disease can be caused by mutations in 3 genes involved in the acidification of the resorption lacuna by osteoclasts: (a). *CAII*, which encodes the enzyme carbonic anhydrase type II, responsible for proton production, (b). *TCIRG1*, which encodes the a3 subunit of the vacuolar ATPase, the osteoclast proton pump, or (c). *CLCN7*, which encodes the chloride channel ClC-7, thought to couple acid secretion and chloride secretion. ADO type II is exclusively caused by mutations in *CLCN7* while ADO type I is now known to be caused by mutations in an osteoblast-expressed gene, *LRP5*, and is no longer considered an osteoclast disease, but a disease of high bone mass (reviewed in (2)). Since the mutations above explain only about 70% of the clinical cases of ARO, it is clear that there must be other genetic origins of this disease. Recently, two additional genes that are rare causes of ARO have been identified. First, a severe form of ARO results from mutations in the *OSTM1* gene, which is also mutated in the naturally occurring osteopetrotic grey-lethal mouse (4) (the name of this mouse derives from its distinctive coat color defect, which occurs due to disruption of the transport of pigment-containing melanosomes in melanocytes). Second, an intermediate form of ARO is

caused by a mutation in the *PLEKHM1* gene, which also underlies osteopetrosis in a naturally occurring rat mutant with abnormally light coat color, the incisors absent (*ia*) rat (5). These two mutations indicate that there are common pathways regulating melanosome transport in melanocytes and the trafficking of acidic vesicles in osteoclasts, raising the possibility that other genes that regulate melanosome transport (and that are mutated in other naturally occurring mouse coat color mutants) may also underlie clinical cases of osteopetrosis in which known causative genes have been excluded.

Although this represents significant progress in understanding the genetic etiology of osteopetrosis in cases in which osteoclast numbers are normal or increased *in vivo*, the cause(s) of "osteoclast-poor" osteopetrosis, in which osteoclasts fail to form *in vivo*, remain elusive. It is plausible that these cases are caused by loss-of-function mutations in osteoclast growth factors, such as macrophage colony stimulating factor (M-CSF) or receptor activator of NFκB ligand (RANKL), or their respective receptors, c-fms and RANK, since mutations in these genes have been found to cause severe osteopetrosis in spontaneous rodent models as well as in engineered models in mice (3;6).

Studies of the Osteoclast Phenotype *In Vitro* Contribute to Understanding Pathogenesis and Treatment Options in Osteopetrosis

The availability of recombinant RANKL has enabled the generation of osteoclasts from the peripheral blood of osteopetrotic patients, and numerous studies have now characterized the osteoclast phenotype of patients with different mutations *in vitro*. These studies not only help to identify how particular genetic mutations may disrupt osteoclast function, but may also reveal whether or not the defect in cases of osteoclast-poor osteopetrosis is osteoclast-intrinsic, and therefore likely to respond to BMT. Early studies performed on patients with "osteoclast-rich" osteopetrosis in whom
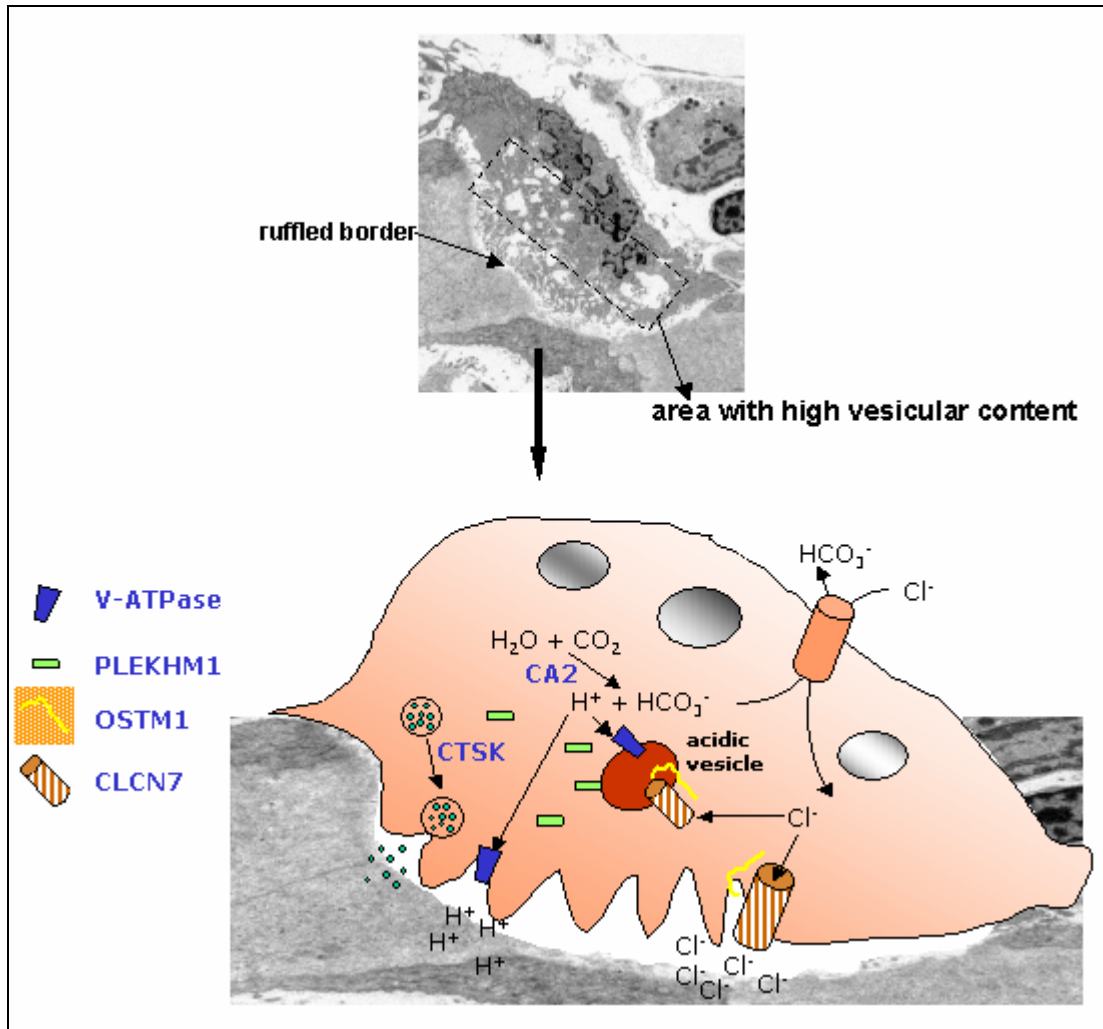
Figure 1. Function of osteoclast genes involved in human osteopetrosis. The top panel shows a transmission electron micrograph of an osteoclast, illustrating the polarized nature of the cell when resorbing and indicating the predominant localization of acidic vesicles and the ruffled border. The schematic diagram in the bottom panel illustrates the genes in osteoclasts involved in the production and secretion of protons. Those shown in blue are known to be involved in human osteopetrosis. The enzyme carbonic anhydrase (encoded by *CA2*) is involved in production of protons. The bicarbonate ion byproduct is exchanged for a chloride ion by the chloride-bicarbonate exchanger. The proton is transported into intracellular vesicles by a proton pump (V-ATPase) with an osteoclast-specific subunit a3 (encoded by *TCIRG1*), coupled to transport of a chloride ion via a channel, CLC-7, encoded by *CLCN7*. These acidic vesicles traffic to the ruffled border and fuse with this membrane, thereby releasing acid into the resorption space and inserting V-ATPase and CLC-7 into the ruffled border membrane. The Ostm1 protein acts as a subunit of the CLC-7 channel and is important for maintaining CLC-7 stability. The protein Plekhm1 lacks a transmembrane domain but also localizes to the outer membrane of acidic vesicles and may play a role in the transport of these vesicles. Loss-of-function mutations in any of these genes/proteins can be a cause of human osteopetrosis. Loss-of-function mutations in cathepsin K (encoded by *CTSK*) lead to pycnodysostosis, a distinct disease, also characterized by osteoclast dysfunction.

the causative mutation was unknown showed that osteoclast formation and, in general, cytoskeletal organization, were unaffected (7;8). However, osteoclasts from osteopetrotic cases exhibited defective ruffled borders (9) and in all cases showed marked deficiencies in bone resorption (7-9). More recent studies have confirmed these earlier findings and have also demonstrated that extracellular acidification (*i.e.*, of the resorption lacuna) is impaired in ARO patients with mutations in *TCIRG1*, and in ADO patients with mutations in *CLCN7* (10-12). However, perhaps surprisingly, endosomal acidification is unaffected in osteoclasts from patients across the mutational spectrum, including mutations in *TCIRG1* or *CLCN7* (5;10-14). Interestingly, three cases of ADOII (*CLCN7* mutations) and one case of ARO (*PLEKHM1* mutation) have been associated with increased levels of intracellular TRAP, possibly due to defective secretion of this enzyme (5;14). Together, these studies suggest that osteopetrosis results from defective trafficking of acidic vesicles towards the ruffled border in osteoclasts, rather than the ability of these vesicles to acidify. In support of this, we have recently found that osteoclasts derived from osteopetrotic patients possess numerous intracellular vesicles, characterized by the accumulation of electron-dense material and resembling large lysosomes, that are not readily seen in control osteoclasts (5).

Interestingly, in ADOII, there is no correlation between disease severity and genotype (indeed some individuals are asymptomatic carriers), indicating that the disease is modulated by secondary genetic factors, or by environmental factors. A recent study has demonstrated that these are likely to be osteoclast-intrinsic genetic factors, since osteoclasts generated from the peripheral blood of patients with ADOII have severely reduced resorptive activity compared to unaffected gene carriers (13). However, these genetic factors have yet to be identified.

There are few studies on the *in vitro* phenotype of osteoclasts from cases of "osteoclast-poor" osteopetrosis, in which osteoclasts are much reduced in number *in vivo*. In three cases, osteoclasts failed to form efficiently when cultured with recombinant M-CSF and RANKL *in vitro*, suggesting the defect is not in these growth factors but is osteoclast-intrinsic (15;16). In a second analysis from one of these patients following bone marrow transplantation, osteoclast formation *in vitro* was restored, also suggesting the defect was osteoclast-intrinsic in this case, although surprisingly the successful engraftment of normal bone marrow failed to recover osteoclast formation in the patient (16). Further studies of osteoclast-poor cases should reveal whether any result from defects outside the osteoclast lineage (*e.g.*, in osteoclast growth factors). This knowledge is clinically important since such cases would not respond to BMT and treatment would be better focused on restoring normal growth factor levels in the patient.

Neurological Pathology in Osteopetrosis

Osteopetrosis patients often suffer from visual impairment and even complete blindness, most likely due to optic nerve compression as a result of defective bone resorption. However, primary retinal degeneration that cannot be explained by bony compression occurs in some patients with malignant ARO (17). Moreover, patients with *CLCN7* mutations may suffer from more general nervous system degeneration that is independent of osteoclast dysfunction, since mice lacking ClC-7 suffer widespread CNS degeneration with features typical of human lysosomal storage disease (18). In addition, neurological defects, including hypomyelination and severe cerebral atrophy, have also been identified in patients with *OSTM1* mutations (19). A recent study by Lange *et al.* has begun to reveal the explanation for the similarity in symptoms between ARO patients with *CLCN7* mutations and those with *OSTM1* mutations. They found that not only do Ostm1 and ClC-7 co-localize on lysosomal membranes and at the osteoclast ruffled border, but that Ostm1 physically interacts with and stabilizes the ClC-7 protein and is therefore required for its normal function (20). Since Ostm1 is highly glycosylated, the authors

postulate that it may shield ClC-7, which, unusually for a lysosomal protein, is unglycosylated, from degradation by lysosomal proteases. Therefore, *OSTM1* mutations most likely manifest their effect by reducing levels of ClC-7. These studies have profound implications for the treatment of patients since they suggest that, whereas the bone defect in patients bearing *CLCN7* and *OSTM1* mutations may be cured by BMT, the neurological degeneration may not.

Future Studies in Osteopetrosis

The majority of genes responsible for human osteopetrosis are now known and their functional analysis has highlighted the critical role of vesicular transport in extracellular acidification and in osteoclast function. Although we are still a long way from fully understanding the mechanistic links between the different genes in osteopetrosis and the way these lead to a similar dysfunctional phenotype, cell biologists have finally started to realize that the osteoclast, a unique type of secretory cell, deserves their attention. With animal models (either natural or engineered) of many of the human forms of the disease already available, and osteoclast formation from patients' peripheral blood mononuclear cells *in vitro* a well-established technique, these studies should prove fruitful. For example, recently, Mitf, the transcription factor mutated in the microphthalmia (*mi)* mouse strain, was found to regulate expression of key genes for osteoclast function, including *CLCN7* and *OSTM1*, demonstrating higher order mechanistic links between osteopetrosis genes (21). The knowledge gained from studies of osteopetrosis have highlighted potential targets for anti-resorptive therapy and have focused attention on targets that may reduce osteoclast activity without a coupled reduction in osteoblast function (22). Indeed, inhibitors of CLC-7 are currently in development (23). Moreover, a better understanding of secretory and endocytic pathways, and their regulation, in osteoclasts is likely to have relevance far beyond the bone field.

Paget's Disease of Bone

Paget's disease of bone (PDB) is a disorder characterized by focal areas of increased bone turnover. Within the pagetic foci, the osteoclasts are notably abnormal: they are increased in size, in number and in their number of nuclei. The increased resorptive activity of these osteoclasts is coupled with increased osteoblast activity, such that the rapid bone deposition is disorganized, architecturally weak and subsequently prone to fracture. The disease is osteoclast-driven, and bisphosphonate therapy inhibits the excessive resorption, followed by normalization of osteoblast function. Other symptoms can include bone pain, deformity, neurological complications, and increased susceptibility to osteosarcoma. PDB is still a common disorder in Caucasian populations, with approximately 3% of individuals over 55 years of age affected. However, many individuals (estimated at 70%) are asymptomatic and recent reports suggest that the incidence is declining (24), although not in all populations (25). Unlike osteopetrosis, no naturally occurring animal models for this disease are known, and all early hypotheses about pathogenetic mechanisms, such as the much-debated viral etiology, have come from clinical and histological observations.

PDB has a clear genetic predisposition. It is inherited in an autosomal dominant way with high penetrance (26). It has a heterogeneous disease pathology, even between affected family members, which highlights the fact that epigenetic factors or combinations of genetic factors affect the severity of disease.

In addition to the late-onset, common form of PDB, three rare forms of earlier-onset and more severe pagetic diseases have been described. Early-onset Paget's disease (ePDB), familial expansile osteolysis (FEO), and expansile skeletal hyperphosphatasia (ESH) share features with late-onset PDB as well as with each other, including focal areas of increased bone resorption, enlarged osteoclasts and the presence of woven bone. Although the three syndromes are very similar, some phenotypic variations

exist, such as the age of onset (typically around the second decade for ePDB, FEO and ESH) and the different bones that are affected (27).

Two more very rare genetic diseases of increased bone turnover are known. Hereditary inclusion body myopathy (IBM) with early-onset PDB (P) and frontotemporal dementia (FD) is a complex, dominantly inherited disorder (abbreviated as IBMPFD) where PDB combines with muscle weakness and early dementia. The myopathy is the main clinical phenotype, but in about half of the patients an "early-onset" (in the 4th decade) PDB is seen (28). Juvenile Paget's disease (JPD), also known as familial idiopathic hyperphosphatasia, was first described in 1956 (29) and is an autosomal recessive disease that presents in early childhood, and is characterized by both increased bone resorption and formation. In JPD, most bones are affected and, like other forms of pagetic bone disease, this increase in bone turnover results in woven bone, which is structurally weak. Trabeculae in the iliac crest from JPD patients exhibit a parallel plate organization rather than the usual meshwork appearance of trabecular bone (30), a phenomenon as yet not understood. JPD is caused by the complete loss of function of osteoprotegerin (OPG) and is so far the only pagetic condition in which the discovery of the causative mutation has led to a better understanding of the pathogenesis of the disease and a new treatment option. We will summarize this evidence briefly below before discussing the other types of pagetic diseases in more detail.

Juvenile Paget's Disease Is Caused by Loss-of-Function Mutations in Osteoprotegerin

JPD is caused by loss-of-function mutations in the gene for OPG (*TNFRSF11B*), a member of the TNF receptor superfamily, which result in either complete deletion of the OPG gene (31) or changes within the gene (32). OPG is a soluble RANKL receptor secreted by osteoblasts (33). It prevents binding of RANKL to RANK, the crucial osteoclast differentiation and survival

receptor, which normally acts via the ligand-initiated formation of signaling complexes, leading to activation of the transcription factors NFκB, AP-1 and NFATc1. These, in turn, regulate the transcription of osteoclast-specific gene expression. Of these signaling cascades, the pathway leading to NFκB activation is perhaps of greatest interest in pagetic diseases (see Fig. 2 and further below).

In JPD patients where the entire OPG gene is absent, serum levels of OPG are undetectable and, as expected, levels of RANKL are elevated (31). The *in silico* predicted effects of the six different reported mutations within the OPG gene (34) on protein expression, secretion, function and disease severity have been confirmed experimentally. For example, OPGΔ182, causing deletion of an aspartate residue, was predicted to result in an unstable loop structure and reduced affinity for RANKL, an effect confirmed in binding studies with synthetic WT and mutated OPG and further verified by reduced inhibitory activity of OPGΔ182 in osteoclast formation studies (35). The deletion/insertion resulting in a premature stop codon at amino acid 325 of OPG, while not affecting the RANKL binding domain, leads to impaired homodimerization (36). OPG in its homodimeric form binds with much higher affinity to RANKL than the OPG monomer (37) and has previously been shown to have higher *in vivo* potency for inhibiting bone resorption (38). Indeed, in this truncated mutant, with an intact RANKL binding domain, RANKL binding is considerably reduced (36).

Rather than using bisphosphonate therapy to inhibit osteoclast function, reconstitution of OPG has now emerged as a treatment option, and successful use of recombinant OPG in the treatment of JPD has recently been reported (39). JPD is therefore a true endocrine disorder, resulting from a loss-of-function of a key osteoclast regulating factor, and as such is mechanistically different from the other pagetic diseases, in which osteoclast-autonomous defects appear most prominent.

The Genetic Basis of Paget's Disease of Bone, and the Role of p62 and VCP

The genetic basis for PDB has been elucidated only in part (recently reviewed in (40)). The protein sequestosome-1 (encoded by *SQSTM1*), also known, and here referred to as p62, is mutated in PDB in approximately 25% of all familial cases, and in up to 10% of sporadic cases examined to date (41). Clearly, additional genes are yet to be discovered, and linkage studies have identified additional susceptibility loci on chromosomes 5q31, 2q36 and 10p13. Polymorphisms in the gene for OPG have
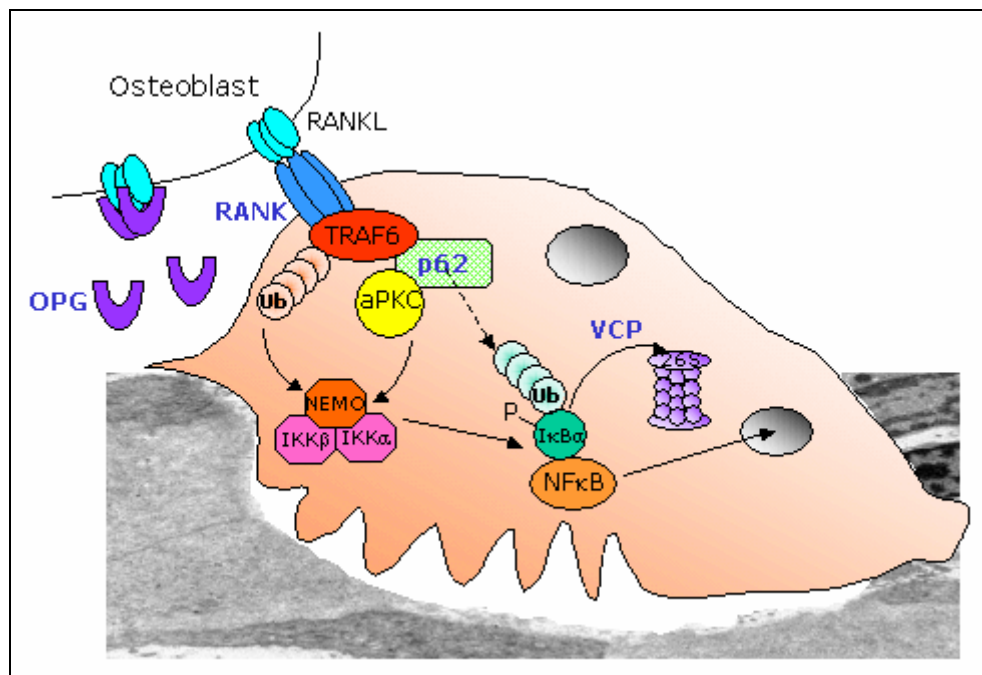

Figure 2. Schematic diagram demonstrating the localization and role of the different proteins (in blue) involved in pagetic diseases. OPG inactivation causes juvenile Paget's disease, and mutations in the signal peptide region of the RANK protein cause familial expansile osteolysis, early onset Paget's disease and expansile skeletal hyperphosphatasia; mutations in p62 are associated with classical Paget's disease of bone. All of these mutations in some way affect the RANK signaling pathway through NFκB. Following RANK ligand binding, trimeric RANK associates with TRAF6 via its cytoplasmic TRAF6 binding domain. p62 also associates with TRAF6 and binds aPKC, which in turn phosphorylates and activates the IKK complex. TRAF6 (via its ring finger domain) undergoes auto-ubiquitination to form K63-linked polyubiquitin chains. Phosphorylation of IκB in the IKK complex is followed by K-48-linked ubiquitination and proteasomal degradation of IκB and subsequent release of active NFκB, which translocates to the nucleus to regulate the transcription of osteoclast specific genes. p62 may also play a role in this pathway as a scaffold protein for TRAF6 ubiquitination and by facilitating the ubiquitination of IκB. VCP has a proven role in shuttling ubiquitinated proteins to the 26S proteasome, as illustrated here for IκBα. For clarity we have not shown the recruitment of the TAK1/TAB2 complex, which is necessary to allow association of ubiquitinated TRAF6 molecules with the IKK/NEMO complex (which is shown) and allows further activation of this complex by facilitating proteasomal degradation of NEMO (70). Pagetic diseases are characterized by the presence of nuclear and cytoplasmic inclusions containing p62, VCP, ubiquitin and proteasomal subunits. Since at present there is no clear evidence how these inclusions are formed and how the proteins travel into the nucleus, they are not shown in this diagram.

been found to increase disease susceptibility, but no mutations in the RANKL/RANK/OPG pathway have been found in classic PDB (unlike the early onset forms, see further below). IBMPFD, a much more rare condition, seems to be linked exclusively to mutations in valosin-containing protein (VCP, encoded by the gene *VCP*) (42). Interestingly, VCP is not genetically linked to classic PDB (43).

p62

p62 is a ubiquitously expressed cytoplasmic and nuclear protein that acts as a dimer. It functions as a scaffold protein with multiple domains to integrate kinase-activated and ubiquitin-mediated signaling pathways downstream from multiple receptors (reviewed in (44)). Of particular relevance to bone physiology is the involvement of p62 with the interleukin receptor and tumor necrosis factor receptor (TNFR) family members, especially RANK. p62 is known to interact with Src family members, atypical protein kinase C members, TRAF6, and ubiquitinated proteins (45). Proteins can be ubiquitinated in a number of different ways: with mono-ubiquitin tags, multiple mono-ubiquitins or various branched chain poly-ubiquitin tags. The type of tag is important for determining the subsequent fate of the protein, be it targeted for proteasomal degradation, accumulation (for example, in "inclusions"), or transport through the cell. All p62 mutations found in PDB lie within or immediately adjacent to its C-terminal ubiquitin-associated (UBA) domain (46). Potential functions of this UBA domain *in vivo* include NFκB activation, the regulation of TRAF6 ubiquitination, protein accumulation and degradation, or other non-ubiquitin-mediated interactions (44;47). As all are important to the normal functioning of osteoclasts, it is possible to envisage scenarios where perturbation of any of these functions might result in the cellular phenotype observed in PDB. Functional studies on the eighteen different p62 mutations in PDB reported to date are only just emerging, while surprisingly little is known about the role of the wildtype protein in bone cells. Studies thus far have focused on its role in the regulation of NFκB, or on its

role in protein degradation by the proteasome.

UBA domain mutations in p62 vary from truncating mutations where most or all of the UBA domain is deleted, to missense mutations that lead to impaired ubiquitin binding, as shown in biochemical studies with expressed protein. Since all patients are heterozygous for p62 mutations and PDB is transmitted in a dominant fashion with high penetrance, the mutated protein must have a still unknown dominant effect. Indeed, patients have been identified with compound heterozygous mutations where the phenotype is no more severe than that seen in individuals heterozygous for the same mutations (48).

Given the dramatic changes in the osteoclast phenotype seen in PDB, a clear effect was predicted on osteoclast formation and function from loss-of-function of the p62 UBA domain. The studies reported thus far (49-51) indeed show that osteoclast precursors overexpressing the most common mutation P392L, or deletion mutants of the entire p62 UBA domain, form increased numbers of osteoclasts with higher numbers of nuclei and increased bone resorption, a finding in keeping with the osteoclast phenotype in patients. In addition, increased numbers of large multinucleated osteoclasts are formed from osteoclast precursors directly obtained from patients with p62 mutations, compared with age and sex-matched controls (49;50). Given the critical role of NFκB in RANKL-induced osteoclastogenesis, these results fit well with the hypothesis that upregulation of NFκB is a key event in the development of a "pagetic" osteoclast. However, the fact that in all studies overexpression of wildtype p62 led to a reduction in NFκB signaling, compared to empty vector controls, remains unexplained and raises the possibility that overexpression itself may have non-specific signaling consequences. Alternatively, it is possible that the UBA domain of p62 plays a critical role in negatively regulating NFκB signaling in osteoclasts, an effect that is perturbed by UBA mutations.

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Surprisingly, mice in which the entire p62 protein is deleted showed no major bone defects under conditions of normal bone remodeling. However, after a resorption-inducing stimulus, osteoclast formation in the knockouts was reduced compared to controls, consistent with a specific role for p62 in osteoclast differentiation (52). Expression of p62 is upregulated during osteoclast formation and has been reported to be almost undetectable in cultures that have not been exposed to RANKL for a relatively long time (52).

A recently described knock-in mouse expressing the P392L mutation under control of the TRAP promoter, to drive expression specifically in osteoclasts, showed increased numbers of osteoclasts with some of the pagetic characteristics seen in patient osteoclasts (50). However, there was no clear evidence of coupled osteoblast overactivity, no nuclear inclusions were seen in osteoclasts, and the characteristic hypersensitivity of osteoclasts to 1,25 dihydroxyvitamin D3 was absent. It should be remembered, though, that this mouse model does not exactly mimic the human disease at the molecular level, as, in patients, a germline mutation in p62 is expressed in a heterozygous manner in all cell types, not just osteoclasts. Mice that express germline p62 mutations are being developed, and we eagerly await learning whether they will show the complete PDB phenotype with aging, or whether additional genetic or epigenetic factors are required.

Valosin-Containing Protein

Studies on the role of mutated VCP have mainly focussed on muscle and neurological features. This protein, a ubiquitously expressed member of the type II AAA ATPase family, which has several domains allowing interaction with different target proteins, acts as a homohexamer and has a complex role in cell cycle control, membrane fusion, organelle biogenesis, and in ubiquitin-dependent protein degradation. Intriguingly, the mutations found in IBMPFD cluster in its ubiquitin-binding region (42), suggesting that a loss of ubiquitin-binding mechanistically links the bone disease seen

in the IBMPFD syndrome with PDB caused by p62 and other mutations. Apart from the fact that VCP is expressed at high levels in osteoblasts (53), little is understood about its specific role in bone, and studies in osteoclasts or osteoclast progenitors are lacking. A major role of VCP is to shuttle polyubiquitinated proteins that are destined for degradation to the proteasome, and in that way to participate in endoplasmic reticulum-associated degradation (ERAD), the pathway by which misfolded proteins are removed from the ER (discussed in (54;55)). It is therefore tempting to speculate that mutated VCP is responsible for the formation of protein aggregates in IBMPFD, even while it remains unclear why this affects only certain tissues and why the onset of this pathology is late in life. Initial studies suggesting that overexpression of mutated VCP indeed leads to formation of protein aggregates *in vitro* (54) have recently been contradicted (55). Similar to studies with p62, it therefore appears that overexpression studies of VCP in "model cells" lead to different results depending on the cell type, constructs and molecular tags used. Thus, while protein aggregates (see further below) are consistently found in muscle and neuronal tissues in patients with IBMPFD and are a hallmark of the disease, a simple cellular model displaying this pathological phenotype, in which to test pathogenetic mechanisms and possible therapies, may, as in classic PDB, not easily be realized. We will return to the possible link between VCP, p62 and protein accumulation and degradation after first discussing the third class of pagetic diseases, namely those caused by mutations in RANK.

Familial Expansile Osteolysis, Early-onset Paget's Disease of Bone, and Expansile Skeletal Hyperphosphatasia Are Caused by Mutations in RANK

FEO, ePDB, and ESH are all caused by heterozygous insertion mutations in the signal peptide region of the RANK gene, *TNFRSF11A*, (56-58), which are predicted to cause a lack of signal peptide cleavage of the RANK protein (56).

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The clinical symptoms in these diseases are similar, and all include early tooth loss and deafness. The diseases vary mainly in age of onset and the skeletal sites affected. In ePDB, caused by a 27 bp (75dup27) insertion mutation, deafness and tooth loss occur when individuals are between 20-30 years old (59). Osteolytic and sclerotic lesions affect the pelvis, skull, mandible and maxilla, and in some cases, the small bones of the hands. FEO, caused by 18 bp insertion mutation(s) (84dup18 or 83dup18), leading to the addition of the same 6 amino acids to RANK, features focal areas of expansile osteolytic bone lesions. ESH, caused by a 15 bp (84dup15) insertion mutation, is characterized by hyperostotic long bones, and biochemical evidence of increased bone remodeling.

The mechanism by which these mutations cause the over-activated osteoclast phenotype has not been fully elucidated. We have recently demonstrated that the mutations alter the subcellular localization of the RANK protein (60). When overexpressed, wildtype RANK is expressed at the cell surface and within the Golgi apparatus, whereas RANK proteins containing the FEO, ePDB or ESH mutations accumulate around the nucleus within an organised smooth endoplasmic reticulum-type compartment (OSER (61)). Even though these results may be influenced by high levels of expression of the mutant proteins in transient overexpression studies, they indicate that these RANK proteins containing signal peptide mutations cannot be processed normally by the ER as predicted (56) and remain trapped in an extension of this compartment. The FEO and ePDB mutations were originally described as activating mutations since constitutive activation of NFκB was seen when the proteins were transiently overexpressed (56). However, we have recently found that stable cell lines expressing just one copy of each of the mutant RANK genes do not show constitutive activation of NFκB (60). Clearly, more work is required to understand how these mutations lead to the hyperactive osteoclast phenotype. Since RANK acts as a trimer, the consequences of a heterozygous mutation on osteoclast behavior may not be modeled adequately by overexpressing the mutant protein in a model cell type. As discussed above for p62 and VCP, studies need to be carried out in a more physiologically relevant setting, including in knock-in mouse models, which are in development.

Do Inclusions Hold the Key to Understanding Pathogenesis?

Early studies in PDB and FEO focused on the histological and ultrastructural abnormalities seen in osteoclasts, especially the common finding of "inclusions" in nuclei and, more unusually, in cytoplasmic areas in affected osteoclasts. Similar observations were made in other disorders of osteoclasts and other cell types (see (62)), especially inclusion body myopathies (63). The similarities between these paracrystalline structures (Fig. 3A) and paramyxoviral nucleocapsids led to the hypothesis that PDB may be a viral disease (64). There is an extensive literature on the putative role of measles virus, or other paramyxoviruses such as canine distemper virus, in PDB, most recently summarized by Reddy (65). Even though pagetic features can be found in cells infected with MV (65) or CDV (66) and the upregulation of genes found to be highly expressed in pagetic osteoclasts can be demonstrated, there is still no conclusive proof, from, for example, proteomic studies or high resolution immunolocalization studies, that the inclusions in PDB and FEO are indeed of viral origin. Nevertheless, the inclusions are a clear signature of the disease and their composition may hold the key to understanding its pathogenesis. Here we would like to discuss the alternative possibility that pagetic diseases, together with inclusion body neuropathies and myopathies, may be members of a larger group of disorders, known as "conformational diseases", many of which are diseases of aging, in which inclusions are commonly found.

p62 and VCP are found by immunostaining in a variety of aggregates or "inclusion bodies", such as the neurofibrillary tangles in Alzheimer's disease, the Lewy bodies in

Parkinson's disease, inclusions in sIBM and Mallory bodies in the liver. The inclusion bodies in sIBM and PDB are indistinguishable at the ultrastructural level, while, for the other conditions, there is insufficient published data on ultrastructure to make comparisons. Progress has recently been made in further identifying the composition of the muscle and neuronal inclusions using biochemical and proteomic approaches (discussed in (45)). Common components of inclusions are ubiquitin, p62,

VCP, subunits of the proteasome, heat shock proteins and tau (67;68;69). These are key components of the ubiquitin proteasome system, the cellular system responsible for the recognition and degradation of misfolded proteins and part of the quality control system in the protein synthesis pathway, confirming that the inclusions are aggregates of proteins destined for degradation. The phenomenon that proteins may aggregate when the proteasomal system is overwhelmed or
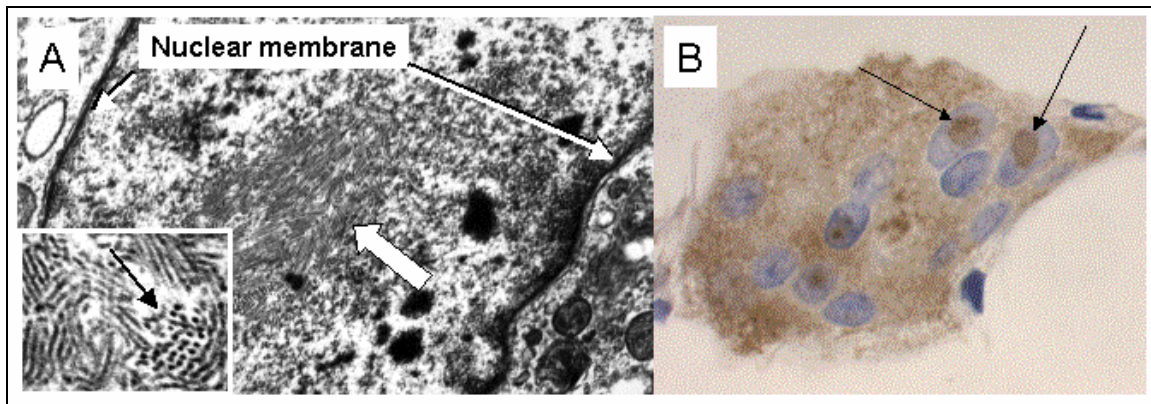

Figure 3. Nuclear and cytoplasmic inclusions in Paget's disease of bone. Panel A shows a transmission electron micrograph at high magnification of a typical intranuclear inclusion (white arrow) in an osteoclast in a patient with classic PDB. The nuclear membrane is indicated. The diameter of the filaments that make up the inclusion (insert, black arrow) is approximately 15 nm. Panel B shows immunostaining for p62 in a bone biopsy of a patient with classic PDB. Note the high cytoplasmic expression of p62 and localized staining in nuclei (arrows). Staining is only observed in part of the nucleus and not in all nuclei in this cell. It is possible, but as yet not proven, that the inclusion seen in A indeed contains p62 and other components of the ubiquitin proteasomal system.

inhibited is increasingly recognized as a mechanism by which cells protect against the potentially toxic effects of accumulation of misfolded or otherwise dysfunctional proteins. Inclusions may present in different forms: while inclusions seen *in vivo* in patients with PDB, FEO, sIBM and IBMPFD are generally highly organized filamentous structures (nuclear and cytoplasmic, Fig. 3A), in other diseases, or in cell cultures, the inclusions may take the form of aggresomes, electron dense cytoplasmic structures resembling precipitated protein, surrounded by a cage of intermediate filaments. A final mechanism by which cells can remove protein is through autophagy, a

process that may be called upon specifically when the ubiquitin proteasome system is impaired (45). This process has recently been found to also involve p62 (45).

We (unpublished data) have recently found high levels of p62 in osteoclasts in bone biopsies of patients with PDB, with clear areas of cytoplasmic and nuclear accumulation (Fig. 3B) and co-localization with ubiquitin and proteasomal subunits, suggesting that osteoclasts, like other cell types, have a nuclear and a cytoplasmic proteasome. In addition, these findings suggested that light microscopic "aggregates" may indeed be the same

structures seen as "inclusions" by electron microscopy (Fig. 3, compare A and B), providing further evidence to suggest that inclusions are similar between the various inclusion body diseases. Technically demanding immuno-EM studies are required to confirm the protein composition of structures that currently can only be identified at the EM level.

At present, it is difficult to reconcile the hyperactive state of the pagetic osteoclasts with the notion that the disease may be caused by deficiencies in the removal of ubiquitinated proteins, since in the other conformational diseases excessive inclusion formation ultimately leads to cell degeneration. In FEO, however, high osteoclast activity in osteolytic lesions is usually followed by a phase in which osteoclasts are absent, presumably through cell death, and it is not uncommon to find active lesions in classic PDB containing osteoclasts with excessive inclusions, many autophagosomes and signs of apoptosis. This may indicate that the hyperactive state is temporary and can be followed by a degenerative state.

Taken together, the recent molecular and histological/ultrastructural findings in pagetic diseases suggest that they share a common pathogenetic mechanism related to protein degradation pathways with other inclusion body disorders. Interestingly, all diseases show pathology in postmitotic cells only and include the only multinucleated cell types in the body: the skeletal muscle cell and the osteoclast. Although NFκB has received the most attention thus far, it remains to be discovered whether this signaling pathway is indeed central to the cellular pathology in the pagetic osteoclast. It is possible, but as yet unproven, that the RANK mutations may activate the ERAD pathway, involving VCP and p62 in protein degradation in the proteasome. The notion that under normal conditions p62 and VCP may act as negative regulators of osteoclast-activation signals is plausible, and could explain how loss-of-function in the UBA domain can lead to a pathological gain-of-function in cellular activity. Questions remain about the way in which mutations in ubiquitously expressed proteins appear to cause such profound cell-specific effects. Animal models for the various genetic forms of pagetic diseases are currently in development and we hope they will display informative phenotypes. In addition, further genes are to be discovered in the remaining susceptibility loci for classic PDB and these may help to link mechanistically the various forms of the disease. The genetic analysis of pagetic diseases thus far has uncovered an unexpected set of new players in osteoclast physiology. Further studies, however complex, are required to make sure this knowledge can ultimately be translated into better treatment, or possibly even prevention, of these diseases.

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