THE ROLE OF OPG, RANK, RANKL IN THE BONE METABOLISM, CARDIOVASCULAR DISEASE AND IMMUNE SYSTEM: CLINICAL AND PHYSIOPATHOLOGICAL IMPLICATION

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[Ruolo di OPG, RANK, RANKL nel metabolismo osseo, nelle malattie cardiovascolari e nel sistema immune: implicazioni cliniche e fisiopatologiche]

SUMMARY

The bone remodelling process is characterized by alternate phases of production and resorption of the mineralized matrix, is possible by two types of cells: osteoblasts and osteoclasts. When these ones are subject to hormonal and cytokines stimulus, they express and produce a lot of proteic molecules that play an essential role in regulating the processes of activation and inhibition of osteoclastogenesis.

Three molecules have been the subject of intense research in the field of bone biochemistry: OPG, RANK and RANKL. OPG is a glycoprotein belonging to the TNFR superfamily. The importance of this molecule is confirmed by the presence of specific domains, that are essential for the osteoclastogenesis inhibition. OPG makes it by binding with RANKL. This event prevents the binding of RANKL to RANK, that is expressed in the osteoclasts, and inhibits the differentiation of pre-osteoclasts in mature osteoclast. RANKL is a peptide produced by osteoblasts cells. We review the physiopathologic implications of this molecules, and their implication in the immune and vascular system.

Infact in the former it has been observed that RANKL expressed in the activated T cells, binds with RANK determining an immunomodulator final effect; in the latter it has been possible to observe that RANK and RANKL are expressed by endothelial cells, and regulate their survival.

Key words: OPG, RANK, RANKL, osteoporosis, immune system

INTRODUCTION

Bone is a specialized, rigid and elastic connective tissue submitted to a continuous process of remodeling characterized by alternate phases of production and resorption of the mineralized matrix. Through this process of remodeling the bone actively participates in the maintenance of the homeostasis of calcium and phosphates.

This function is made possible by two types of cells: the osteoblasts and the osteoclasts.

The osteoclasts are specialized cells derived from monocyte/macrophage cells, that are differentiated from cells of mesenchimal origin that adhere to the bone matrix and secrete acids and lithic enzymes that, in a specialized extracellular compartment, degrade it.

Parole chiave: OPG, RANK, RANKL, osteoporosi, sistema immunitario
The osteoblastic cells derive from bone marrow cells through a series of progenitors. They become mature osteoblasts forming bone matrix, and then, when they remain trapped in their products of secretion turn in to osteoclasts. Other cell types such as monocytes/macrophages and endothelial cells contribute to the bone remodeling through direct contact with osteogenic cells or through the release of soluble cytokine or growth factors.

The cytokines, in particular way, play a role of protagonists in the interactions and biochemical communication between osteoblasts and osteoclasts. It has been shown that the contact of the osteoblasts with immature osteoclasts clearly leads to the differentiation of osteoclasts into mature cells.

Osteoclastic cells reabsorb the mineral component of bone tissue under control of hormones or growth factors.

Osteoblastic cells, once stimulated by the contact with osteoclasts or by osteoclastic soluble factors, secrete osteoid matrix on the resorption sites, starting the formation of the bone.

This delicate equilibrium can be broken and that could lead to the development of various skeletal diseases, in which the bone matrix can be decreased, as in the osteoporosis (a skeletal disorder characterized by the reduction of the bone strength and predisposition to an increased risk of fracture); or increased as in the osteopetrosis.

The molecules protagonists of the interaction among these different cell types have been recently discovered as several proinflammatory cytokines (such as IL-6, TNF-alpha and IL-1 that stimulate the osteoclastogenesis) and proteic molecules such as osteoprotegerin (OPG), receptor activator of nuclear factor kB (RANK) and its ligand RANKL that represent a key triad of the biology of the bone and that present large areas of research to understand the pathogenetic mechanisms of several bone diseases.

**OPG**

OPG is initially a peptide composed of 401 aminoacids that is cleaved to became a mature protein composed of 380 aminoacids. The human gene of OPG is located on the chromosome 8. The promoter sequence of human OPG’s gene contains binding elements for the transcription factors cbfa-1, that has been showed to increase the OPG’s gene transcription.

This molecule belongs to the TNFRs superfamily (receptors of the tumor necrosis factor), and is a basic glycoprotein.

OPG was discovered by two research teams in the United States and in Japan in the late eighties. Simonet WS. et al, working on transgenic mice to identify the TNF receptor related molecules, discovered the existence of a cleaved molecule.

This molecule, subsequently called OPG, similar to the receptor TNF, has been held responsible for an osteopetrosic characteristic phenotype. At the same time it was shown, in fact, that the osteopetrosis is associated with a diminution of osteoclastogenesis and osteoclastic activation.

This fact prompted reflection on the possible role that OPG could have in regulating osteoclastogenesis.

Yasuda et al., observing the mechanisms of stimulation and inhibition of osteoclasts, discovered a molecule able to inhibit osteoclastogenesis in the stromal cells of the bone marrow, OCIF (osteoclastogenesis inhibition factor).

Following studies showed that OPG and OCIF were the same molecules. Currently this molecule is called OPG and belongs to the super-family of the TNF receptors.

In detail, OPG is composed of 7 structural domains that influence his biological activities: among these the 1 to 4 N-terminal domains are rich in cystein and they are essential for the inhibition of osteoclastogenesis, and they show some correspondence with the extracellular domains of other members of the TNFR family.

Domain 7 is an eparine-binding domain able to interact with several proteoglycans; domains 5 and 6 in the carboxy-terminal region contain two homologous regions whose physiological meaning is not clear, but is highly structural analogous with some domains that have been found in the citoplasmatic region of some mediators of apoptosis as TNFR 1, DR3, CD95/Fas and to the TNF receptors apoptosis inducing ligand (TRAIL).

Although OPG’s mRNA has been identified in several tissues like lung, heart, kidney, liver, stomach, bowel, brain, thyroid and bone, only in the latter it has been studied accurately.

The same mice also showed calcifications of great arteries, and aortic dissecation.
RANKL

RANKL, (receptor activator of nuclear factor κB-ligand) is a peptide composed of 317 amino acids and exists in two forms: one linked to the cytoplasmic membrane, the other soluble (34).

The mRNA of RANKL is express in the bone and in the bone marrow, in the lymph nodes, in the thyme, in the spleen, in the fetal liver and in the Peyer’s glands (34,2). RANKL was identified soon after OPG, by the Simonet WS et al and Yasuda et al. While they were using OPG to find his ligand, they found a molecule initially called OPG-L, currently known as RANKL (34,60) that also belongs to the superfamily of the TNF receptors.

The major role played by this molecule is to stimulate the differentiation of preosteoclastic cells into mature osteoclasts (34,39), to stimulate the activity of the osteoclasts and to inhibit their apoptosis (16).

In fact, in vitro studies have shown that RANKL is essential for the differentiation of the osteoclastics precursors in mature cells (34,60).

A further confirmation of the crucial role played by RANKL in the osteoclastogenesis is shown by studies on transgenic knock-out mice. These ones developed a serious osteopetrosis and defect in dental eruption (33) caused by the absence of a limiting factor of the bone matrix production. RANKL binds its receptor RANK that is express by a small number of cells included mature osteoclasts and progenitors, activated T lymphocytes and dendritic cells of myeloid origin (DCs) (2,19).

RANK

The last element of the triad, in order of identification, is RANK the receptor. RANK is a peptide of 616 aminoacids, with a signal peptide of 28 aminoacids, an extracellular domain, a transmembrane domain of 21 aminoacids and large C-terminal citoplasmatic domain (2).

RANK is expressed in the cells of the monocyte/macrophage line, included the preosteoclastic cells, T and B cells, dendritic cells and fibroblasts (2,27). The activation of RANK after its binding RANKL leads to the expression of specific genes in the osteoclast during his differentiation, which results in promoting bone resorption.

Interaction RANK/RANKL: intracell signal transduction and its regulation

The RANK-RANKL binding stimulates the osteoclasts gene expression that leads to resorption activation and therefore the osteoclasts survival at the beginning of a new cycle of bone remodeling.

The RANK signal is mediate from cytoplasmatic factors that activates all the messengers that control these functions. During the osteoclastogenesis and the osteoclasts activation at least 5 separate signals pathways are induced and these are mediated by kinase proteins: NF-κB inhibitor kinase (IKK), c-Jun N-terminal Kinase (JNK), p38, extracellular signal regulated Kinase (ERK) and the Src pathway. The first step of the RANK signal translation is the link of the cytoplasmatic factor associated with TNFR known as TRAF to specific sites inside the citoplasmatic domain of RANK (27,14,17).

TRAF6 assembles the signal proteins that regulate the osteoblasts specific gene expression leading to differentiation and activation. The best systems studied are the activation of the transcription factors of NF-κB and protein activator 1 (AP1), whose activities are quickly induced after the binding with the ligand. The activation of these transcription factors can be induced from signals pathways mediated by kinase proteins like IKK1/2 (NF-κB) and JNK1 (AP-1) (30,15). Recently TGF-beta inducible kinase (TAK1) and TRAF-binding adapter protein (TAB2) has been found in activated receptor complex (35,43). Experimental evidence suggests that TAK1 is involved in the activation of NF-κB and AP-1 through IKK1/2 and JNK1 (40). Besides the JNK’s activation in these cells, a MAPK-related MKK7 kinase is necessary. Although is not clear if TAK1 directly acts on IKK1/2 or on MKK7 or if some other kinases intervene. The stress-activated kinase protein p38 is also involved in the signal transduction induced by RANK and that is apparently activated through the fosforilation of MKK6 (37,41).

The stimulation of p38 leads to the activation of the transcription factor me/Mif, that regulates the expression of TRAP and CATK genes, all required by the mature osteoclast (41).

ERK/1 kinase is also activated by RANK/RANKL binding and it seems to be influenced by the MEK1 activation (39,26).
The ERK inhibition strengthens the RANKL induced osteoclasts differentiation, suggesting that the ERK pathway is involved in the osteoclastogenesis inhibition.

It has been showed that the protein Src, required for the osteoclastic activation, bind TRAF6 and allows the RANK mediated signal translation through fosfatidilinositol3OH Kinase (PI(3)K) and through the serine/threonine kinase protein (AKT)\(^{57}\). Both these proteins are known to induce cell survival, the rearrangement and the motility of the cytoskeleton. Moreover PI(3)K is inhibited by the SHIP-kinase.

The signal that starts from RANK culminates in thegeneric expression change that characterizes the osteoclastic activity\(^{11,49}\).

**Cytokine-mediated modulation of the RANK-RANKL system**

RANKL, is express on the surface of preosteoblastic and stromal cells, and binds RANK on the osteoclastics precursors. Within this interaction the presence of M-CSF\(^{153}\) seems to develop an essential role. From these interactions a sequence of events starts, each one modulated by different levels of control. In facts the activation on the surface of the osteoclasts of IL-1 receptors, c-Fms, TNF-alpha, PGE-2 and TGF-beta, supports in vitro osteoclastogenesis and can stimulate the bone resorption in vivo.

Despite this, all the signals started by RANK are inhibited by OPG in vitro and in vivo because OPG prevents the binding of RANKL to RANK. The induction of the osteoclastogenesis started by RANKL leads to the induction of interferon-beta that is secreted in autocrine way to inhibit (downregulation) the expression of c-Fos, a critical factor of translation involved in the development of the osteoclasts\(^{96,122}\). Gamma-interferon has also a negative effect on the function of RANK, and can inhibit the osteoclastogenesis in vitro. This event appears to be interesting since the INF-gamma is used in the treatment of the osteopetrosis because of its increasing effect on the bone resorption. It has been shown too that IL-4 has an inhibitory effect on osteoclastogenesis.

This event seems to be regulated by STAT (signal transducer and activator of transcription)\(^{10}\). **Role of OPG/RANKL/RANK in the bone metabolism, in the immune system and in the vascular system**

**In the bone metabolism**

The osteoblasts and their precursors express RANKL. It stimulates the receptor RANK set on the osteoclast precursor and on the mature osteoclasts and it activates the intracell systems which promote osteoclast differentiation and activation and the reorganization of the cytoskeleton that increases the bone resorption and bone loss. The stromal cells and the osteoblasts secrete OPG that acts as a competitive receptor and stops RANKL.

In this system the expression of RANKL is stimulated by IL-1, IL11, IL-17, TNF-alpha, PTH, Prostaglandine E2 and Glucocorticoids, while it is inhibited from IL-4, TGF-beta, 17 beta estradiol. The production of OPG is increased by IL-1, IL-13, IL-18, TNF-alpha, TGF-beta, BMP-2, 17-beta estradiol, and by mechanical solicitation.

It is decreased by PTH, Prostaglandine E2, glucocorticoids, Ciclosporine A\(^{24}\). The loading forces applied on the surface of the bone are followed by an increase of OPG’s mRNA synthesis, while the expression of OPG from the bone marrow cells, decreases with the age; and this leads to consider OPG as a mediator of immobilization in senile osteoporosis.

**In the immune system**

RANKL is express and secreted by activated T cells and it acts on the osteoclasts (for instance promoting bone resorption during the inflammatory process that involves the bone tissue), T cells and dendritic cells (DC) having the antigen where it increases the activity and the survival of every cell of the immune system. The DC can modulate these processes through the secretion of OPG. In this system the expression of RANKL is induced by IL-1, IL-7, IL-17, TNF-alpha. It is inhibited by 17-beta-estradiol. The production of OPG is induced from CD40 Ligand\(^{24}\).

In the functioning of the immune system, it has been observed that the RANKL/RANK binding increases the survival in the DC through the induction of Bcl-xl. It also increases the immunostimulatory functions in the DC and it modulates the activated T cells\(^{57,44}\). All of this shows the important role of immunomodulation developed by RANKL.

This has been confirmed by the study on RANKL knock out mice that shows agenesis of the...
lymph nodes and alterations of the spleen and the Peyer’s glands. The deletion of the RANK gene leads to the birth of mice phenotypes like those Knock-outs for RANKL, and demonstrates the importance of RANK-RANKL system in the regulation of the immune system.

OPG is held to be essential in this regulation from the moment it was observed that OPG is directly involved in the maturation of the B cells and in an efficient synthesis of antibodies.

An important aspect of OPG role in the immune system is that related to the cytotoxic ligand TRAIL, a powerful activator of the apoptosis of sensitive cells.

**In the vascular system**

RANKL is express from the endothelial cells that also produces the specific receptor RANK. The interaction RANKL / RANK regulates the survival of the endothelial cells. RANKL can be blocked by OPG, that is secreted by the endothelial cells and by the muscular smooth cells.

In this system the expression of RANKL is increased by: IL-1, and TNF-alpha. The production of OPG in the muscular smooth cells is increased by: PDGF-BB, and it is inhibited by: Glucocorticoids, Cyclosporin A, Troglitazone.

The production of OPG in the endothelial cells, is increased by IL-1, and TNF-alpha.

**Clinical implications**

After having observed the presence of these three molecules in various cell typologies and on many tissues, research has focused on the possibility of dosing these single molecules with the purpose to use them as markers of activity, in different morbid cases, above all tumoral, that involve the bone and other tissues.

In fact it has been observed that tumoral cells in the bone microenvironment promote an inflammatory response that leads to an increase of activated osteoclasts and therefore to bone resorption. The tumoral cells also create a favourable environment for osteoclasts development.

The inflammatory response and the tumour environment establish a vicious circle between bone and tumour cells. The latter release growth factors, hormones, eicosanoids and cytokine in the bone microenvironments. These factors stimulate the osteoblastic stromal cells that increase the differentiation and RANKL-mediated activation of the osteoclasts.

RANKL and OPG have already been individualized in a lot of tumour cells. In fact Brown et al. have shown that RANKL was found in 10 samples out of 11 of carcinoma of the prostate and that the proportion of tumour cells expressing RANKL was significantly increased in all the bone metastasis.

Furthermore, RANKL was express in squamous cells carcinoma and has been found in over the 90% of the tumour cells in metastatic lesions of breast [28], lung and thyroid adenocarcinoma, but RANKL mRNA has not been found in tumour cells of either breast or melanoma.

Other authors (Good et al.), more recently, have shown that primitives benign bone tumors, primitive malignant tumors and bone metastasis are all positive for RANKL. Moreover, while the seric levels of OPG show few variations in the osteolytic disease, the levels of RANKL are subject to notable changes in comparison to the control groups. The relationship RANKL/OPG is notably increased in bone metastasis and in tumors associated with severe osteolysis. This shows how tumour cells induce an imbalance in the relationship RANKL/OPG for RANKL. It has also been observed that tumoral microenvironments can release high-levels of OPG to balance the high concentrations of RANKL produced by tumour cells. The same happens in patient affected by Paget’s disease in which high-levels of OPG have been found. It is probable that this could be a protective mechanism of the skeleton to compensate for increased bone resorption.

Similar protective mechanisms have also been found in the osteoporosis. In fact in this disease relatively constant seric levels of OPG have been found and no significant alterations have been observed with respects to RANKL.

In human osteogenic osteosarcoma in which the apposition of bone matrix prevails, a notable increase in the OPG levels has been observed, in accordance with the results obtained by studying transgenic mice selected for an over-expression of OPG that, in fact, showed serious osteopetrosis.

The relationship RANKL/OPG is also considered a predictive index of survival in multiple myeloma in which the seric levels of RANKL are elevated and they correlate with the bone disease. In fact the relationship RANKL/OPG is an index of the activity of the illness and a marker of bone resorption.

OPG is also express on the endothelial cells.
and in the smooth muscular cells of arterial walls\(^{37}\). With this presupposition the study of the levels of RANKL and OPG in the vascular physiopathology led to find out that those animals with a deficit of OPG develop calcifications of the renal arteries and the aorta.

It has been observed in animals that the treatment with OPG prevents arterial calcifications caused by warfarin or high doses of vitamin D\(^{47}\).

However it is not clear how OPG protects the vascular walls.

Some studies suggest that RANKL and OPG modulate the apoptosis of the endothelial cells and that can have effects on the integrity of the vascular lumen\(^{39}\).

It is surprising, however, that the patients affected by coronaropathy have serum levels of OPG higher than healthy subjects and the relative risk of cardiovascular mortality is increased in those subjects\(^{9, 31}\). Then, while the animal model suggests a protective role for OPG in the vascular system, the role of this molecule in males is not clear yet.

**Therapeutic implications of the OPG/RANKL/RANK System**

To the light of the above observations, the research has focused on possible therapeutic uses of molecules of synthesis that mimics the action of OPG, or that competes with RANKL, or with RANK.

Some studies on animals have shown that OPG opposes osteoporosis through the inhibition of the endosteal osteoclasts, and it prevents bone erosion in arthritis or the calcification of the vessels, stopping a process similar to the osteoclastogenesis\(^{47, 42}\).

It has also been observed that OPG when produced in bone tumors, stops tumour osteolysis through the interaction with RANKL. Furthermore OPG inhibits the osteolysis, increases survival in the animal model of multiple myeloma, and inhibits the development and growth of various animal tumors.

This explains the great interest in the synthesis of molecules of recombinant OPG, or of other molecules able to stop the interaction between RANKL and RANK. For this reason a study has been conducted on a group of postmenopausal women who were administered a single subcutaneous dose of OPG-Fc (fusion molecule) produced using genetic engineering techniques to appraise its effects on the bone resorption\(^{5}\).

The results have shown a good tolerance of the injected molecule and an amazing effectiveness in greatly reducing bone resorption for a long period of time. Subsequently the study was continued on patients affected by multiple myeloma and by carcinoma of the breast, with osteolytic lesions. This treatment has also shown good compliance of the subcutaneous injection of a single dose of OPG-Fc. A notable reduction of the indexes of bone resorption has been observed too. But despite the enthusiasm resulting from these findings, a complication has arisen when the ability of OPG to tie TRAIL (TNF-related apoptosis-inducing ligand) was evaluated\(^{22}\).

In fact, the bond of OPG to TRAIL stops the inhibitory effect of TRAIL on tumour sensitive cells. The limit of this administration is tied up to the pharmacodynamics of OPG-Fc, since the half-life of this molecule requires monthly administrations to maintain a considerable suppression of the bone resorption\(^{5}\).

Bekker PJ. has produced a monoclonal human antibody against RANKL, called AMG 162 (denosumab)\(^{6}\). This molecule possesses a longer half-life in comparison to OPG-Fc, since the halflife of this molecule requires monthly administrations to maintain a considerable suppression of the bone resorption\(^{6}\).

The protective effectiveness of denosumab in fractures, is therefore object of large trials in women and men that are submitted to subcutaneous administrations every six months. Considering the wide range of conditions in which RANKL is involved in bone loss such as osteoporosis, rheumatoid arthritis, bone metastasis, multiple myeloma and treatments inducing bone loss (like long exposures to the glucocorticoids), it seems clear that denosumab has a promising role as a potential therapy for the above listed diseases.

The effectiveness of such a molecule has been tested on a group of postmenopausal women, and the results have been: a good compliance and the absence of adverse reactions.

Following a similar thought, Zhang J. team has created RANK-Fc, using genetic engineering techniques. These researchers have appraised the
possibility of this molecule to prevent the progression of prostate carcinoma stabilized in animal model.

The results showed that RANK-Fc reduced the osteoblastic lesions induced by the tumor, and also reduced the prostate specific antigen in the serum, such as the volume of the bone tumour mass. However no positive result has been observed using RANK-Fc on carcinoma prostate cells injected under the skin, confirming the role played by this molecule only in the bone resorption. So we can conclude that RANK-Fc is very useful because it does not stop TRAIL-related apoptosis but in a way stops specifically RANKL-induced osteoclastogenesis. Meanwhile researchers are still trying to synthetize some polymers that could be less toxic.

Cheng et al. sets a small peptide called OP3-4, that mimics the actions of OPG in stopping RANKL/RANK interactions and modulates the signal of RANK altering its biological function(64).

Discussion

The recent discovery of the rank-rankl-opg system has brought, in a short time, to considerable interdisciplinary examinations that have greatly improved our knowledge of intercell systems of communication. This new knowledge has prompted investigation in large fields of medicine that involves the bone metabolism diseases characterized by both bone loss or formation, tumour, inflammatory and autoimmune bone disease and obstructive vasculopathies.

Particular interest has been arouse by the possibility to find a molecular pathway that links immune system, bone metabolism and vascular system and that could start up new physiopathological, clinical and therapeutic scenarios.

While the pharmacological research has reached advanced phases in finding therapeutic perspectives, based on the RANK RANKL OPG system, especially in the field of bone disease, the real role of this system in the pathogenesis of vascular calcifications and on the consequence that this means of intercellular communication could have on immune system, still needs further investigations(65).

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