

Bone remodeling, humoral networks and smart biomaterial technology for osteoporosis

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1. ABSTRACT

One of the unfortunate sequelae of increased life expectancy is a growing number of age-related degenerative diseases, a prime example being osteoporosis. This form of metabolic bone disease and related comorbidities consume tremendous resources and costs from a nation's health care system. Osteoporosis results from genetic, age-related, and hormone-dependent causes as well as a compendium of secondary pathophysiological states. The presence of osteoporosis as a comorbidity confers a significant negative prognostic element following orthopedic procedures. *In vitro* and *in vivo* studies of osteoporotic bone implicate microarchitectural bone rarefaction, microenvironmental and functional disturbance of osteoblast-osteoclast coupling, and abnormal tissue and signalling molecule repertoires, each having detrimental effects on the regenerative and osteointegration processes. This review explores the pathophysiology of bone remodeling from a macro- and micro- systems biology standpoint with a focus on cytokine interactions. Furthermore, therapeutic interventions exploiting vulnerable nodes in these physiological networks will be posited. One exciting development in this area is the use of novel biomaterials.

2. INTRODUCTION

Osteoporosis is caused by genetic, age-related, hormone-dependent, or iatrogenic (drug-related) factors as well as a host of pathophysiological states, including malabsorption and chronic kidney disease, and harmful life styles (e.g., smoking and alcoholism). Osteoporosis is associated with increased risk for fracture and also contributes to orthopedic implant or reconstructive surgery failures. As a negative prognosticator for many illnesses, osteoporosis confers significant quality of life compromise, economic burden and excess morbidity and mortality on health care systems globally.

Contemporary innovations for the treatment of musculoskeletal pathology include new surgical techniques, biological therapies and smart biomaterials. These so-called "smart" biomaterials are produced by modifying the physical properties of the scaffolds using peptide sequences, and most importantly, by developing materials that can deliver proteins to enhance tissue regeneration and actively participate in the formation of functional tissue (1, 2). Specifically, biological stimulators, such as cells, growth factors and signaling molecules, alone or in combination with bioactive and biomimetic materials have

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been studied in clinical trials. They have been shown to treat many traumatic or degenerative pathologies, such as fractures, congenital skeletal malformations, cartilage or ligament lesions, osteoporosis, and other bone remodeling disorders.

Biomaterials that are successfully cultured with healthy bone cells show reduced osteoconductive properties when cultured with pathological bone cells isolated from osteoporotic bone (3, 4). *In vitro* and *in vivo* studies of osteoporotic bone implicate microarchitectural bone rarefaction, microenvironmental and functional disturbance of osteoblast-osteoclast coupling, and abnormal tissue and signalling molecule repertoires, each having detrimental effects on the regenerative and osteointegration processes. It is proposed that achieving some threshold resonance between biological interventions and the complex interactions of humoral regulatory factors in the skeleton is necessary for therapeutic success (5).

Unfortunately, comorbid and patient idiosyncratic factors can mitigate the utility of biological therapies and biomaterials. What are these precise mitigating factors? How do they interact with physiological networks involving bone? What fashion can therapeutic skeletal interventions be tailored to optimize outcome?

This article will address these questions. First, bone remodeling physiology will be reviewed with an emphasis on humoral (cytokine and hormonal) regulatory networks. Then, the pathological state in osteoporosis will be discussed setting the stage for potential biological interventions. Third, biological therapies and various biomaterials tailored to exploit vulnerable nodes in the systems physiology of bone will be explored.

3. PHYSIOLOGIC BONE REMODELLING

3.1. General aspects

Bone is a dynamic tissue. It requires continuous remodeling to supply and fulfill structural and metabolic functions. There are several key physiological functions that bone remodeling subserves. Generally considered as the most important, bone provides the internal mechanical support of the body and anatomically, the sites of tendon and muscle attachment for locomotion. Accordingly, bone protects vital organs body and encases the bone marrow. However, bone also plays an important metabolic role. Bone is the main store of inorganic ions, calcium (Ca) and phosphorus (P) principally, and it is involved in mineral homeostasis (6-8). Additionally, recent evidence now implicates bone as a source of humoral factors that can affect pancreatic beta-cell function and insulin secretion, and via complex networking, other organs participating in the control of body composition and intermediary metabolism (9, 10).

The dynamic nature of bone was firstly proposed in 1892 and is widely referred as Wolff's law: "As bones are subjected to stress demands in weight bearing posture, they will model or alter their shape accordingly" (11). In other words, even though skeletal mass and histomorphology are well defined, bone will remodel in

response to mechanical forces, biochemical changes, and biological signals.

Approximately 10% of the skeleton is renewed each year by remodelling (12). This process is necessary to repair skeletal microfractures of the skeleton, prevent accumulation of older and weaker bone, and maintain mineral homeostasis. This process is complex and characterized by the coordinated actions of osteoclasts and osteoblasts, organized in basic multicellular units (BMU) that follow a cycle of resorption, reversal, formation, and resting phases. Remodeling begins with the migration of partially differentiated mononuclear pre-osteoclasts to the bone surface where they form multinucleated osteoclasts (13,14). The signal that initiates the resorption phase is thought to be the mechanical stress that alters local bone architecture and is sensed and transduced by osteocytes (15). After osteoclastic resorption, which takes about 2 weeks, there is a reversal phase when mononuclear cells on the bone surface provide signals for osteoblast differentiation and migration. The reversal phase lasts up to 4-5 weeks. In the formation phase, osteoblast lay down bone until the resorbed bone is completely replaced by new bone; this phase may last for about 4-6 months. Eventually, bone surface is covered with flattened lining cells and a resting period follows (16). This dynamic process occurs in BMUs at multiple sites simultaneously throughout the skeleton and is a localized rather than a systemic process (17).

3.2. Systemic regulation: the role of hormones

There are several control points that are susceptible to systemic and local humoral factors and produce a specific skeletal phenotype (6). Direct systemic regulation of bone remodeling is carried out by various hormones. These include, but are not limited to, parathyroid hormone (PTH), calcitonin (CTN), insulin, insulin-like growth factors-1 and -2 (IGF-1 and -2), growth hormone (GH), pituitary glycoproteins (thyroid stimulating hormone [TSH], follicle-stimulating hormone [FSH]), glucocorticoids (cortisol), sex steroids (androgens and estrogens), thyroid hormones and 1,25-dihydroxyvitamin D (125-D) (13).

PTH is the primary regulator of calcium homeostasis. It maintains serum calcium concentrations by stimulating bone resorption, facilitating renal calcium absorption, and increasing renal production of 125-D. PTH stimulates bone formation when given intermittently and bone resorption when secreted continuously (18, 19).

CTN, a natural product of C-cells in the thyroid gland, in pharmacologic doses, is a potent osteotropic factor which blocks basal osteoclast activity by mediating loss of the osteoclast ruffled border, by retraction of cytoplasmic extensions and decreased motility, and secretion of proteolytic enzymes through its receptor on osteoclasts (20). Under normal physiological conditions, CTN exerts little regulatory control over bone remodeling.

The GH/IGF-1 and -2 systems are important for skeletal growth, especially at the cartilaginous end plates

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and during endochondral bone formation. They are among the major determinants of adult bone mass through their effect on regulation of both bone formation and resorption (21).

125-D, the active metabolite of vitamin D, is essential in enhancing the absorption of Ca and P from the gastrointestinal tract, and in this way, it promotes bone mineralization. 125-D also regulates renal Ca handling and can affect PTH secretion. In addition, 125-D possesses important anabolic effects on bone, thus exerting a dual effect on bone turnover (6).

Glucocorticoids exert both stimulatory and inhibitory effects on bone cells. They are essential for osteoblast maturation by promoting their differentiation from mesenchymal cells but they also decrease osteoblast activity. Furthermore, glucocorticoid excess sensitizes bone cells to other regulators of bone remodelling and augments osteoclast recruitment (22, 23).

Estrogens prevent osteoclast formation and reduce osteoclast lifespan. They stimulate bone formation by enhancing osteoblast proliferation and by decreasing apoptosis. Estrogens affect gene expression for bone-specific enzymes, bone matrix proteins, hormone receptors, transcription factors, and they also up-regulate the local production of osteoprotegerin (OPG), IGF-1, IGF-2, and transforming growth factor-beta (TGF-beta) (24). Androgens are essential for skeletal growth and maintenance via their anti-resorptive effect via androgen receptor signal transduction, which is present in all types of bone cells (25).

The remodeling process is also influenced by the hypothalamic-pituitary-thyroid (HPT) axis. Osteoclast activity is increased by thyroid hormone but only through coupling with osteoblasts, which is mediated by cytokine signaling (interleukin-6 (IL-6), IL-8, prostaglandin E2, and IGF-1) (26). In addition, thyroid-stimulating hormone (TSH) reduces the formation, function and survival of osteoclasts and thus exerts an anti-resorptive effect. After ovariectomy, TSH can prevent bone loss and even restore lost bone (27). Thyroid hormone excess opposes the effects of TSH in bone remodeling and can cause secondary osteoporosis and fracture (28-33). Even when the effects of suppressed TSH occur with elevated thyroid hormone levels, there is increased bone turnover and decreased bone mass (34).

3.3. Local regulation: the role of cytokines

The role of cytokines as intermediaries in the control of bone remodeling stems from the observation that mature osteoclasts lack receptors for most hormone regulators, namely PTH and 125-D (35). This premise was validated by identification of the receptor activator of nuclear factor κ B ligand (RANKL; a cytokine) as the principal intermediary of osteoblast-osteoclast coupling via binding with receptor activator of nuclear factor κ B (RANK), which is highly expressed on the osteoclast membrane (36).

Cytokines are most often defined as a group of regulatory factors with the following attributes:

- low molecular weight (<80 kDa),
- very potent (acting in the 10^{-13} – 10^{-10} M range),
- generally glycosylated peptides,
- produced transiently by most nucleated cells,
- manifest pleiotropic, mainly autocrine or paracrine, overlapping biological activities.

Even though cytokines are primarily involved in the inflammatory response, they provide integration between immune and endocrine systems in many tissues or organs (25). The interconnected network between the skeletal and immune systems is referred to by the emerging interdisciplinary research field of osteoimmunology (37, 38).

Activation of the remodeling cycle starts with osteoblastic degradation of unmineralized osteoid between the osteoblastic cell layer and the mineralized bone. This is necessary since osteoclasts cannot adhere to unmineralized bone (12, 13). Then, osteoblasts increase their expression of RANKL and macrophage colony stimulating factor (M-CSF). M-CSF binds to its receptor c-Fms on preosteoclastic cells, which is necessary for osteoclastogenesis because it is the primary determinant of the pool of these precursors cells. RANKL binds to its cognate receptor RANK expressed on osteoclast progenitor cell. Together, this leads to expansion of the osteoclast progenitor pool, increased survival of these cells, differentiation into mononucleated progenitor cells, fusion into multinucleated osteoclasts, and then activation.

Simultaneously, osteoblasts decrease their expression of OPG, a secretory dimeric glycoprotein and soluble receptor belonging to the tumor necrosis factor (TNF) receptor superfamily. OPG is an inhibitor of RANK activation via decoy receptor binding to RANK. This reduces the effect of RANKL on osteoclastogenesis and in effect constitutes an efficient negative layer of control.

After the retraction of osteoblasts, activated osteoclasts attach to bone by vitronectin receptors, which have binding sites containing the amino acid sequence Arg-Gly-Asp (RGD) in osteopontin and bone sialoprotein on mineralized bone surfaces. The most common cell-binding domain used as a candidate peptide to improve cell adhesion onto biomaterial surfaces is the Arg-Gly-Asp (RGD) sequence. RGD recognition of the adhesive protein RGD sequence by cell integrins provides a signal for cell adhesion, spreading, and growth (39). Osteoclasts develop a ruffled border and, by means of a proton pump and a chloride channel, generate an acid environment in Howship's resorption lacunae. This dissolves hydroxyapatite crystals. The demineralized organic matrix of bone will subsequently be degraded by proteolytic enzymes, including highly collagenolytic cathepsin K.

Subsequently, osteoblast precursor cells are recruited to the lacunae, where they differentiate into fully

Table 1. Cytokine mediators of local regulation of physiologic bone remodelling

Cytokine	Functions	References
RANKL	receptor activator of nuclear factor KB Ligand. RANKL, produced by osteoblast, is the key cytokine for the induction and maintenance of osteoclasts by binding to its receptor RANK	36
RANK	RANKL-membrane receptor highly expressed on osteoclasts	36
OPG	osteoprotegerin. OPG is a soluble decoy receptor for RANKL and is a potent inhibitor of osteoclastogenesis	36
M-CSF	macrophage colony stimulating factor. M-CSF is secreted by osteoblast and together with RANKL is essential for osteoclastogenesis	36
IGF-I, IGF-II	insulin-like growth factor I and II. IGFs are considered coupling agents, linking bone formation to bone resorption, thus recruiting and activating osteoblasts	8; 11
TGF-BETAs	transforming Growth Factor beta family. TGF-beta's are released during resorption phase and are able to promote osteoblast recruitment and activation. TGF-beta's are important anabolic agents in bone.	13
IL-1, IL-6	Interleukin-1 and 6. These two molecules are pro-inflammatory cytokines and induce osteoclast resorption by enhancing RANKL production	3
TNF-ALPHA	TNF-alpha modulates bone remodelling primarily by stimulating M-CSF production and by directly increasing RANKL expression.	4

active osteoblasts that will fill the resorption lacunae with new bone.

It has been suggested that IGF-I and TGF-beta superfamily members are also “coupling factors” that link bone formation to bone resorption since they are released during the resorption process and function in the recruitment and activation of osteoblasts in BMUs (12). In fact, TGF-beta peptides are chemotactic agents for bone cells and the exposure of osteoblast precursors to these factors induces proliferation. Since it is unlikely that the TGF-beta superfamily members act alone, other growth factors as IGF-1 and -2, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), are known as bone growth stimulants. Lean *et al.* (15) demonstrated that IGF-I is the paracrine factor secreted by osteocytes in response to mechanical forces, thus acting as an initiator of the bone remodeling.

The RANKL-RANK-OPG system is essential for osteoclast differentiation. Their expression is regulated by hormones and other cytokines that stimulate or inhibit bone resorption. IL-1, TNF-alpha, and cytokines in the IL-6 family of cytokines, enhance RANKL expression (40). IL-6 is produced by osteoclasts in response to PTH and 125-D. PTH also inhibits OPG secretion (41). Although adequate levels of 125-D are necessary to provide calcium for bone formation via intestinal absorption, high levels induce bone resorption by increasing RANKL gene expression in osteoblasts (42). Estrogens also regulate RANKL and OPG expression indirectly via various cytokines. In fact, estrogens are a potent inhibitor of IL-1beta and TNF-alpha production in bone marrow cells and monocytes (43). Estrogens also inhibit the production of IL-6 in stromal cells and osteoblasts (44).

In general, cytokines exert regulatory control over bone remodeling by serving as intermediaries for the indirect effects of hormones while also mediating the complex cell-cell communications between osteoblasts and osteoclasts. Table 1 summarizes the principal cytokines involved in physiologic bone remodeling.

3.4. Integration of systemic and local factors with whole body function: a systems approach

Several recent discoveries involve complex physiological networks that involve bone remodeling. In addition to the more intuitive direct physiological

relationships described in the above sections, body composition, intermediary metabolism, pancreatic beta-cell function, and intestinal endocrine function each participate in potentially important network connections. Fu *et al.* present a model wherein leptin production by adipocytes influences hypothalamic sympathetic nervous system activity (45). Downstream agonism of type-2 beta-adrenergic receptors on osteoblasts drives an incoherent feed-forward loop involving cyclic adenosine monophosphate (cAMP) response element binding (CREB), the clock genes *per* and *cry*, and activator protein-1 (AP-1) to modulate osteoblast proliferation. The integrative effects of fat and body composition (leptin production), brain (sympathetic nervous system) and bone (osteoblast proliferation) also impact pancreatic beta-cell activity since osteocalcin (from osteoblasts) promotes insulin production (46). Another mechanism that modulates osteoblast proliferation involves the production of serotonin by intestinal enterochromaffin cells (47). Furthermore, mathematical analysis of a multiorgan physiological network centered on bone can yield emergent, nonintuitive relationships that can prove to be useful in clinical medicine. More specifically, the full potential of biomaterial use in bone may require a systems approach to bone physiology.

4. BONE REMODELLING AND OSTEOPOROSIS

4.1. General considerations

Dysregulated bone remodeling (bone resorption > bone formation) can lead to bone loss, or osteoporosis (48). The currently accepted definition of osteoporosis is ‘a systemic skeletal disease characterized by a reduction in bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture risk’ (49).

Osteoporosis may be either a primary or a secondary form. Primary osteoporosis is the more common form and is due to the typical age-related loss of bone from skeleton. It is classified as type-1 and type-2. Type-1, or postmenopausal osteoporosis, affects women within 15 to 20 years of menopause. Women manifest two phases of type-1 age-related bone loss. The first, at menopause, is caused by estrogen deficiency and results in an uneven increase in bone resorption, as compared with formation, that mainly affects trabecular bone. This is called ‘high-turnover’ osteoporosis. After 4-8 years, ‘low-turnover’

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osteoporosis begins, with a slower and constant loss of both trabecular and cortical bone, primarily caused by decreased bone formation (50).

Estrogen deficiency is thought to underlie type-1 osteoporosis, rendering the skeleton more sensitive to PTH, resulting in increased Ca resorption from bone. This in turn decreases PTH secretion, 125-D production, and Ca absorption. This ultimately causes loss of trabecular bone, leading to vertebral crush fractures and distal forearm fractures. Quantitative measurements of the amount of bone resorption in post-menopausal women is equal to 425 mg/day of calcium, while bone formation is only 387mg/day; this results in a net daily loss of 38 mg of Ca (49).

Type-2, or senile osteoporosis, occurs in women or men more than 70 years of age. Type-2 osteoporosis is generally referred to as a low-turnover osteoporosis and it is usually associated with decreased bone formation along with decreased ability of the kidney to produce 125-D. This vitamin D deficiency results in decreased Ca absorption, secondary hyperparathyroidism, and increased bone resorption. Cortical and trabecular bone is lost and this leads to increased risk of hip, long bone, and vertebral compression fractures (51).

Men exhibit only the slow phase of bone loss, most likely due to decreased levels of bio-available testosterone and estrogen associated with increased levels of sex-hormone binding globulin (SHBG). Decreased estrogen causes an increased bone resorption, while decreased testosterone is responsible for decreased bone formation (52).

Secondary osteoporosis results from the presence of other diseases or altered conditions that predispose to bone loss and is classified as type-3. This form of osteoporosis occurs equally in men and women and at any age. Secondary osteoporosis is associated with a variety of conditions, including endocrine imbalances (as Cushing's syndrome, hypo- and hyper-thyroidism, and hypogonadism); cancer (notably multiple myeloma); gastrointestinal disorders (especially celiac and inflammatory bowel diseases due to malabsorption); drug use (such as corticosteroids, cancer chemotherapy, anticonvulsants, heparin, barbiturates, benzodiazepines, valporic acid, gonadotropin-releasing hormone, and excessive use of aluminium-containing antacids); genetic disorders (cystic fibrosis and osteogenesis imperfecta); rheumatic and autoimmune diseases (lupus and rheumatoid arthritis); chronic kidney disease; and other specific conditions (alcoholism, depression, immobilization, and poor nutrition, including malnutrition due to eating disorders) (16, 53-56).

Among the parameters that can be measured today, bone mineral density (BMD) is the most used predictor of osteoporotic fractures. The World Health Organization, WHO, has defined osteoporosis as the state of having a BMD greater than 2.5 standard deviation below peak adult bone mass, whether or not a fragility fracture

has occurred, based on epidemiological studies on the risk of osteoporotic fractures (57-59). Osteopenia is defined as a BMD between 1 and 2.5 SD below that of a young normal adult. Using standardized bone density measurements of the total hip, "normal" bone is greater than 833 mg/cm², "osteopenia" is between 833 and 648 mg/cm², and "osteoporosis" is lower than 648 mg/cm². In order to distinguish the asymptomatic condition of having low bone mass without fractures, the WHO has suggested that the term "severe osteoporosis" has to be reserved for patients with a BMD and a fragility fracture (51). The current emphasis on BMD in the diagnosis and treatment of osteoporosis limits awareness of the importance of bone quality. It has been shown that loss of connectivity within the network of trabecular bone is also a fracture risk factor independent of BMD (60).

Currently the WHO is designing an absolute fracture-risk model that will help clinicians determine who is at risk and when drug therapy should be initiated. Secondary findings from the Improving Measurements of Persistence of Actonel Treatment (IMPACT) trial showed that 38 percent of subjects, ages 65-80, with a diagnosis of osteoporosis had no risk factors (61). Optimal fracture-risk assessment in the individual patient can be achieved by using diagnostic correlates from DXA and currently available biotechnology in a translational medicine approach. The use of bone-related biomarkers and other laboratory tests not traditionally associated with bone health can improve therapeutic management and identify individuals with elevated fracture risk independent of reduced BMD (62).

As the average age of the world's population shifts upward, the incidence and prevalence of osteoporosis increases. According to the ESOP study (63), an epidemiological survey on osteoporosis in Italy, osteoporosis is an under-diagnosed and under-estimated disease. About 23% of women older than 40 and 14% of men over 60 are affected by osteoporosis, while osteopenia is present in 42% of women and 34% of men.

4.2. Local dysregulation: the role of cytokines

Bone hyperresorption during estrogen withdrawal is mediated by elevated FSH levels and activation of cytokine-induced increases in osteoclastogenesis (64). Activated osteoclasts are usually found in the presence of accessory (inflammatory) cells. Moreover, both human and animal studies demonstrate that hypogonadal bone loss may involve activation of osteoclasts by pro-inflammatory cytokines (65). Specifically, Pacifici *et al.* (66), showed that in women, ovariectomy was associated with an increase in IL-1 and TNF-alpha secretion by peripheral blood mononuclear cells, with a concomitant increase in bone resorption. In the women who received estrogen replacement therapy after ovariectomy, IL-1 and TNF-alpha secretion returned to pre-operative levels (66). It was also demonstrated that the production of IL-6 (67), IL-1, TNF-alpha (68), and M-CSF (macrophage-colony stimulating factor) (69) is enhanced in bone marrow stromal cells and osteoblasts from ovariectomized mice. IL-6 production can be directly

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Table 2. Cytokine mediators of local dysregulation of osteoporosis

Cytokine	Dysregulation	References
M-CSF	M-CSF, together with IL-1, IL-6 and TNF-alpha, is enhanced in bone marrow stromal cells and osteoblasts increasing bone resorption in estrogen deficiency osteoporosis	61
IGF-I, IGF-II	It has been suggested that decreased expression of TGF-beta's and IGF-I in osteoblasts leads to a decreased stimulation of osteoblast proliferation and differentiation during estrogen deficiency.	8
TGF-BETAs	The expression of TGF-beta's seems to be decreased in osteoblasts and bone marrow macrophages of ovariectomized rats	8, 71
IL-1, IL-6	IL-1 and IL-6 expression is enhanced in bone marrow macrophages inducing an increased bone resorption in estrogen deficiency osteoporosis	65
TNF-ALPHA	TNF-alpha expression is enhanced in bone marrow stromal cells and macrophages inducing an increased bone resorption in estrogen deficiency osteoporosis	60

regulated by hormones: its hepatic production is increased by PTH (70). Ovariectomy also leads to enhanced expression of IL-1 and TNF-alpha in bone marrow macrophages (69). It should be noted that the increased production of M-CSF in stromal cells from ovariectomized mice may be an indirect effect caused by increased IL-1 and TNF-alpha. Weitzmann *et al.* (71) also demonstrated that ovariectomy increases the TNF-alpha-producing subset of T lymphocytes by approximately twofold. Attesting to the relevance of T cells in estrogen-deficiency-induced bone loss, athymic T-cell deficient mice are completely protected against the bone loss induced by ovariectomy (71).

Data from Eghbali-Fatourehchi *et al.* (72) showed enhanced RANKL expression in bone marrow stromal cells isolated from post-menopausal women, suggesting that upregulation of RANKL on bone marrow cells is an important determinant of increased bone resorption induced by estrogen deficiency. D'Amelio P *et al.* (64) recently found a similarly enhanced RANKL expression in peripheral blood mononuclear cells, including T cells of postmenopausal women with osteoporosis, compared with healthy postmenopausal and premenopausal women. This suggests that bone resorption induced by estrogen depletion is mediated by the immune system and supports the notion of a distinct osteoimmune dysregulation in postmenopausal osteoporosis

The neutralization of elevated levels of IL-1, TNF-alpha, and IL-6 reduces bone loss in ovariectomized animals. Several studies found that by administering neutralizing specific antibodies, binding proteins, or receptor antagonists for IL-1, TNF-alpha, or IL-6, osteoclast formation and bone resorption were decreased after ovariectomy in mice and rats (68, 73, 74). This implicates IL-1, TNF-alpha and IL-6 in the mechanisms by which estrogen withdrawal induces an increased expression of RANKL and M-CSF, increased osteoclast activity, and increased bone resorption. Table 2 reports the cytokines and their mechanism of action in osteoporosis.

The role of the RANK-RANKL-OPG system in osteoclastogenesis, was demonstrated in a series of gene

ablation studies. Mice lacking functional RANKL develop a form of osteopetrosis and lack osteoclasts (75). On the contrary, mice with the OPG-deficient gene develop osteoporosis with an increased number of osteoclasts (76) and mice with OPG-overexpressed gene develop osteopetrosis (77).

Other cytokines are also involved in the pathophysiology of bone remodeling. IL-7, IL-11, IL-15, IL-17, and prostaglandin E-2 (PGE-2) have been shown to stimulate bone resorption, while IL-4, IL-10, IL-13, TGF-beta, and interferon-gamma (IFN-gamma) to inhibit bone resorption (78,79). In addition, Shaughnessy *et al.* (80) demonstrated that the neutralization of IL-11 activity decreases osteoclast formation and increases cancellous bone volume in ovariectomized mice.

Steroid hormone receptors, are cytosolic proteins which dimerize after ligand binding and translocate into the nucleus where they induce or inhibit the transcription of genes. There are 2 different estrogen receptors (ER). ER-alpha is expressed in many cells including osteoblasts and osteoclasts, ER-beta is expressed in epithelial and mesenchymal tissues, including osteoblasts. Although most of the action of the two ERs are due to their function as ligand-dependent transcription factors, the promoters of IL-1, TNF-alpha, and IL-6 lack classic ER response elements.

Stein and Yang (81) reported that ERs are able to interfere at a molecular level with the transcription of the genes encoding various cytokines. For instance, the IL-6 promoter is inhibited by estrogen in the absence of a functional ER binding site (81). This inhibition is mediated by NF-kB and CCAAT/Enhancer binding protein beta (C/EBP beta) (81). However, it has been shown that ERs interact with several other transcription factors and it has been proposed that this is the mechanism by which the steroid hormone inhibits cytokine receptors. For example, it has been shown that ligand-activated ER-alpha and -beta can block the ability of NF-kB to bind to response elements in the IL-6 gene (81, 82). It is likely that this repression by ERs is also important for the control of the IL-1 gene, which contains several NF-kB sites in the promoter. It has also been shown that activation of ERs represses the stimulatory effect of the transcription factor AP-1 on the TNF-alpha gene (83). It is important to note that attempts to assess circulating levels of these cytokines in postmenopausal women and to then correlate them to clinical markers of osteoporosis have resulted in conflicting results. This most likely reflects the relevance of local cytokine production rather than circulating cytokine levels.

5. THERAPEUTIC CONSIDERATIONS: BIOLOGICAL THERAPY AND BIOMATERIALS

5.1. Current chemotherapeutics

Contemporary management of osteoporosis consists of nonpharmacological and pharmacological measures. Among the non-pharmacological, basic recommendations include changes in lifestyle, nutrition (for deficiencies or insufficiencies in calcium and vitamin D) and physical activity (84). Pharmacological therapies

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directly affect the bone tissue and can be divided into therapies that decrease bone resorption (antiresorptive therapies) and those that increase deposition of bone extracellular matrix (anabolic therapies) (85). Antiresorptive therapies alter osteoclast activity and diminish bone turnover; they include bisphosphonates, selective estrogen receptor modulators (SERMs), estrogen, and calcitonin. Anabolic therapies stimulate bone formation by promoting osteoblast activity and number; the only FDA approved anabolic agent is teriparatide, or human recombinant parathyroid hormone.

Bisphosphonates are the most commonly used treatments for osteoporosis. They are non-hormone phosphate-based compounds that increase bone mineral density by two key properties: their high chemical affinity for bone hydroxyapatite crystals and their inhibitory effects on osteoclast activity (85). Mineral binding affinities differ among the clinically used bisphosphonates and may influence their differential distribution within bone, their biological potency, and their duration of action (86). Bisphosphonates can reduce the pathological fracture risk by 50% after 1 year of oral administration (87).

SERMs are antiresorptive agents that activate estrogen receptors in bone. They are currently used in postmenopausal women with favorable effects on bone mineral density and bone mass (88). Their use has widely supplanted hormone (estrogen) replacement therapy for osteoporosis.

Synthetic CTN is administered in patients who are more than five years postmenopausal with low bone mass. It binds directly to osteoclasts and decreases their activity and number (85). CTN is also helpful in the treatment of bony pain secondary to fractures: although the analgesia mechanism is not well-understood, the neuroactivity of CTN may be cytokine-mediated (89).

Strontium ranelate is a recently developed drug with both antiresorptive and anabolic properties. It inhibits osteoclast activity and accelerates apoptosis, thus decreasing bone resorption. It also seems to increase preosteoblast differentiation. Strontium ranelate was recently approved for use in Europe, but is not currently available in the United States (90, 91).

5.2. Biological therapies

Biological therapies are those using compounds naturally found in the human body. For many human diseases, biological therapies are still under clinical investigation and hold great promise. Fortunately, in the management of osteoporosis, one biological therapy has already been FDA-approved for use in osteoporotic patients and has proven benefit (92). Teriparatide consists of an amino acid sequence of human parathyroid hormone synthesized with recombinant DNA technology. It has been found to be a useful stimulator of bone formation, reflected by increasing BMD owing to improving both cortical and trabecular bone formation.

Nearly every known modulator of osteoclast activity exerts its effect through the RANK-RANKL-OPG

system (65). It is therefore not surprising that this system represents an attractive therapeutic target for the treatment of osteoporosis. Eghbali-Fatourehchi *et al.* (72) demonstrated that RANKL expression is significantly higher in post-menopausal women, compared with pre-menopausal women and post-menopausal women treated with estrogen. Estrogens are known to increase OPG secretion and estrogen depletion after menopause leads to a decrease in OPG and to an increase in RANKL, causing bone resorption and loss (17).

In recent years, agents currently under study include OPG, RANK and RANKL antibodies or inhibitors of RANKL, such as Denosumab (63). This biological agent is a specific antibody raised against RANKL that blocks the binding of RANKL to RANK. Denosumab has been tested in clinical trials with promising results (17). The Phase II trial showed an increase of bone mineral density in Denosumab-treated postmenopausal women compared with placebo-treated ones and a similar BMD to ones treated with bisphosphonate therapy (93).

Other biological therapies for osteoporosis under investigation include OPG and RANK receptors. In animal studies, recombinant OPG was shown to be highly effective in inhibiting bone loss by binding to RANKL and decreasing osteoclastogenesis (75). In human studies with OPG, a single dose was shown to reduce bone resorption markers by 80% (94). However, a problem with OPG therapy is that it has a short (20-30 minutes) half-life and is not specific for RANKL. OPG also binds TNF-related apoptosis-inducing ligand (TRAIL), which is important in natural immunity (94). Another promising target for inhibiting the activity of mature osteoclasts is the H^+ -ATPase pump, which is necessary for osteoclasts to secrete H^+ and acidify the resorptive area (95). Thus, a reduction in osteoclastic resorption could be achieved by inhibiting H^+ -ATPase activity (95). Owing to the rapidity rate of discovery for anti-resorptive or anabolic therapy targets, it is expected that a library of novel biological agents will expand our armamentarium for the treatment of osteoporosis.

5.3. Biomaterials

When osteosynthesis devices or cementless implants are inserted in bone for fracture fixation, joint replacement and prosthetic dentistry, mechanisms underlying the osseointegration process involve a cascade of various cellular and extracellular events (96). The role of osteoporosis in the success of bone implants needs further investigations, however microarchitectural deterioration and biological drawbacks affecting cells of both osteoblastic and osteoclastic lineages, extracellular matrix proteins, local and systemic regulatory factors can negatively influence the early bone response and the bone remodelling processes around implants (3, 96, 97).

Many *in vitro* and *in vivo* experimental researches and clinical studies demonstrated that aging and estrogen deficiency cause a decrease of the osseointegration rate and bone density around common orthopaedic implant

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materials (3, 98-101). It is supposed that even the ideal clinically used biomaterial could fail if implanted in a compromised microenvironment. As an example, human Demineralized Bone Matrix (DBM) that is the gold standard of osteoinductive biomaterials, decrease the osteoinductive potential when implanted in female rats with low estrogen levels (102).

Therefore, new solutions acting on biomaterials are required to stimulate bone formation and osteoformation of implants and many strategies have been proposed to decrease failure risks of implantation surgery in patients affected by osteoporosis and needing reconstructive therapies because of fragility fractures, degenerative diseases or edentulism. These strategies could be divided into 2 main groups:

- strategies directly focused on modifying biomaterial features as design, surface chemistry and bioactivity, roughness and topography
- strategies focused on local and systemic substances/therapies delivered by biomaterials which can be used as adjuvants to stimulate endogenous tissue healing

Implant design may maximize the bone-implant interface, while chemical and topographical properties of the implant surface can be critical for protein adsorption which mediates cell adhesion, can recruit bone forming cells and stimulate cell proliferation and extracellular matrix protein synthesis. Cells interact with the environment (and also with biomaterial surfaces) through transmembrane and intracellular receptors that mediate cell division, maturation, gene expression, collagen and other extracellular matrix (ECM) protein synthesis (103). Regarding implant chemistry, the use of ceramic coatings (i.e. hydroxyapatite, tricalcium phosphate) on metallic materials is suggested to reverse the negative effects of osteoporotic conditions both in animal models and in patients (104, 105). Changes in cell-protein-material interface communications, dissolution and release of specific key ions that act on local cells to up regulate gene expression or influence cell differentiation have been advocated to explain the stimulatory effect of hydroxyapatite and other ceramic coatings (103). However, in some studies the use of an osteoconductive hydroxyapatite coating was not sufficient in restoring normal physiological bone ingrowth in estrogen deficient subjects (106, 107).

The sensitivity of bone cells is not limited to chemical phenomena but also to the surface topography (103). The implant surface roughness combined with a proper implant chemistry was demonstrated to be an important “geometrical mediator” of bioactivity for bone cells and tissue also in the case of aged and osteoporotic bone. In the cases of insufficient bone quantity implants with a high roughness profile have demonstrated superior outcomes compared to smooth surfaces (108). High roughness ($R_a \sim 12-18\mu m$) biomaterials with and without HA coating, behaved similarly without differences in the osteointegration rate in young, aged and estrogen-deficient animals (109).

The amount of growth factors (GF) and the number of bone cells capable of being stimulated by a bioactive surface decrease as a consequence of patient aging and estrogen deficiency and at present many researchers are working also on the combination between biomaterials and drugs or signalling molecules such as ions, peptides and proteins (110).

Biphosphonates have been used locally applied or immobilized on the surface of implants or by systemically administered in osteoporotic animals and patients submitted to implantation surgery (107, 111-113). Also drugs capable of enhancing bone formation such as statins, were tested and improved bone healing around titanium in osteoporotic rats (114). It has been suggested that several statin drugs, including Simvastatin, increase the mRNA expression of bone morphogenetic protein 2 (BMP-2) in osteoblasts, with a subsequent increase in bone formation, when injected subcutaneously over the murine calvaria (115).

Strontium (Sr), that is an inorganic ion and a natural component of food and beverages, has the ability to enhance bone volume and prevent bone loss (116); thereby Sr has become increasingly attractive for the prevention and treatment of osteoporosis. There are *in vitro* and *in vivo* studies that elucidate the ability of Sr treatment to promote osteoblast-related gene expression and alkaline phosphatase of osteogenic-differentiating mesenchymal stem cells (117) and, when coadministered with calcium in ovariectomized goats, to increase new bone matrix formation in a dose-dependent manner (118). Thus, the growing evidence of the beneficial effect of Sr on bone justifies the increasing interest toward Sr incorporation in biomaterials, as calcium phosphate bioceramics and cements (119-121).

Combination of biomaterials with growth factors, mesenchymal stem cells, different forms of biophysical stimulation, hormones and other signalling molecules demonstrated to have a beneficial effect on the process of osteointegration due to the ability to induce osteoblast development, bone regeneration and vascularization (122-127). However, the effect on bone formation around implants inserted into osteoporotic bone was investigated in few studies on bFGF, BMP-2 and Prostaglandin E_2 EP4 selective agonist (128-130).

During the last years also new strategies are growing thanks to the development of biotechnology. Biomimetic coating methods are inspired by the natural process of biomineralization. The deposition of calcium phosphates apatite crystals onto metallic surfaces with different techniques (electrodeposition, immersion in SBF or in supersaturated CaP solution) forms a biomimetic coating of biological apatite which serves as biomimetic matrix for osteogenic cell attachment and growth. Biomimetic innovative biomaterials were successfully tested *in vitro* and *in vivo* healthy preclinical models (108, 131, 132) but no data are available on the efficacy of these materials in osteoporotic bone of pathological animal models.

Future trends of innovative research investigate on nanomaterials that matching natural bone have been proposed as the next generation of orthopaedic implant materials with the aim to improve surface properties of the implant by possessing a greater surface area, to create an environment more conducive and to promote osteoblast function (133). Cells of our body are predisposed to interact with nanostructured surfaces, because all living systems are governed and are composed by molecular building blocks at nanometer scales, as proteins, nucleic acids, lipids and carbohydrates (134). Nanobiomaterials, possessing higher surface area to volume ratio than conventional microscale biomaterials, have demonstrated to provide advantageous interactions with the protein that control cellular function, in particular fibronectin, vitronectin, laminin and collagen (135). Although nanobiomaterials seem to be an attractive strategy, no data are available when implanted in compromised osteoporotic bone in preclinical studies; also understanding the molecular mechanism of healthy and pathological cell-nanomaterial interactions, with particular attention also at improving proper vascularization of the tissue ensuring adequate blood supply for successful biomaterial/tissue integration could be expected. Tissue engineering based therapies, by combining both smart biomaterials tailored for compromised bone tissue, cells and growth factors or drugs for rebalancing the bone remodelling process, could be an innovative tool to promote osteointegration in osteoporotic conditions, although, to date, few attempts are available on fracture healing of osteoporotic rats (136).

6. CONCLUSIONS

All the current available studies support the systems biology view that manipulating the implant biomaterial surface in the setting of bone growth, bone remodelling, and local microenvironment networks could lead to an enhanced and accelerated osteointegration rate in osteoporotic patients.

However, the high costs of these delivery systems, issues of release control, the uncertainty concerning the most effective dosage, and the possibility of collateral effects of the proposed signalling molecules, are still a limitation for the use of biomaterials in clinical trials. Furthermore, contemporary clinical strategies that demonstrate a beneficial effect on fracture healing and osteointegration in healthy conditions were not investigated in pathological animal models and clinical osteoporotic conditions. It is precisely these studies – the use of smart biomaterial technology in the setting of complex pathological networks involving bone – that need to be designed.

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Abbreviations: Ca: calcium; P: phosphorus; BMU: basic multicellular units; PTH: parathyroid hormone; CTN: calcitonin; IGF: insulin like growth factor; GH: growth

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hormone; TSH: thyroid stimulating hormone; FSH: follicle stimulating hormone; 125-D: 1,25 dihydroxyvitamin D; OPG: osteoprotegerin; TGF: transforming growth factor; HPT: hypothalamic-pituitary-thyroid; IL: interleukin; RANKL: receptor activator of nuclear factor kB ligand; RANK: receptor activator of nuclear kB; M-CSF: macrophage colony stimulating factor; TNF: tumor necrosis factor; RGD: Arg-Gly-Asp; FGF: fibroblast growth factor; PDGF: platelet-derived growth factor; cAMP: cyclic adenosine monophosphate; CREP: cAMP response element binding; SHBG: sex-hormone binding globulin; AP: activator protein; BMD: bone mineral density; IMPACT: improving measurements of persistence of actonel treatment; IFN: interferon; ER: estrogen receptors; NF-kB: nuclear factor-kappa B; C/EBPbeta: CCAAT/Enhancer binding protein beta; SERM: selective estrogen receptor modulators; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; DBM: demineralized bone matrix; ECM: extracellular matrix; BMP: bone morphogenetic protein; Sr: strontium.

Key Words: Bone, Osteoporosis, Hormones, Cytokines, Osteoblasts, Biomaterials, Review

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