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Paracrine regulation of osteoclast formation and activity: Milestones in discovery

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Abstract

The molecular and physiological mechanisms of control of osteoclast formation and activity have been explained with the discovery of three members of the Tumour Necrosis Factor superfamily. Receptor activator of NF-kB ligand (RANKL) is the type II membrane protein in cells of the osteoblastic lineage which interacts with its receptor, RANK, on hemopoietic precursors to promote osteoclast formation and maintain their viability and activity. The process is further regulated by the decoy receptor, osteoprotegerin (OPG), also produced by stromal/osteoblastic cells, and which binds to RANKL to prevent RANKL stimulation of osteoclast formation. These discoveries fulfilled predictions that came from more than 20 years of research in bone cell biology in predominantly rodent systems. The hypothesis that the osteoblast lineage directed osteoclast function introduced the concept of intercellular communication in bone. It needed new methods to be developed to test it, and there were many who contributed to this. With a number of identifiable milestones from the early 1980s on, a highly convincing case was made for the existence of what turned out to be RANKL and RANK. As it happened, OPG came first, and the background biological information was so instructive that it was obvious that OPG would lead to the final answer. By that stage the necessary methods were all in place, and in a short time all the key molecular regulators were identified. Ultimate proof of their physiological importance came from genetic experiments in mice.

Keywords: TNF Ligands and Receptors, RANKL, Osteoprotegerin, Bone Homeostasis

Introduction

The cells responsible for bone resorption were identified by Kölliker[1] who named the multinucleated cells he observed on bone surfaces as "ostoklasts", and suggested that they were responsible for bone resorption. Ultimately known as osteoclasts, they were recognised to possess unique ultrastructural characteristics which both distinguished them from other cell types and enabled them to be motile and efficiently resorb bone[2,3]. Apart from their multinuclearity, a striking feature of the osteoclast is the presence of the "ruffled border", which is a complex structure of deeply interfolded finger-like projections of the plasma and cytoplasmic membranes adjacent to the bone surface, through which

bone-resorbing acids and enzymes pass[4-6]. Adjacent to and surrounding the ruffled border is the clear zone. This is an area of cytoplasm devoid of cellular organelles except for numerous cytoplasmic actin filaments. The clear zone is also known as the "sealing zone", since the plasma membrane in this region comes into very close apposition with the bone surface to ensure osteoclast attachment, and to separate the bone-resorbing area beneath the ruffled border from the unresorbed area, which maintains a favourable microenvironment for bone resorption[2,6]. Osteoclasts bring about dissolution of bone mineral by creating an acid microcompartment under the ruffled border, adjacent to the bone surface[7].

They are rich in tartrate-resistant acid phosphatase (TRAP), which is a commonly used histochemical marker for osteoclasts, although not exclusive to those cells. It is nevertheless a convenient marker for *in vitro* generated cells when combined with identification of calcitonin receptors[8] and the ability to form resorption pits when grown on thin slices of cortical bone or dentine. Some other properties are indicated in Figure 1, including possession of vitronectin receptors, cathepsin K, vacuolar ATP-ase, and chloride-7 channels. This combination of properties provides the phenotype that equips osteoclasts uniquely to resorb bone.
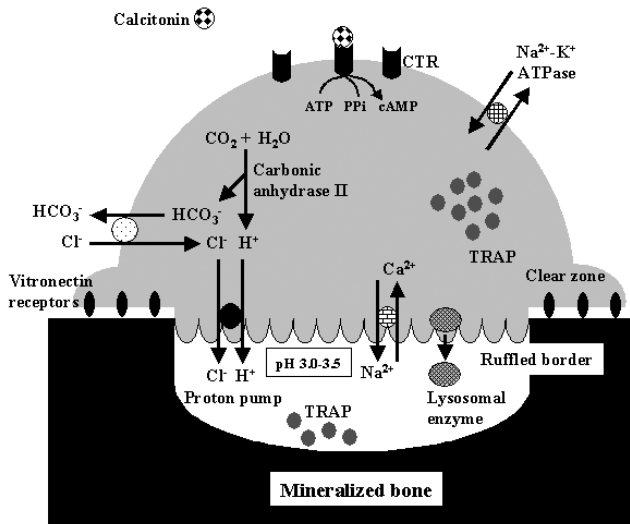
Figure 1. Morphological features, and some of the enzymes and exchange systems involved in osteoclast function.

Origin of osteoclasts

Autoradiographic evidence led Tonna[9] to conclude that osteoclasts arise from fusion of osteoblasts and that osteoclasts can dissociate again into osteogenic precursor cells. Young[10] believed that osteoclasts and osteoblasts originate from the same progenitor cell, the osteoprogenitor cell, and at a later stage may return to the osteoprogenitor pool. In 1974, Rasmussen and Bordier[11] proposed that endosteal mesenchymal cells differentiate into pre-osteoclasts which may then form an osteoclast by fusion. At a certain time and place the osteoclast then dissociates into pre-osteoblasts, giving rise to osteoblasts and osteocytes. These views of a connective tissue cell origin of osteoclasts were subsequently superseded in the face of compelling evidence for a hemopoietic origin of osteoclasts.

Studies using a variety of model systems including quail-chick chimera experiments, parabiosis experiments and the restoration of bone resorption in osteopetrosis by bone marrow and spleen cell transplantation, showed that osteoclasts are supplied to bone via the circulatory system, and are formed by fusion of mononucleated precursors derived from hemopoietic progenitor cells[3,12-15]. The important finding common to these experiments was that the precursors of the osteoclast could travel via the blood to an area where osteoclasts were needed, whereas osteoblasts were recruited from local precursors. This suggested that local precursors could not differentiate into osteoclasts and consequently that the lineages of osteoblasts and osteoclasts are different[15]. Although such experiments did not show definitively that the osteoclast is derived from the hemopoietic stem cell, since bone marrow is diverse and contains stromal cells in addition to hemopoietic cells, the accumulated evidence strongly suggested that the osteoclast is derived from the fusion of mononucleated precursors of hemopoietic origin.

Regulation of osteoclast development

The life span of multinucleated osteoclasts *in vivo* appears to be up to two weeks, with a half-life of around six to ten days[16], thus they need to be replenished continuously for bone resorption to be maintained. Osteoclast formation is a multistep process involving proliferation and recruitment of osteoclast progenitor cells, differentiation of these progenitors and fusion of mononucleated osteoclast precursors to form multinucleated, mature osteoclasts. Many hormones and cytokines are involved in their development. Since osteoclasts are ultimately derived from hemopoietic stem cells, it is not surprising that the colony-stimulating factors and cytokines known to promote and co-ordinate hemopoietic progenitor cell proliferation and differentiation play a very important role in the formation and/or activation of osteoclasts also.

In the development process, progenitor cells are capable of proliferation but not self maintenance and have restricted differentiation capacities, often committed to a single line of differentiation. Osteoclast progenitors are cells without readily recognisable characteristics, which do not express acid phosphatase activity *in vivo* and are very sensitive to irradiation. It is thought that osteoclast progenitors are promonocytes or monoblasts[17]. Osteoclast precursors, or pre-osteoclasts, are mononucleated, post-mitotic cells which may be separated on the basis of enzyme activity into early precursors, which are TRAP-negative, and late precursors, which are TRAP-positive[18]. Pre-osteoclasts also possess increasing numbers of CTRs[8]. In the final step, multinucleated osteoclasts consist of fused mononucleated pre-osteoclasts, which may fuse with one another or with existing osteoclasts. In addition, existing multinucleated osteoclasts may fuse with each other to form even larger cells. Mature osteoclasts express TRAP and, alone among the monocyte/macrophage series, possess numerous CTRs[8].

Cells of the osteoblast lineage produce stimulator(s) of osteoclast activity

The discovery in the late 1990s of the crucial physiological roles of TNF ligand and receptor family members in the physiological control of osteoclast formation and activity found its origin in concepts developed a little over 20 years ago, with the hypothesis that cells of the osteoblast lineage are responsible for directing the process.

The observations that isolated osteoblasts of various origins responded to bone-resorbing hormones and possessed receptors for these factors, in addition to the lack of evidence demonstrating receptors or direct responses to these hormones in osteoclasts, led to the concept that bone-

resorbing factors must act firstly indirectly on osteoblasts, most likely bone lining cells. This was proposed to release factors that influence the bone-resorbing activity of osteoclasts[18,29]. Furthermore, since osteoclasts are derived from hemopoietic progenitors and not from a local bone cell, Chambers[21] came to the same conclusion for other reasons. He argued that since the osteoclast derives from a "wandering" cell, it made sense to have its activity programmed by an authentic bone cell, i.e., the osteoblast.

In the next few years, evidence was gathered from many laboratories in support of this idea of an osteoblast-derived bone resorption-stimulating factor. Chambers developed the method of growing cells isolated from long bones of newborn rat (or occasionally mouse) on slices of bovine bone or dentine for up to 24 hours, and measuring the number and/or areas of resorption pits that were generated by the isolated osteoclasts[22-27]. Dose-dependent stimulation of resorption could be achieved with treating agents such as PTH, $1.25(OH)_2$ vitamin D, prostaglandins etc., and this assay system was considered to be one in which osteoclast activation was being measured. Other laboratories used this method successfully also[28,29], but the cells isolated in this way from newborn bone were very heterogeneous in content, a feature compounded by the fact that different investigating laboratories varied in their approaches to isolation. Some chopped the newborn rodent bones finely, some split them open, washed the marrow out and scraped the endosteal surfaces, yet others removed the cells by fluid shear. The various methods ensured great variation in the level of "purity" of these isolated osteoclast cultures. An approach introduced by Chambers[30] was to wash the cultures thoroughly after they had been allowed a short time (15–30 mins) to settle, and to compare the responses of these cultures to those that were allowed a long time to settle, and therefore had a much higher level of contamination with other cells - osteoblasts and fibroblasts predominantly. The short settlement cultures were regarded as "functionally pure", since they showed no increase in resorption when treated with the resorbing agents such as PTH etc., whereas all of these treatments were effective in the more heavily contaminated cultures. This provided clear evidence that osteoclasts, in order to respond to resorbing agents, needed the presence of other cells - in this case presumed to be osteoblasts.

In some of these early studies of "osteoclast activation", osteoblasts were found to release a factor(s) into the culture supernatant, activating osteoclastic bone resorption in response to stimuli such as $1.25(OH)_2D_3$ and PTH[24,31]. No progress was made towards isolating an active principle from conditioned medium, and it is possibly explained by the presence in conditioned medium of growth-promoting activity capable of increasing the number of non-osteoclasts in the cultures during the experimental period. That possibility raised a technical issue that bedevils this experimental approach – was this assay simply an osteoclast *activation* assay? In some circumstances it clearly was not, in that osteoclast numbers increased if the numbers of non-osteoclasts were too great, either because of the method used in isolation, or because the culture period was too long. This is illustrated best in studies of prolonged cultures of "isolated" rat osteoclasts, growing on bone for several days[32]. Osteoblast numbers increased 3-fold from 24 to 48 hours, and cultures in which osteoclastic resorption was not responsive to PTH within the first 24 hours, became responsive thereafter, as a result of continuing increases in osteoclast numbers after 24 hours. We considered it possible therefore, that even in cultures of less than 24 hours, the varying methods used to prepare freshly isolated rodent osteoclasts could yield osteoclasts at different stages of maturation, as well as different numbers of osteoblasts[33]. We therefore questioned the use of this as an assay purely of osteoclast activity unless meticulous care were to be taken, and suggested that in many cases it would yield data that reflected osteoclast formation, but taking place in a culture system much more difficult to standardise than the co-cultures to be reviewed below.

It is easy to make such critical comments in retrospect, but there is no doubt that these experiments did much to strengthen the view that the cells of the osteoblast lineage did indeed promote osteoclast activity and hence the concept at that stage of "osteoclastic resorption-stimulating activity (ORSA), which owes much in its development to the work of Chambers. Perhaps the most significant observation made in the course of those experiments, not recognised as such at the time, but clearly so with hindsight, was that contact with osteoblasts led to osteoclast activation in culture. Calcitonin, in picomolar concentrations, induced a state of immotility in osteoclasts which was demonstrable within a few minutes of its administration[22], but direct contact with osteoblasts released osteoclasts from this quiescent state within ten minutes of contact, a response requiring cell-cell contact, since separation of the two cell types by a filter failed to induce this escape. That observation can readily be explained now by the action of RANKL upon osteoclast activity.

Overall, the concept of the existence of an osteoclast resorption stimulating activity took us some steps further in understanding osteoclast regulation, but important questions remained by the mid-1980s. Did a single stromal/osteoblastic cell factor exist which is responsible for osteoclast activation, and if so is it cell-associated or secreted? Would the formation of osteoclasts be at least as important as regulation of their activity? The next real advances came with the development of methods to study osteoclast formation *in vitro*.

Contact-dependent stimulation of osteoclast formation by osteoblastic cells

Several *in vitro* systems provided strong evidence that accessory cells are necessary for the generation of osteoclasts from hemopoietic precursors. Burger et al.[34], using a co-culture system in which hemopoietic cells from embryonic mouse liver were co-cultured with fetal long bone rudiments from which the periosteum had been stripped, showed
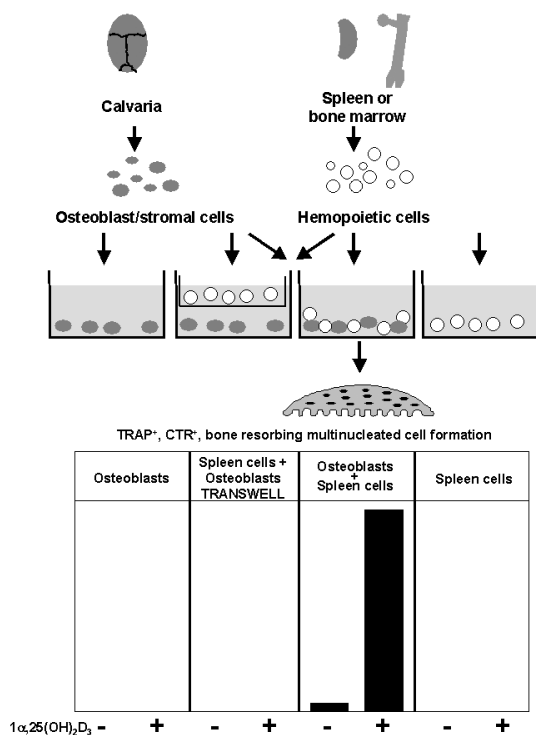
Figure 2. Summary of the approach and data in Takahashi et al.[37]. Osteoclasts were formed in co-cultures of spleen cells with calvarial osteoblasts only when the two cell types were grown on the same surface and treated without being separated by a filter.

that living bone cells are required for osteoclast development. However, it was the development of murine bone marrow cultures that led to major advances, with reproducible assays of osteoclast formation[35-38]. These were used first to show that treatment with bone resorbing agents such as 1.25(OH)$_2$ vitamin D could promote osteoclast formation in a dose-dependent manner, with osteoclast quantitation carried out by counting TRAP-positive multinucleated cells that were also CT receptor positive by receptor autoradiography. In the course of these studies, Takahashi et al.[35] made an observation that turned out to be a crucial one. They noted consistently that more than 90% of the TRAP-positive mononucleated cell clusters and multinucleated cells formed in mouse marrow cultures in response to bone resorbing stimuli were located near colonies of alkaline phosphatase-positive mononucleated cells (possibly osteoblasts). This led them to the idea that osteoblastic cells are involved in osteoclast formation, in addition to the evidence produced in the few earlier years of their influence on osteoclast activity. They set out to determine whether close contact between osteoclast progenitors and osteoblastic cells was necessary in order for osteoclast formation to occur.

They did so and established beyond doubt that osteoclast formation requires a contribution from cells of the osteoblast lineage. In doing this, they provided the concepts and tech-

niques that set the scene for the discovery of osteoclast control by RANKL, RANK and OPG. Their first, relatively simple experiment was remarkably informative. Takahashi et al.[36] prepared osteoblast-rich cultures from newborn mouse calvariae and grew them in co-culture with mouse spleen cells, and on treatment with 1.25(OH)$_2$D$_3$, osteoclasts were formed. Most importantly though, separation of osteoblastic cells from spleen cells by a 0.45 μm membrane filter in co-cultures prevented osteoclast-like cell formation[37], indicating that direct contact is required between the two cell types in order for osteoclast formation to occur (Figure 2). Similar results were obtained with the bone marrow-derived stromal cell lines MC3T3-G2/PA6, ST2 and KS-4[39,40], any of which could be substituted for primary osteoblastic stromal cells in co-cultures with spleen cells, to result in the formation of osteoclast-like cells in the presence of 1.25(OH)$_2$D$_3$. Importantly, those studies highlighted the fact that the ability to promote osteoclast formation was a property of the osteoblast/stromal lineage, and not one which would ever have been applied to mature, bone-forming osteoblasts. A later illustration of this point came from the finding that genetic ablation of mature osteoblasts in the mouse had no influence on the ability of the mice to form osteoclasts[41].

Studies of osteoclast generation in mouse cells from several laboratories used convincing criteria to characterise the multinucleated cells formed in culture as authentic osteoclasts[35,42,43]. These criteria included TRAP staining, CT receptors and the formation of resorption pits on thin slices of bone or dentine. Although Kurihara et al.[44] proposed that osteoblastic stromal cells are not required for osteoclast differentiation, authentication of the osteoclast-like cells formed in that study was not as rigorous, and the weight of evidence at that time indicated that osteoblastic stromal cells are necessary for the process of osteoclast formation from hemopoietic precursors.

How hormones and cytokines influence contact-dependent regulation of osteoclasts by osteoblastic cells

With increasing acceptance of the concept that cells of the osteoblast lineage control osteoclast formation and activity by a contact-dependent mechanism, it was important to understand how this process was regulated. Prostaglandin(PG)-induced osteoclast formation in mouse bone marrow cultures was found to be mediated by a mechanism involving cAMP[45]. The potencies of the PGs in this respect was greatest for PGE$_1$ and PGE$_2$, followed by PGF$_{2\alpha}$, which correlated closely with their relative potencies in increasing cAMP production in osteoblastic and bone marrow cells and in increasing bone resorption in organ culture[19]. Likewise, PTH and PTHrP, acting through their common receptor, promoted osteoclast formation in marrow cultures by a cAMP-dependent mechanism[46], and the effect of interleukin-1 (IL-1) resulted from the generation of PGE$_2$ as an intermediate effector[47].
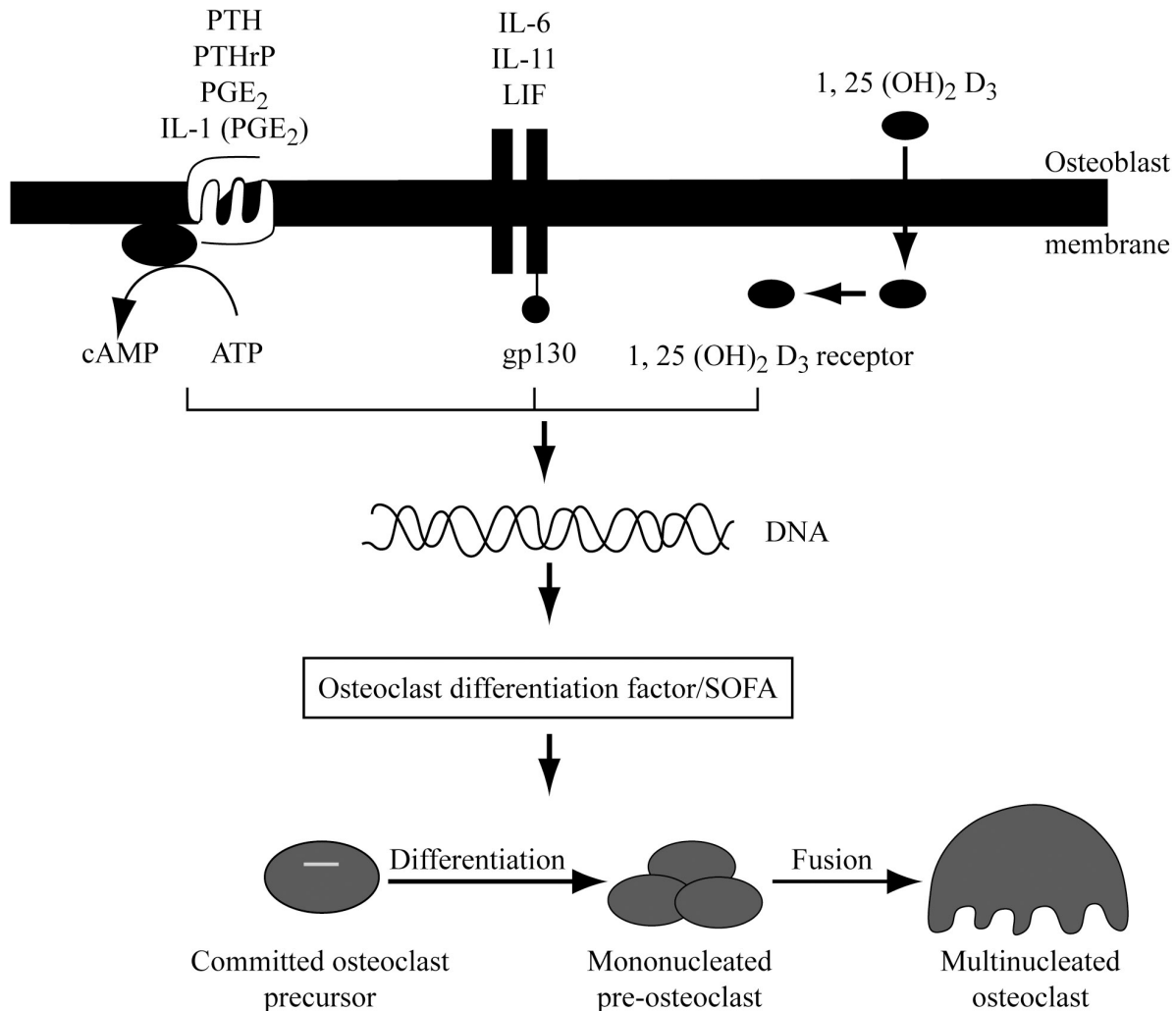
Figure 3. The stimulators of osteoclast formation are all predicted to promote formation of ODF/SOFA, but they fall into three distinct classes as defined by their signalling mechanisms. It was proposed that their actions converged in the nucleus to achieve synthesis of the osteoclast regulator molecule.

A second signalling mechanism for regulation was provided by the steroid hormone, $1.25(OH)_2D_3$, which had very similar effects on osteoclast formation in marrow cultures and in co-cultures of osteoblastic with hemopoietic cells[37]. $1.25(OH)_2D_3$ uses an entirely different signalling system, in which it combines with its receptor and translocates to the nucleus to influence transcriptional events.

Finally, a membrane bound receptor complex involving a 130 kDa glycoprotein (gp130)[48] provides for osteoclast formation under the influence of the group of cytokines that use this signalling mechanism. In mouse co-cultures, simultaneous treatment with IL-6 and its soluble receptor (sIL-6R) induced osteoclast formation, but when added separately they were ineffective[48]. The other cytokines in this group, IL-11, leukemia inhibitory factor (LIF) and oncostatin M (OSM), all of which use gp130 as a common transducer, also stimu-

lated osteoclast formation[48]. In following up this observation, using cells from IL-6R-overexpressing transgenic mice in crossover cocultures with cells from wild-type mice, expression of IL-6R by osteoblastic cells was shown to be indispensable for the induction of osteoclasts[49]. This clear demonstration that IL-6 stimulation of osteoclast formation required the cytokine to act upon the osteoblast, despite the fact that osteoclasts possessed its receptor, illustrated the power of using *ex vivo* experimentation with cells from genetically modified animals to study osteoclast formation.

Thus, the concept of stromal/osteoblastic regulation of osteoclastogenesis was firmly established, and its regulation by a number of circulating and local factors. Despite the fact that they fell into three main classes with respect to their initial signalling mechanisms (Figure 3), it seemed that a common pathway for these agents was the membrane stromal factor called

variously "stromal osteoclast-forming activity" (SOFA)[50] or "osteoclast differentiation factor (ODF)[38]. It was assumed that these agents must converge in their actions at some stage before finally generating the crucial membrane factor[33,51].

Were there candidate molecules for ODF/SOFA?

Overwhelming though the evidence was that such a molecule existed, there were no credible candidates before the discovery of RANKL. In particular, of the many multifunctional cytokines that had some role in osteoclast formation, none fulfilled the requirements. One variant of osteopetrosis resulted from a mutation in the coding region of the M-CSF gene in the *op/op* mouse[52,53], and M-CSF was found to play a role in both proliferation and differentiation of osteoclast progenitors[54]. On the other hand M-CSF inhibited the bone resorbing activity of isolated osteoclasts[55], and osteoclasts were found to be rich in M-CSF receptor[56]. Bone resorption in organ culture was reduced by M-CSF, GM-CSF and IL-3[57], and all three cytokines inhibited the generation of osteoclasts in mouse bone marrow cultures. The conclusion from these and other observations was that none of these hemopoietic growth factors fulfilled criteria expected of one which is specific for osteoclast formation, and certainly not the predicted ODF/SOFA.

Identification and isolation of an "osteoclast colony-stimulating factor" was claimed[58], but the biological assay used in that isolation work was the mixed bone marrow culture system, containing both stromal and hemopoietic elements. The material isolated therefore had no actions which could distinguish it from any of a number of cytokines and hormones known to be capable of promoting osteoclast formation with the mediation of stromal cells/osteoblasts. No evidence was provided in that or in subsequent work from the same group that the isolated factor could promote osteoclast formation from purely hemopoietic cells. Indeed Lee et al.[59] found it to promote formation of TRAP-positive cells from bone marrow cells cultured in agar, as did IL-3 and stem cell factor, results similar to those of Kurihara et al.[44], using spleen cells from 5-FU-treated mice. On the other hand, when strict criteria for osteoclast identification were used, none of the CSFs were able to induce osteoclast differentiation in semi-solid cultures of mouse bone marrow cells.

A clue that might have been helpful had it not been overtaken by subsequent events was the evidence that the c-*fos* gene product is required for osteoclast development[60]. Spleen cells from c-*fos*-/- mice were incapable of forming osteoclasts when co-cultured with normal osteoblasts, but could be rescued by infection with a c-*fos* expressing retrovirus. This indicated that c-*fos* had a part to play in the cascade that followed ODF signalling and resulted in osteoclast formation. This subsequently proved to be so, with the discovery of the essential role of c-*fos* in RANKL action[61].

Although the hypothesis was compelling, there were no further major advances by the mid-1990s. Some groups tried to identify ODF/SOFA using methods of differential gene display. These efforts were not successful, although on occasions resulting in discovery of interesting new regulators of osteoclast formation, as we did with IL-18[62]. As is so often the case, the answer came as a result of a mixture of hard work with the methods already available, as well as with some degree of serendipity. Four groups independently cloned the molecule known now as RANKL, and it is instructive to review briefly how each group arrived at that point.

Final steps in the discovery process; the TNF ligand-receptor superfamily

Each of four research groups arrived independently and at about the same time at the identification and cloning of RANKL. Two of these groups in the final stages of their work had the specific aim of identifying the long sought-after membrane promoter of osteoclast formation. The other two were immunology groups who in studying the T cell-dependent immune response, identified RANKL in the process, but only subsequently became aware of its role in bone.

The group at the Snow Brand Milk Products Company, Japan, had found that a human embryonic lung fibroblast cell line IMR90, secreted into the medium an activity that inhibits osteoclast formation in mouse marrow culture. They saw this as an opportunity to identify a key player in osteoclast control, which they began to call "osteoclastogenesis inhibitory factor (OCIF)", and set out to purify it. The bioassay they used to monitor purification, i.e., inhibition of osteoclast formation in mouse bone marrow cultures treated with $1.25(OH)_2D_3$, was technically demanding and time-consuming, with a very slow turnaround time (greater than 7 days). None of these features was favourable for protein purification, and the fact that they succeeded in purifying and sequencing the heparin-binding protein, OCIF[63], must be regarded as an outstanding technical achievement. Using this sequence they cloned OCIF and soon showed that its cDNA sequence was identical with that of OPG[64], which had been cloned by Simonet et al.[65] (*v infra*) as a novel member of the TNF receptor family.

Because OCIF/OPG strongly inhibited osteoclast formation in co-cultures or marrow cultures treated with $1.25(OH)_2D_3$, PTH, or IL-11, it seemed to them evident that OCIF would achieve its inhibition of osteoclast formation by binding to the responsible effector molecule, i.e., ODF/SOFA. They had the means at their disposal to address this question, knowing that certain mouse marrow stromal cells would be expected to express ODF/SOFA strongly on the cell surface when given appropriate stimuli, the most effective of which would be combination of dexamethasone and $1.25(OH)_2D_3$[38]. They used expression cloning of the ligand for OCIF/OPG with a cDNA library of mouse ST2 cells that had been treated in this way, and identified a cDNA encoding a 316 amino acid type II transmembrane protein of the TNF ligand family[66]. Expression of the protein confirmed its ability to promote osteoclast formation.

A different path was followed by the Amgen group. In the course of a fetal rat intestine cDNA sequencing project they noted an underlined expressed sequence tag (EST) with features suggesting that it might be a member of the TNF receptor family, based on known domain structures[67]. This was confirmed when a full length clone was prepared and sequenced, revealing a 401 amino acid glycoprotein with features of a secreted member of the TNF receptor family[65]. Being in the happy position of being able to have transgenic mice generated when new molecules of sufficient interest were discovered, they did this, and found that hepatic expression of the novel protein yielded mice that survived with profound osteopetrosis. This they showed to be due to inhibition of late stages of osteoclast differentiation, and furthermore, recombinant protein inhibited osteoclast formation *in vitro* and increased bone density when administered to normal mice. They named the protein "osteoprotegerin (OPG)", and they too recognised that it could provide a crucial approach to unravelling the molecular mechanisms of control of osteoclast formation.

Using recombinant OPG-Fc fusion protein as an immunoprobe, they identified a mouse myelomonocytic line that expressed on its surface a molecule which could be readily detected. An expression library prepared from these cells was constructed and screened for binding in pools of transfected COS7 cells. A single plasmid clone was identified, and when expressed, gave rise to on OPG-binding protein on the surface of the expressing cells. They called this 316 amino-acid protein OPG ligand (OPGL), and showed that there was 87% conservation between mouse and human protein sequences[68]. OPGL was able to promote osteoclast formation from hemopoietic precursors in the presence of M-CSF, and to stimulate bone resorption and elevate the blood calcium levels when administered *in vivo*[68].

The publication by Simonet et al.[65] of the identification of OPG was a landmark event in the field, but although Tsuda et al.[62] did not win the publication "race", their independent contributions were equally outstanding. From the ways in which each of these groups discovered OPG/OCIF, and with any appreciation of the concepts that had developed over the previous decade or more of osteoclast control, it was quite apparent that this discovery would prove to be central to completing the picture of the control of osteoclastogenesis.

Remarkably enough, two other groups were successful in identifying and cloning RANKL, each of them[69,70] in fact publishing this work some months before either Lacey et al.[68] or Yasuda et al.[65]. Wong et al.[69] identified and characterized a TNF-related activation-induced cytokine (TRANCE) during a search for apoptosis-regulatory genes in murine T cell hybridomas, finding it to be predominantly expressed on T cells and in lymphoid organs and controlled by the T cell receptor through a calcineurin-regulated pathway. The putative receptor for TRANCE was detected on mature dendritic cells[71]. Wong et al.[69,71] were not aware at that time of any involvement of TRANCE in bone biology, and in their survey of tissue distribution of TRANCE mRNA in mouse tissues, bone was not examined. It might be noted that this omission
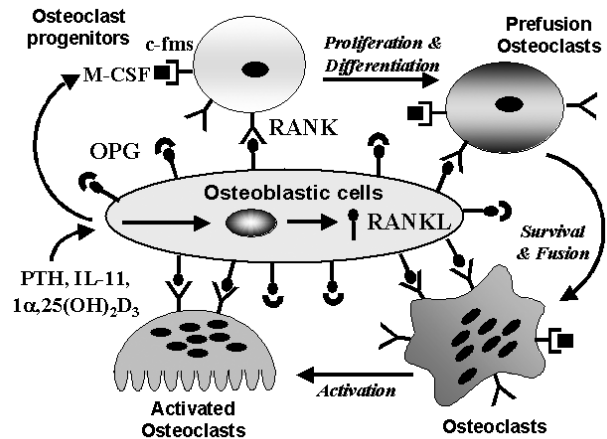

Figure 4. The stimulators of Fig 3 now known to promote production of RANKL, which binds to RANK and promotes osteoclast formation and maintains viability and activity. OPG binds RANKL to limit its activity.

remains the case almost always, when new molecules of whatever variety are discovered, unless it takes place in the context of investigators who have a direct interest in bone.

In studying the processing and presentation of antigens by dendritic cells to T cells, Anderson et al.[70] characterized receptor activator of NF-kB (RANK), a new member of the TNF receptor family derived from dendritic cells, and its ligand RANKL, which they recognized to be identical to TRANCE[69]. A soluble form of RANKL augmented the ability of dendritic cells to stimulate T cell proliferation in a mixed lymphocyte reaction and increased the survival of RANK-positive T cells. Again, Anderson et al.[70] were not aware at the time of their first publication of any link between RANK/RANKL and bone. Interestingly though, the type I membrane protein, RANK, contained four extracellular cysteine-rich domains, as was the case with OPG, published earlier that year[65].

Osteoclast regulation and function

These discoveries filled in the gaps that had been eluding us for many years[72]. The concepts that drove the research to such outcomes had been developed over years of study of bone cell biology, relying virtually entirely on rodent systems, predominantly *in vitro*, but drawing on *in vivo* observations also. The ODF/SOFA hypothesis predicted a control mechanism that was sufficiently important from the evolutionary point of view that it was likely to be highly conserved, and that has certainly proven to be so, both in respect of the overall mechanism and of the conserved sequences of the central molecules. By treating with RANKL and M-CSF it was now possible for the first time to prepare osteoclasts in relatively large numbers without the participation of stromal/osteoblastic precursors, including the preparation of

human osteoclasts from peripheral blood[73,74]. The physiology of the bone resorption regulatory system was in a short space of time laid out before us with convincing evidence of the essential regulatory function of RANKL, not only in promoting osteoclast formation, but also their survival and activity[75], as was predicted from the earlier demonstration of activation of osteoclasts through contact with osteoblastic cells[22,75]. The concepts resulting from these discoveries are summarised in Figure 4.

The most compelling evidence of all came from the validation studies in genetically manipulated mice. Overexpression of OPG resulted in mice with osteopetrosis because of failure to form osteoclasts[65], whereas genetic ablation of OPG led to severe osteoporosis[77,78]. Genetic ablation of RANKL resulted in osteopetrosis because RANKL is necessary for normal osteoclast formation[79]. Genetic ablation of RANK led to osteopetrosis also because it is the receptor for RANKL[80]. Finally, the link to the immune system expressed itself both in the RANKL- and in the RANK-null mice, each of which have severe abnormalities in that system, with failure of lymph node development and impaired immune responses. Other links may emerge of course, since RANKL is produced in many tissues during development and after birth[81].

Nomenclature

The discovery of three new TNF superfamily members that provided the final common pathway to the physiological control of osteoclast formation and activity was certainly a source of great excitement, and provoked a great flurry of research activity – there were many obvious things to do, and some surprises that emerged shortly after the discoveries. Many of these will be discussed in other articles in this issue of the journal.

With such exciting developments, and with the several independent discoveries all taking place at about the same time, and some in the context of a research area other than bone, what resulted was a potentially bewildering array of names and acronyms for these new effectors. The American Society for Bone and Mineral Research took the initiative of setting up a Special Committee on Nomenclature, comprising representatives of the discoverers, a number of scientists with long experience in the field, and editors of endocrine and bone journals. After meeting several times and consulting widely beyond the bone field, the Committee recommended using the names of RANK for the membrane receptor, RANKL for the ligand, and osteoprotegerin (OPG) for the decoy receptor[82].

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