Duchenne muscular dystrophy: RANK/RANKL/OPG (receptor activator of nuclear factor-kB/RANK ligand/osteoprotegerin) system and glucocorticoids

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked inherited disorder. Patients present with decreased bone mineral density (BMD) due to glucocorticoid therapy and progressive muscle weakness. Bone remodeling allows bone volume and structure to be maintained and controlled by local and systemic factors. These include the receptor activator of the nuclear factor-kB (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system, a determining pathway in the balance between bone formation and resorption. Disruptions in this complex, caused by factors such as glucocorticoids, can affect bone metabolism. The extensive action of the RANK/RANKL/OPG pathway suggests an influence on dystrophic muscle pathophysiology. This review aimed to highlight some aspects of the RANK/RANKL/OPG system, the effect of glucocorticoids on this pathway, and the pathophysiology of the patient with DMD.

Keywords: Duchenne muscular dystrophy. RANK ligand. Osteoprotegerin (OPG). Receptor activator of nuclear factor-kappa B (RANKL). Glucocorticoids.

Distrofia muscular de Duchenne: el sistema RANK/RANKL/OPG (receptor activador del factor nuclear-kB/ligando de RANK/osteoprotegerina) y glucocorticoides

Resumen

La distrofia muscular de Duchenne (DMD) es un trastorno hereditario ligado al cromosoma X. Los pacientes presentan una disminución de la densidad mineral ósea (DMO) debido a los efectos adversos del tratamiento con glucocorticoides y a la debilidad muscular progresiva. El remodelado óseo permite mantener el volumen y la estructura ósea, proceso controlado por factores locales y sistémicos. Entre ellos destaca el sistema del receptor activador del factor nuclear-kB (RANK), su ligando natural RANKL (RANKL) y la osteoprotegerina (OPG), una vía determinante en el equilibrio entre la resorción y formación ósea. Las alteraciones en este complejo, originadas por factores como los glucocorticoides, pueden afectar el metabolismo óseo. La amplia acción de RANKL y OPG ha sugerido una influencia en la fisiopatología de la DMD. El objetivo de esta revisión fue destacar algunos aspectos del sistema RANK/RANKL/OPG, el efecto de los glucocorticoides en esta vía y la fisiopatología del paciente con DMD.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic, degenerative, progressive, recessive inherited disorder caused by mutations in the DMD gene encoding the dystrophin protein. These patients show metabolic alterations such as hyperinsulinemia, insulin resistance, elevated inflammatory markers, alterations in body composition, and decreased bone mineral density (BMD). DMD is associated with an increased risk of bone fragility due to the adverse effects of prolonged glucocorticoid therapy and loss of mobility, sometimes resulting in osteopenia and osteoporosis.

Two processes constantly renew bone: bone modeling and bone remodeling. Both processes involve osteoclastic bone resorption and osteoblastic bone formation. Osteoclasts, multinucleated cells derived from the myeloid hematopoietic lineage, are the primary cells involved in bone resorption, whereas osteoblasts, which carry out bone formation, originate from multipotent mesenchymal stem cells. Bone remodeling occurs continuously to repair skeletal damage, prevent the accumulation of fragile and hypermineralized bone, and maintain homeostasis. During bone remodeling, osteoclastic resorption is tightly coupled to osteoblastic bone formation. These processes are regulated by the receptor activator of the nuclear factor-kappa B (RANK) signaling pathway, which natural ligands are RANKL and osteoprotegerin (OPG). As this process is essential for skeletal health, alterations in the RANK/RANKL/OPG complex can disrupt the balance between bone resorption and bone formation, leading to increased bone loss or excessive bone formation. Increased osteoclastic activity with consequent bone loss has been observed in different pathological conditions, including DMD.

In patients with Duchenne disease, the loss of mobility, together with the administration of systemic corticosteroids, induces a decrease in bone mineral density and favors the development of osteoporosis. This study aimed to highlight some aspects of the RANK/RANKL/OPG system in the remodeling process, the effect of glucocorticoid treatment on this signaling pathway, and the bone health of DMD patients.

Osteoporosis in DMD

Severe osteoporosis with clinically significant bone fragility has been reported in pediatric patients with DMD. Currently, osteoporosis is recognized as an essential complication of DMD because it requires follow-up for early diagnosis and interventions to prevent clinically critical sequelae. In this context, the two main risk factors for osteoporosis are progressive muscle...
weakness with loss of load-bearing capacity and bone toxicity from glucocorticoid therapy\textsuperscript{18}. Clinically, osteoporosis manifests with long bone and vertebral fractures (VF). The latter are frequent, especially in patients treated with glucocorticoids. VFs in the pediatric population with DMD are associated with chronic back pain and spinal deformity, while long bone fractures have been associated with premature and permanent loss of ambulation. Up to 60\% of individuals with DMD have lower extremity fractures, often in the distal femur, tibia, and fibula\textsuperscript{6}.

**Bone remodeling**

The bone system is constantly renewed by two processes: bone modeling and bone remodeling\textsuperscript{7,8}, which involve osteoclastic bone resorption and osteoblastic bone formation. Osteoclasts, multinucleated cells derived from the hematopoietic myeloid lineage, are the primary cells involved in bone resorption, while osteoblasts, which carry out bone formation, originate from multipotent mesenchymal stem cells\textsuperscript{9}. The modeling, resorption, and formation processes occur independently at different sites in the skeleton, producing essential changes in bone architecture. Bone modeling begins in the early stages of skeletal development and modifies the size and shape of the bone. During this process, resorption and bone formation must be uncoupled; bone is removed from one anatomical site, and new bone is formed at another.

An important example of modeling is preserving skeletal shape during linear growth\textsuperscript{7}. Most bone modeling is completed with skeletal maturity, but this process may continue even until adulthood due to an adaptive response to mechanical loading or exercise\textsuperscript{19}. In contrast, bone resorption and bone formation are closely coupled spatially and temporally in remodeling so that bone volume and overall structure remain unchanged. During remodeling, small regions of bone are resorbed by osteoclasts and replaced by osteoblasts; this close coordination between resorption and formation ensures the maintenance of structural integrity and allows for the replacement of up to 10\% of the skeleton each year\textsuperscript{7}.

Bone remodeling maintenance requires balancing bone formation, resorption, and direct communication between the different bone cells. Cells of the osteoblast lineage (osteoblasts, osteocytes, and bone lining cells) and bone resorption cells (osteoclasts), together with their precursor cells, are organized into specialized units called bone basic multicellular units (BMUs)\textsuperscript{20}. Several factors control the bone remodeling process. Prominent among these regulatory factors is the RANK/ RANKL/OPG system, which sends local and systemic signals that determine the maintenance of equilibrium and the timing of bone resorption and formation within the remodeling cycle\textsuperscript{7-9}.

An increased rate of bone remodeling has been reported in various pathological conditions affecting bone tissue, including postmenopausal osteoporosis, hyperparathyroidism, and rheumatoid arthritis. A decrease in BMD has also been described in different pediatric disorders requiring glucocorticoids, such as leukemias, juvenile rheumatoid arthritis, juvenile systemic lupus erythematosus, asthma, and neuromuscular disorders such as DMD\textsuperscript{6,10,21}.

**RANKL/RANK/OPG system**

In the late 1980s, understanding of the molecular mechanisms that regulate the formation and activation of osteoclasts advanced rapidly with the discovery of the RANKL/RANK/OPG signaling system, demonstrating that this pathway is key to bone resorption\textsuperscript{22}. This system induces the differentiation and activation of osteoclasts and osteoblasts, balancing the remodeling cycle between formation and resorption. RANKL is secreted by osteoblasts, while RANK, its receptor, is located in pre-osteoclastic cells. The RANKL/RANK interaction induces the formation of multinucleated mature osteoclasts, leading to bone resorption\textsuperscript{8,9,22,23}.

A third component, OPG, is also produced by osteoblasts and its binding to RANKL inhibits the RANKL/ RANK interaction and thus osteoclastogenesis.

The binding of RANKL to its natural receptor RANK promotes the activation of the intracellular nuclear factor-kB signaling pathway. As a result, the differentiation of preosteoclasts into mature osteoclasts is generated, facilitating bone resorption\textsuperscript{24}. Osteoclast precursors express the macrophage colony-stimulating factor (M-CSF) receptor that promotes the expression of RANK when stimulated. Osteoblasts control osteoclast precursor differentiation by producing RANKL, a cell surface molecule that is the main effector of the RANK receptor, and OPG, a soluble decoy receptor for RANK\textsuperscript{5,22,24}.

The binding of RANKL to the extracellular domain of RANK leads to the expression of specific genes involved in osteoclast differentiation, survival, and bone resorption (Figure 1A). The initial step of this signaling pathway is binding TNF receptor-associated factor (TRAF) within the cytoplasmic domain of RANK\textsuperscript{8,22}, leading to NF-kB activation and translocation to the
Glucocorticoids affect bone formation because they inhibit osteoblastogenesis, osteoblasts’ production of bone matrix components, and insulin-like growth factor-I (IGF-I). They also stimulate osteoblast apoptosis and promote osteoclastogenesis by increasing RANKL and decreasing OPG expression in stromal and osteoblastic cells. Finally, inhibition of bone formation will lead to a decrease in bone remodeling and a sustained increase in the risk of fractures. These effects are more significant at the trabecular bone level, so changes in these patients are observed mainly in the vertebrae.

Other mechanisms of glucocorticoids that probably contribute to the decrease in bone tissue are the development of hypogonadism, reduced physical capacity, inhibition of intestinal calcium absorption, and increased renal calcium losses. Glucocorticoids have catabolic effects on muscle, in addition to acting on bone and mineral metabolism, producing weakness due to muscle atrophy caused by accelerated muscle protein catabolism. Therefore, glucocorticoid-induced myopathy may contribute to bone deficit.

Effect of glucocorticoids on RANKL/RANK/OPG system

RANKL and OPG mediate a primary effect of glucocorticoids on the bone system. Glucocorticoids directly stimulate osteoclast differentiation by increasing RANK signaling and indirectly inhibiting interferon-β production. The latter suppresses osteoclast differentiation and stimulates RANKL production and OPG inhibition by osteoblastic cells, thus promoting osteoclastogenesis and bone resorption. These factors can contribute to the initial phase of glucocorticoid-induced bone loss (Figure 1B). When this occurs, patients may present with an impaired state of bone remodeling. Humphrey et al. evaluated the effect of different glucocorticoid receptor ligands on OPG and RANKL production and expression in human osteoblastic cell lines, and demonstrated that deflazacort was at least 10-fold less potent as an inhibitor of OPG production compared to prednisolone. Despite this difference in potency, deflazacort was as effective as prednisolone and dexamethasone in inhibiting OPG production at the maximum dose tested (10 μM). Furthermore, deflazacort stimulated RANKL expression by 1.2-fold, whereas prednisolone did so by up to 6-fold.

RANK/RANKL/OPG and Duchenne muscular dystrophy

Muscle hypertrophy or atrophy and gain or loss of bone mineral density occur in parallel in various

nucleus. TRAFs constitute a family of adaptor proteins, and most of them participate in the activation of the transcription factor NF-kB and the members of the mitogen-activated protein (MAP) family. Different studies suggest that TRAF plays a critical role in the differentiation and activation of osteoclasts.

At least seven signaling pathways are activated by RANK-mediated protein kinase: four regulate osteoclastogenesis, and three activate osteoclasts. OPG acts as a decoy receptor blocking the binding of RANKL to RANK; thus, it is a negative regulator of RANK signaling, capable of inhibiting osteoclastogenesis. Osteoblasts also produce OPG in response to anabolic agents such as estrogens and transforming growth factor β (TGF-β), which is related to bone morphogenic proteins (BMPs) and the Wnt/b-catenin pathway. In some studies, osteoporosis has been reported in OPG-deficient mice, while overexpression of OPG or its exogenous administration at high doses induces osteopetrosis-like changes, highlighting the physiological relevance of OPG.

Effect of glucocorticoids on the skeletal system

Glucocorticoids are a group of drugs widely used in different pediatric diseases due to their anti-inflammatory action. However, their use is associated with numerous adverse effects, including Cushing’s syndrome, diabetes, abdominal obesity, glaucoma, cataracts, growth retardation, hypertension, and musculoskeletal system disorders such as myopathy, osteonecrosis, and osteoporosis, which are mainly related to high doses and prolonged administration times.

Furthermore, glucocorticoid-induced osteoporosis is currently the leading iatrogenic cause of osteoporosis, a condition characterized by low BMD and impaired microarchitecture leading to decreased bone strength. Decreased BMD has been described in different pediatric pathologies with glucocorticoid administration, such as leukemias, juvenile rheumatoid arthritis, chronic renal failure, juvenile systemic lupus erythematosus, asthma, and neuromuscular disorders such as DMD.
physiological or pathological conditions. In addition, endocrine, mechanical factors, inflammation, and nutrition can simultaneously affect skeletal muscle and bone metabolism.

Skeletal muscle and bone form a single functional unit. This muscle-bone unit adapts synchronously during development and periods of modified mechanical loading, such as exercise or prostration or disease.

Figure 1. The bone remodeling process is controlled by various factors, among which the RANK/RANKL/OPG system is the most important. This system induces the differentiation and activation of osteoclasts and osteoblasts, balancing the remodeling cycle between bone formation and resorption. A: RANKL is secreted by osteoblasts, whereas RANK, its receptor, is located on preosteoclast cells. The RANKL/RANK interaction induces the formation of multinucleated mature osteoclasts, leading to bone resorption. A third component, OPG, is also produced by osteoblasts and functions as a decoy receptor by blocking the binding of RANKL to its cellular receptor RANK, acting as a negative regulator of RANK signaling, with the ability to inhibit osteoclastogenesis. B: glucocorticoids stimulate RANKL production and OPG inhibition by osteoblastic cells, which promotes osteoclastogenesis and bone resorption. All these factors may contribute to the initial phase of glucocorticoid-induced bone loss.

c-fms: colony-stimulating factor-1 receptor; MCSF: macrophage colony-stimulating factor; OPG: osteoprotegerin; RANK: receptor activator of nuclear factor-kappa B; RANKL: RANK natural ligand.
(prolonged bed rest, aging, spinal cord injury, critical illness, neuromuscular disease)\(^{44-47}\).

DMD is one of the best examples of synchronization between degeneration, muscle atrophy, and bone loss\(^{18,49}\). Muscle-bone interactions involve bone-derived osteokines, muscle-derived myokines, and dual-origin cytokines that trigger common signaling pathways leading to fibrosis, inflammation, and protein synthesis or degradation. In this regard, myokines can be interleukins, growth factors, or released peptides that can influence remote tissues such as bone. For example, interleukin 6 (IL-6), a proinflammatory cytokine, is among the most studied myokines for its effects on metabolism. IL-6 is a crucial mediator of bone homeostasis and an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Significantly higher IL-6 concentrations have been reported in patients with DMD and mice with X-linked muscular dystrophy (mdx) compared to healthy controls\(^{50}\). Alternatively, blockade of the IL-6 receptor with tocilizumab (a monoclonal antibody directed against the IL-6 receptor) has decreased muscle damage, fibrosis, and regeneration and increased muscle fiber diameter\(^{51}\). Like muscle cells, bone cells release osteokines such as osteopontin (OPN), a known inhibitor of bone mineralization. OPN is also expressed in inflammatory cells, such as macrophages, and its expression is significantly increased during inflammation. OPN is known to promote fibrosis. OPN ablation shifts dystrophic macrophages to a pro-regenerative phenotype, decreasing serum CK concentrations and changes in muscle mass and strength\(^{54}\).

The RANK/RANKL/OPG system regulates bone matrix modeling and remodeling pathways and contributes to muscle pathophysiology in DMD\(^{52}\). Furthermore, RANK, RANKL, and OPG mRNAs are present in skeletal muscle, and RANKL/OPG proteins are found in the myoplasm\(^{41,53}\). Dufresne et al. demonstrated that RANK is expressed in the sarcolemma membrane, potentially interacting with bone-derived RANKL\(^{41}\), and that fully differentiated myotubes secrete OPG, supporting the notion of bidirectional signaling between bone and muscle\(^{53}\). Meanwhile, Zhou et al. compared the expression of bone regulatory myokines in skeletal muscle. These authors compared the mdx model, the dKO model (mice lacking expression of both dystrophin and utrophin, which experience a more severe disease course that phenotypically resembles human DMD), and an age-matched (20-24 weeks) control group (wild-type mice, WT). Among the genes analyzed, the RANKL gene presented a significant increase in mRNA levels in dystrophic muscles, indicating a steady increase in protein concentrations in skeletal muscle tissues of the mdx and dKO dystrophic models compared to WT controls.

Conversely, myostatin showed significant down-regulation in mRNA levels in dystrophic muscles, suggesting a decrease in protein levels in mdx and dKO skeletal muscles compared to WT controls. Regarding the bone phenotypes of the dystrophic mice, computed microtomography analysis of the lumbar vertebrae showed that both mdx and dKO mice significantly decreased bone fraction, number, and thickness of trabecular bone and BMD, and an increase in trabecular spacing compared to WT controls. A significant decrease in mid-femoral bone areas was also observed in dystrophic mice compared to WT controls\(^{54}\).

RANKL participates in different signaling pathways\(^{28}\), including those dependent on NF-κB and Ca\(^{2+}\). Calcium is the primary regulator of multiple intracellular processes in muscle, including myosin-actin cross-linking, protein synthesis and degradation, mitochondrial adaptation, and fiber type switching. The latter is achieved through controlling proteases and transcriptional factors, such as the nuclear factor of activated T cells 1 (NFATc1)\(^{55}\). In osteoclasts, the RANKL/RANK interaction activates Ca\(^{2+}\) and TRAF/NF-κB-dependent signaling pathways, deregulated in DMD\(^{52}\).

A recent study demonstrated that concentrations of several members of the TNF receptor family are significantly elevated in the serum of mdx mice, including the RANK protein, suggesting that it may be involved in the DMD pathophysiology\(^{56}\). To determine the action of the RANK gene in dystrophic skeletal muscle, Dufresne et al. observed a significant improvement in the strength of the long extensor muscles of the fingers in mdx mice with muscle-specific deletion of RANK; however, it had no protective effects against eccentric contraction. These data indicate that the RANK/RANKL/OPG pathway may participate in the pathophysiology of dystrophic muscle.

Furthermore, research in this model has shown that RANK suppression and the use of OPG-Fc mitigate muscular dystrophy by significantly improving muscle strength\(^{56}\). Hamoudi et al. hypothesized that prolonged silencing of the RANKL/RANK signaling pathway would significantly improve DMD. They determined the efficacy of different doses of anti-RANKL in adult mdx mice with utrophin haploinsufficiency (mdx/utrn\(^{+/-}\)). The results showed that a 4 mg/kg/3d dose of anti-RANKL significantly improved the specific strength of the dystrophic extensor digitorum longus and soleus muscle compared to mdx/utrn\(^{+/-}\) mice treated with phosphate-buffered saline (PBS). However, the treatment did not protect the dystrophic muscles from loss of strength.
induced by repeated eccentric contraction. Also, hydroxyproline content (bone turnover marker) decreased by approximately 50% after anti-RANKL treatment. Serum CK levels were 6-fold higher in PBS-treated mdx/utrn+/- mice compared to WT mice. Treatment with anti-RANKL significantly reduced serum CK levels by approximately 35% compared with PBS-treated mdx/utrn+/- mice. The authors concluded that RANK/RANKL protein levels are higher in the microenvironment of dystrophic muscles and that inhibition of their interaction with anti-RANKL antibody results in decreased muscle damage, fibrosis, edema, inflammation, and inhibition of NF-κB activity. Anti-RANKL treatment also effectively preserved muscle function and increased bone stiffness in dystrophic mdx/utrn+/- mice.

Recent studies suggest that circulating levels of OPG could be involved in glucose homeostasis, in addition to being markers of bone metabolism. Bonnet et al. studied the effects of OPG-Fc, considering that RANKL inhibitors could positively influence muscle mass and strength, especially in osteoporosis or sarcopenia, with effects mediated by glucose regulation. The authors evaluated the impact of OPG-Fc administration (4 mg/kg) in muscle for four weeks in the HuRANKL+/- model (characterized by RANKL overexpression) and a Pparb/-/- model, which developed a combination of osteo/sarcopenia associated with impaired glucose homeostasis. The authors reported a decrease in muscle mass and strength in the HuRANKL+/- group compared to the WT group. In contrast, restoration of cortical bone volume, an increase in limb strength proportional to muscle mass growth, and a marked improvement in insulin sensitivity and muscle glucose uptake was observed in the HuRANKL+/- OPG-Fc group compared to the HuRANKL+/- group, which only received saline. In the Pparb/-/- model that received OPG-Fc, an increase in muscle volume and a tendency to gain strength were also observed compared to the control group. The authors concluded that the RANK/RANKL/OPG system plays a crucial role in bone and muscle metabolism.

As far as we know, few studies have evaluated the RANKL-OPG pathway in pediatric pathologies. Previous reports have shown that blood levels of RANKL are elevated in inflammatory and chronic processes and during treatment with glucocorticoids.

To date, information on children with DMD is limited to the work of Akhtar et al., who quantified the levels of RANKL, OPG, and the RANKL/OPG ratio in 50 children with DMD and 50 controls (mean age 11.2 ± 0.67 years). In patients with DMD, an inverse relationship between RANKL levels and age was reported (p = 0.0001), whereas controls showed no change (p = 0.52).

Alternatively, OPG and the RANKL/OPG ratio also decreased with increasing age (p = 0.026 and p = 0.002, respectively). Seventy-eight percent of glucocorticoid patients tended to have lower RANKL levels than those with no glucocorticoid intake (0.26 pmol/L vs. 0.40 pmol/L, p = 0.05). A possible explanation for these results was that children on glucocorticoid treatment were older (mean age 12.3 years vs. 7.4 years; p < 0.004), which is consistent with the inverse correlation between age and serum RANKL concentrations (r = -0.6, p ≤ 0.0001). Additionally, RANKL and RANKL/OPG levels were significantly higher in the ambulatory compared to the wheelchair-user group, probably because children with ambulatory DMD were younger (mean age 13.4 years vs. 7.6 years; p < 0.001). However, no correlation was found between RANKL and RANKL/OPG and densitometry Z-index and the presence of fractures.

Conclusions

Understanding the RANK/RANKL/OPG signaling system has elucidated its importance in the health of the skeletal system and its role in the development of different bone diseases. For example, severe osteoporosis with clinically significant bone fragility has been reported in pediatric patients with DMD. The weakness observed in DMD is partly the result of decreased gait, chronic glucocorticoid use, and changes in the molecular structure of the muscle-bone interaction, highlighting the action of RANK/RANKL/OPG. This system is determinant in maintaining the balance and timing of bone resorption and formation within the remodeling cycle. In addition, the study of the broad biological action of RANKL in other organs and tissues has suggested its activity in the pathophysiology of DMD. As a result, several strategies have focused on using anti-RANKL molecules, RANKL deletion, and OPG, in search of alternatives for treating DMD.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.
Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

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