New trends in bone research

ABSTRACT—Recent advances in bone research have employed novel cell and molecular biology techniques to determine some of the fundamental mechanisms regulating bone function. Endocrine control of bone cell development and matrix turnover have been defined at a molecular level by studying the interaction of steroid/thyroid hormones with gene promoters. New steroid/thyroid hormone receptors have been cloned, suggesting that our current view of hormonal regulation of bone metabolism is far from complete. The function of one particular steroid hormone receptor, the vitamin D receptor, has come under close scrutiny following the observation that polymorphic variations in this receptor are linked to differences in bone mineral density. Detailed studies of bone cell differentiation have shown that cytokines may be particularly important targets for hormonal control in bone. The role of cell adhesion molecules in regulating bone resorption has also been explored; modulation of their activity may be of benefit in the treatment of diseases such as osteoporosis. Pharmacological intervention via the newly cloned calcium-sensing receptor offers another site for regulation of bone turnover.

The study of bone metabolism has inevitably been driven by the need to treat bone disease; the use of oestrogens and bisphosphonates as treatment for osteoporosis and Paget's disease represents the fruition of some of these lines of research. The last ten years of research in this field have been characterised by the use of new technology to determine some of the fundamental mechanisms that regulate bone function. Of particular interest is the use of molecular biology to define the gene regulatory pathways which control bone homoeostasis. This article reviews recent observations from these studies and points to some future avenues of work.

Background

The maintenance of skeletal integrity requires coordinated control of bone remodelling, a dynamic process reflecting the equilibrium between bone formation and resorption. Regulation of bone remodelling is mediated by a variety of local and systemic factors which act either directly or indirectly on bone cells. Prominent amongst the circulating agents modulating bone metabolism are the classical bone hormones such as vitamin D, oestrogens, parathyroid hormone (PTH) and calcitonin, although it is now clear that thyroid hormone, glucocorticoids and the retinoids also exert powerful influences on bone function [1]. Much of the interest in endocrine factors has focused on their impact on diseases such as osteoporosis. For example, the uncoupling of bone formation and resorption that leads to osteoporotic bone loss can be counteracted by oestrogen replacement, although as yet the precise nature of this effect remains unclear. Hence, attention has turned to the role of growth factors and cytokines as locally produced regulators of bone turnover [2]. How they act is also still to be fully defined, but it seems likely that they represent a tissue- or environment-specific response to the relatively non-specific changes in circulating hormones.

Successful study of the physiological role of bone-associated factors will, to some extent, depend on better understanding of the activities of bone cells. For example, it will be important to define the differentiation pathways for bone forming (osteoblast) and bone resorbing (osteoclast) cells. Likewise, the interaction between resorbing osteoclasts and the bone matrix surface represents a tempting target for therapeutic intervention, as does the control of bone matrix production by bone forming osteoblasts. Our knowledge of these functions is far from complete but has greatly improved over the last few years, and reflects the diverse approaches to research in this field.

Vitamin D function

The concept of vitamin D acting as a secosteroid was proposed over 20 years ago. Since then a variety of studies has emphasised the endocrine nature of the active form of vitamin D, 1,25-dihydroxyvitamin D_3, (calcitriol) [3]. The most compelling argument for its hormonal activity is the presence in target tissues of high affinity intracellular receptors for calcitriol. Cloning and sequencing the gene for this receptor, the vitamin D receptor (VDR), has highlighted its homology with other members of the steroid/thyroid hormone nuclear receptor superfamily [4] and, in particular, the thyroid and retinoic acid receptors. In common with other nuclear receptors the VDR acts as a ligand-dependent transcriptional regulator [5]. The mechanism of this action has been subject to considerable scrutiny, and has disclosed a variety of genes responsive to calcitriol. These include classical mineral homoeostasis proteins such as PTH and the calbindins, as well as bone matrix components such as osteocalcin and osteopontin. However, it is now evident that calcitriol can influence expression of a much wider range of proteins. Recent studies by Teitelbaum and colleagues [6] have characterised the vitamin D responsiveness of the cell adhesion molecule (CAM)

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$\alpha_v\beta_3$ integrin, which is present on osteoclasts. This molecule appears to play a crucial role in directing osteoclast bone resorption and is therefore a potential target for agents designed to inhibit bone loss. Studies by our group have shown that calcitriol is a potent regulator of leukocyte-specific integrins [7]. This has obvious implications for the well documented interactions of vitamin D with the immune system [8], but may also influence bone cell development as osteoclasts and monocytes have a common haematopoietic lineage.

An important feature of these studies has been the identification of the precise gene promoter sequences which bind the VDR and direct ligand-dependent changes in transcription. In common with other members of the nuclear receptor superfamily, the VDR binds to short nucleotide sequences upstream of the target gene. These sequences, or hormone response elements (HREs), may either be palindromic (inverted repeats) or direct nucleotide repeats, and they provide a degree of hormone specificity which is enhanced by highly selective ligand binding to the receptor. Specific but flexible transcriptional effects are also facilitated by the requirement for dimerisation of steroid/thyroid hormone receptors. Carlberg and colleagues have described VDR homodimer formation [9] but it is evident that the VDR, like the retinoic acid receptor, is promiscuous and interacts with other receptors. Heterodimerisation with the retinoid X receptor appears to be the predominant mechanism for VDR-responsive genes. However, recent reports have highlighted VDR interaction with the thyroid hormone receptor, T_3R, in which hormone-selective responses are directed by the orientation of the dimerisation pattern [10]. Much of this work has been confined to artificially designed HREs but has been strengthened by the isolation and sequencing of additional target genes for calcitriol. Newly characterised promoters include the calcitriol metabolising enzyme 24-hydroxylase [11] and the aforementioned osteoclast integrins [6], both of which appear to be regulated by novel HREs. The mechanism of action of steroid hormones such as calcitriol is thus far more complex than originally thought; indeed, it is probable that additional accessory proteins create further complexity by directing cell-specific responses to calcitriol.

The relevance of these studies to pathophysiological conditions was originally illustrated by the autosomal recessive disorder hereditary vitamin D resistant rickets (HVDRR). Sequence analysis of the VDR gene in HVDRR patients has revealed 12 separate point mutations affecting either the hormone or the DNA binding abilities of the receptor [12]; particularly susceptible are the conserved amino acids of the zinc finger motif which forms part of the DNA binding domain of the receptor. Detection of mutations in this region has opened the way for detailed analysis of the changes in molecular interactions which occur as a result of base-pair alterations [13]. In addition, the severe nature of the rickets associated with this disorder emphasises the impact of abnormal VDR gene expression on mineral homoeostasis.

Gene polymorphisms and bone disease

With the above studies in mind, it has been interesting to consider the recent series of articles describing the use of VDR gene polymorphisms as markers of osteoporotic bone disease. Eisman and colleagues reported VDR genotypes due to polymorphic variations in the VDR gene which correlated with circulating levels of osteocalcin, a known marker of increased bone turnover [14]. Further investigation using monozygotic and dizygotic twin studies indicated that a particular genotype (designated *BB* as a result of *Bsm 1* restriction enzyme digestion pattern) was a reliable predictor of bone density [15]. This was a remarkable observation since almost two-thirds of women who suffer osteoporotic fractures do so as a result of low bone density. Taking into consideration two further polymorphisms (at *Apa 1* and *Taq 1* restriction sites), the authors estimated that as much as 75% of the genetic component of osteoporosis could be predicted from VDR genotyping. These findings have considerable implications in a disease which is under strong genetic control. As discussed previously, the diverse effects of calcitriol on bone and mineral metabolism are highly dependent on expression of a functionally active VDR, and receptor polymorphisms may therefore influence a variety of signal pathways. More specifically, there is some evidence for the successful use of vitamin D as treatment for osteoporosis [16]. Early identification of high risk groups from phenotypic analysis may help to optimise this type of treatment.

Inevitably, there have been reservations concerning the impact of VDR polymorphisms on the development of bone disease. In part, these have come from groups which have not been able to show any correlation between VDR genotypes and bone mineral density [17]; a prominent criticism has been the restricted nature of the population initially studied, which was Caucasian-Australian. However, further investigations have included groups such as a Japanese population from which it was possible to predict bone density using VDR genotyping (although the distribution of polymorphisms within this population was different) [18]. More recently, Eisman and colleagues have also demonstrated an association between VDR genotype and bone mineral density in a population of postmenopausal British women [19]. In addition to continued population studies, an important consideration is the molecular and physiological impact of VDR genotypes on bone function. The polymorphisms occur predominantly in the 3' untranslated region of the VDR cDNA, a region thought to confer messenger RNA (mRNA) stability. Using artificially constructed 3' regions (mini-genes) Morrison *et al* reported that a haplotype of the *Bsm 1, Apa 1* and *Taq 1* polymorphisms was characterised by increased transcriptional

activity or enhanced mRNA stability [15]. It remains to be seen what effects, if any, these variations have on expression of the VDR protein, and, in turn, whether this affects responses to calcitriol. Similarly, in view of the pleiotropic effects of vitamin D, it is probable that polymorphic variations in the VDR will be subject to cell-specific regulatory mechanisms. The identification of other polymorphisms segregating with high or low bone mineral density will no doubt complicate this field still further. Indeed, recent reports have suggested a relationship between the incidence of osteoporotic fractures in postmenopausal women and type I collagen gene polymorphisms [20]. The success of such genotyping studies is highly dependent on their potential as predictors of bone loss. Early detection of subjects predisposed to osteoporosis is likely to lead to better and more varied application of anti-osteoporotic therapy.

Osteoclast function

The question of genetic contribution to bone disease remains a distracting irrelevance for many clinicians and researchers. This is perhaps best understood in view of the fact that our knowledge of the function of bone cells is far from complete, even though they are the principal targets for disease therapy. The regulation of osteoclast and osteoblast function continues to be the prime interest of many groups, and has been complemented more recently by exciting studies of matrix-embedded osteocytes: they include the elegant studies that have sought to define the factors involved in osteoclast differentiation. Following the initial observation that bone matrix components stimulate the release of interleukin (IL)-1 by mononuclear cells [21], Manolagas and colleagues used a variety of bone cells to show that IL-6 was a target for the anti-osteoporotic effects of oestrogen [22]. A central theme of both studies was the proposal that osteoclasts and granulocytes/macrophages arise from a common haematopoietic progenitor. Thus, using cultures of marrow cells from bone tissue, it was possible to assess osteoclastogenesis in animal models of osteoporosis and in human surgical samples. Ovariectomised mice showed substantial increases in the numbers of granulocyte/macrophage colony forming units from bone marrow as well as from spleen. This correlated with greater numbers of osteoclasts per femur and, more importantly, the effect was prevented when the animals were treated with either oestrogen or antibodies to IL-6 [22]. The authors proposed that IL-6 was the causative factor in directing osteoclast formation in oestrogen depleted animals, although results from sham-operated mice showed that the IL-6 neutralising antibodies were without effect on osteoclastogenesis. More recent studies have highlighted IL-11 as the critical cytokine for osteoclast development in oestrogen-replete states [23]. The interrelationship between oestrogen, cytokines and bone resorption forms a potential target for anti-osteoporotic therapy. However, the future development of this avenue of research is likely to be further complicated by investigation of the receptor for IL-6, which has a soluble component.

Another feature of osteoclast function which may provide a target for anti-resorptive agents is the interaction of these cells with bone matrix components. The importance of agents which modulate osteoclast activity was recognised many years ago and led to the development of bisphosphonates as treatment for metabolic bone disease [24]. Bisphosphonates are analogues of pyrophosphates, with poor uptake from the gut but preferential accumulation at the ruffled border, the resorptive membrane of osteoclasts. The stability of bisphosphonates and their local accumulation interferes with osteoclast function and inhibits bone resorption, although their precise mode of action at a molecular level remains to be determined. Bisphosphonate analogues were originally used as treatment for Paget's disease and hypercalcaemia of malignancy but have achieved more recent success as therapy for osteoporosis.

Reservations about the metabolic fate of bisphosphonates and their long-term efficacy have stimulated interest in new antiresorptive agents. In particular, interfering with osteoclast adhesion mechanisms represents an approach to the regulation of osteoclast activity which may be more specific than bisphosphonates. Molecules such as the β_1 and β_3 integrins play an important role in osteoclast adhesion to bone matrices. This mechanism depends on the recognition of short amino acid patterns within extracellular matrix components. Artificial peptides containing these patterns, known as Arg-Gly-Asp (or RGD) peptides, inhibit osteoclast function *in vitro;* for example, echistatin, an RGD-containing peptide isolated from snake venom, inhibits osteoclast activity by antagonising β_3 integrin-mediated functions [25]. Similar effects have been reported for antibodies directed against osteoclastic integrins, further emphasising the importance of CAMs as targets for therapeutic intervention in osteoporosis [26].

A potentially fruitful area for future research concerns the eventual fate of resorbing osteoclasts. It seems likely that cellular regulation via apoptotic mechanisms, which has been widely described for other tissues [27], will also apply to bone marrow cells. Initial studies indicate that oestrogen promotes osteoclast apoptosis *in vitro* [28], but the full implications of these findings have yet to be explored.

Osteoblast function

The relative abundance of osteoblasts in bone tissue and the availability of osteosarcoma cell lines have contributed to better characterisation of bone formation than of resorption. Detailed reviews of osteoblast differentiation pathways and gene regulatory mechanisms have been published [29,30]. Two recent

developments suggest that knowledge of osteoblast function is far from complete. The first is the isolation of new members of the steroid/thyroid receptor superfamily from osteoblastic cell lines, of which one shows strong homology with the retinoic acid receptor, and the other is closely related to the peroxisomal proliferator receptor [31,32]. The receptors were isolated by homology with known steroid/thyroid receptor gene sequences, and neither was expressed exclusively in bone tissue. Their identification suggests that new bone regulatory factors and genes will soon be discovered. Another facet of osteoblast gene function which may help our understanding of the differentiation of these cells has been the description of a homeobox (*hox*) domain in the gene for the non-collagenous matrix protein osteocalcin [33], which is prominent amongst the bone-related genes characterised in osteoblasts. Towler and colleagues recently reported that osteocalcin requires a *hox* domain binding motif for high level activity. *Hox* domains are crucial for coordinated expression of the thousands of proteins required for embryonic development [34]. The presence of such a domain within the osteocalcin gene promoter suggests that this gene is extremely important for skeletal formation.

Parathyroid hormone, parathyroid hormone-related peptide and calcium sensing

PTH regulates serum calcium and phosphate by direct action on bone cells, by modulating renal tubular reabsorption and by stimulating renal production of calcitriol [35]. The overall effect of PTH is to decrease serum phosphate and increase serum calcium. Recent interest in PTH function has centred on the regulation of *PTH* gene expression. Calcitriol, which down-regulates PTH synthesis, interacts with the PTH promoter via a classical VDR-HRE mechanism [36]. In contrast, calcium, which can increase or decrease PTH synthesis, appears to act at a post-transcriptional level [37]. The actions of the PTH protein are mediated by an extracellular receptor which stimulates intracellular accumulation of cAMP and increases intracellular free calcium [38]. This receptor is also specific for PTH-related peptide (PTHrP), which has considerable homology with PTH over the first 34 amino acids. PTHrP was originally identified as the protein responsible for the hypercalcaemia of malignancy [39]. It is expressed in almost all tissues, but its function has yet to be fully defined. Kronenberg and colleagues have made use of mice homozygous for a PTHrP null mutation to highlight the requirement for this hormone in normal skeletal development [40].

The relationship between PTHrP/PTH and calcium homoeostasis has also achieved prominence following the cloning and characterisation of an extracellular calcium-sensing receptor from parathyroid tissue [41]. The receptor has a calcium binding domain linked to a seven-amino membrane-spanning peptide similar to the G protein-coupled receptor superfamily. Mutations in the human calcium receptor are associated with hypercalciuric hypercalcaemia and neonatal hyperparathyroidism [42]. The calcium receptor is obviously important as an extracellular messenger for the control of PTH/PTHrP synthesis. However, several other tissues, such as kidney and bone cells, also have a substantial requirement for calcium sensing. In particular, the action of PTH/PTHrP in stimulating osteoclast activity via indirect actions on osteoblasts may play an important part in the resorption process [43]. Thus, the calcium receptor is yet another potential target for therapeutic agents which not only affect PTH synthesis but may also influence bone function. The development of calcium mimetics to antagonise osteoclast calcium responses is in its initial stages and seems likely to lead to an entirely new field of bone research.

Future avenues of research

It is to be hoped that the diverse and novel approaches to bone research described in this review should continue to reap rewards in the coming years. Techniques such as differential display polymerase chain reaction (PCR) [44], *in situ* PCR [45] and expression cloning (successfully used to identify the PTH/PTHrP receptors and the calcium receptor) will help to identify new bone genes and improve the characterisation of existing bone regulators. Many aspects of bone and mineral homoeostasis are still to be clarified. The imminent identification of the gene for hypophosphataemic rickets may lead to the isolation of a new phosphate regulating factor. It remains to be determined whether or not this is a circulating hormone, as proposed by Kumar and colleagues in their studies of oncogenous osteomalacia [46]. However, the elucidation of this gene will doubtless point to the contribution of phosphate to bone status.

At a cellular level, the role of matrix-embedded osteocytes in regulating bone turnover is likely to receive greater attention. The location of these cells, and their sensitivity to biomechanical stress suggests that they may be crucial to the signalling required for bone maintenance. It is also interesting to note that recent studies have reported the expression of oestrogen receptors on osteocytes, indicating a possible role in the pathophysiology of postmenopausal bone loss [47]. Other aspects of bone function which remain puzzlingly elusive include the cloning of the gene for 1 α-hydroxylase, the enzyme responsible for calcitriol production. The continuing evolution of bone research evident over the last few years suggests that these and other questions will soon be answered.

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