SHORT COMMUNICATION

Osteoprotegerin Protects against Muscular Dystrophy

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Receptor-activator of NF- κ B, its ligand RANKL, and the soluble decoy receptor osteoprotegerin are the key regulators of osteoclast differentiation and bone remodeling. Although there is a strong association between osteoporosis and skeletal muscle atrophy/dysfunction, the functional relevance of a particular biological pathway that synchronously regulates bone and skeletal muscle physiopathology still is elusive. Here, we show that muscle cells can produce and secrete osteoprotegerin and pharmacologic treatment of dystrophic *mdx* mice with recombinant osteoprotegerin muscles. (Recombinant osteoprotegerin-Fc mitigates the loss of muscle force in a dose-dependent manner and preserves muscle integrity, particularly in fast-twitch extensor digitorum longus.) Our data identify osteoprotegerin as a novel protector of muscle integrity, and it potentially represents a new therapeutic avenue for both muscular diseases and osteoporosis. (*Am J Pathol* 2015, 185: 920–926; <http://dx.doi.org/10.1016/j.ajpath.2015.01.006>)

The discoveries of the receptor-activator of NF- κ B ligand (RANKL), the receptor-activator of NF- κ B (RANK), and the soluble decoy receptor osteoprotegerin (OPG) triad (RANK/RANKL/OPG) have been among the most important advances in the field of bone biology and disease.¹ RANK, RANKL, and OPG are members of the tumor necrosis factor superfamily and form a key cytokine system for regulating osteoclast differentiation and function.^{2,3} RANKL is expressed by osteoblasts whereas RANK, its receptor, is expressed on pre-osteoclastic cells.⁴ RANK/RANKL interaction induces the formation of multinucleated mature osteoclasts, ultimately causing bone resorption. OPG is a soluble decoy receptor for RANKL that inhibits RANKL/RANK interaction, osteoclastogenesis, and osteoporosis.⁵ OPG is also a decoy receptor for the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), and increases cell survival by preventing the activation of pro-apoptotic TRAIL death receptors-4-5 (DR4 and DR5).⁶ Overexpression of OPG in mice results in osteoporosis, whereas OPG-null mice suffer from severe osteoporosis.^{7,8} Therefore, the OPG:RANKL ratio in the immediate vicinity of bone cells is a decisive factor in the regulation of bone turnover.⁹

Skeletal muscles represent up to 35% to 45% of total body mass and are responsible for many vital functions such as movement, breathing, thermoregulation, and metabolism. Interestingly, osteoporosis and muscle atrophy/dysfunction occur simultaneously in a number of conditions, including spaceflight, extended bed rest, aging, and some muscular and neuromuscular diseases.^{10–12} For example, Duchenne muscular dystrophy (DMD) is a severe genetic disease characterized by critical muscle degeneration combined with low bone density.^{13,14} These observations suggest the existence of cross-talks and mutual cooperative interactions between bone and skeletal muscle. However, a possible common signaling pathway simultaneously controlling these different tissues has not been identified to date. The first objective of the present study was to investigate the ability of skeletal muscle in culture to secrete OPG. The second objective was to characterize the

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Figure 1 Lipopolysaccharide (LPS)-stimulated myotubes produce osteoprotegerin (OPG). The concentration of soluble OPG secreted by 1 μ g/mL LPS-stimulated myotubes was measured by an enzyme-linked immunosorbent assay. A threefold increase of OPG concentration in the supernatant was observed 24 hours after LPS stimulation. Data are shown as means $\pm$ SEM. * P < 0.05, one-way analysis of variance and Tukey *post hoc* tests. CM, culture media.

effect of OPG-immunoglobulin fragment complex (OPG-Fc) treatment on muscle function of dystrophic mice. Here, we show that muscle cells can produce and secrete OPG. Importantly, we show for the first time that OPG-Fc treatment strikingly improves skeletal muscle integrity and function in the *mdx* model of muscular dystrophy. OPG-Fc completely restores the function of the dystrophic extensor digitorum longus (EDL) muscle. The discovery of a common pathway linking bone and muscle wasting may provide powerful tools for developing more effective treatments for a wide variety of myopathies and muscle dystrophies.

Materials and Methods

Animals

Male wild-type (C57BL/10ScSnJ) and *mdx* dystrophic mice (C57BL/10ScSn-*Dmd*^{*mdx*}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at our animal facility. OPG-Fc (0.3 and 1 mg/kg per day for 10 days i.p.) was purchased from R&D Systems (Minneapolis, MN) and injected daily from days 25 to 35 after birth. The fusion to the Fc region prolongs OPG half-life in circulation and 1 mg/kg per day OPG-Fc is considered a low dose in rodent models of osteoporosis.¹⁵ Control mice were injected daily with the same volume of phosphate-buffered saline (vehicle). At the end of the different experimental procedures, mice were euthanized by cervical dislocation under anesthesia. All of the procedures were approved by the Université Laval Research Center Animal Care and Use Committee based on the Canadian Council on Animal Care guidelines.

Isometric Contractile Properties

Mice first were injected i.p. with 0.1 mg/kg buprenorphine to palliate the poor analgesic effect of pentobarbital. Mice

then were anesthetized i.p. with 50 mg/kg pentobarbital sodium 15 minutes later. Soleus (SOL) and EDL muscles were dissected and incubated in a buffered physiological salt solution (Krebs-Ringer), attached to an electrode and a force sensor as previously described.¹⁶ Diaphragm (DIA) muscle strips were dissected carefully to assess contractile properties as described by Lynch et al.¹⁷ Time-to-peak twitch tension (in milliseconds), half-relaxation time (in milliseconds), twitch tension (in grams), maximum tetanic tension (in grams), and maximum specific tetanic tension (in Newtons/cm²) values were obtained using a 305B-LR dual-mode lever arm system controlled by dynamic muscle control and data acquisition software (Aurora Scientific Inc., Aurora, ON, Canada). The cross-sectional areas were estimated by dividing the wet weight by the optimal muscle length multiplied by the muscle density (1.06 g/cm³) multiplied by the fiber-to-muscle length ratio for SOL and EDL muscles.¹⁸ Functional measurements were analyzed with Dynamic Muscle Data Analysis software version 6.1 (Aurora Scientific, Inc.).

Immunohistochemistry, Immunofluorescence, and Staining

Transverse sections of SOL, EDL, and DIA muscles (10 μ m) were cut (Leica Microsystems CM1850, Nussloch, Germany) in duplicate from the proximal and distal portions of the muscles. Sections were incubated overnight at

Figure 2 Osteoprotegerin (OPG)-fragment complex treatment mitigates loss of force in dystrophic muscles. Maximal specific force of soleus (SOL), extensor digitorum longus (EDL), and diaphragm (DIA) muscles from 5-week-old C57BL/10J or *mdx* mice treated from days 25 to 35 with vehicle or 0.3 or 1 mg/kg per day OPG-fragment complex i.p. OPG-fragment complex treatment rescues muscle force in a dose-dependent manner, especially in fast-twitch EDL muscle. Uninjured muscles from C57BL/10J mice were used as controls. Data are presented as means $\pm$ SEM. n = 6. * P < 0.05, ** P < 0.01, and *** P < 0.001, analysis of variance with Tukey *post hoc* test. PBS, phosphate-buffered saline.

Figure 3 Osteoprotegerin (OPG)-fragment complex (Fc) treatment greatly improves muscle integrity and reduces muscle inflammation. Immunohistochemistry analyses (**A**) show that the numbers of neutrophils (anti-Ly6C/Ly6G; **B**) and macrophages (anti-F4/80; **C**) are reduced significantly in extensor digitorum longus (EDL) muscles. Representative hematoxylin and eosin staining (**D**) and quantification of muscle damage using ImageJ software version 1.49i (**E**) show that 1 mg/kg per day OPG-Fc greatly reduces cell infiltration and muscle damage. Healthy muscles from C57BL/6J mice are shown as controls. Data are presented as means $\pm$ SEM. $n = 6$. * $P < 0.05$ and *** $P < 0.001$, analysis of variance with Tukey *post hoc* test. PBS, phosphate-buffered saline.

4°C with the following antibodies: rat anti-mouse F4/80 (1:100; Serotec, Oxford, UK) or Ly-6G and Ly-6C (Gr-1) antibodies (1:300; BD Bioscience, Mississauga, ON, Canada) to identify macrophages and neutrophils, respectively. Biotinylated secondary antibodies for immunohistochemistry were purchased from Vector Laboratories (Burlington, ON, Canada) and peroxidase conjugate and diaminobenzidine chromogen were used to show neutrophils and macrophages (Cedarlane, Burlington, ON, Canada). Hematoxylin and eosin staining (Sigma-Aldrich, St. Louis, MO) was used to visualize muscle integrity and quantify the extent of muscle damage. The damaged area was defined as an area not occupied by normal or regenerating muscle fibers. Sections were examined with an inverted microscope (Nikon, Mississauga, Canada) and analyzed with ImageJ software version 1.49i (NIH, Bethesda, MD).

Cell Culture

C2C12 myoblasts (ATCC, Manassas, VA) were cultured in high-glucose Dulbecco's modified Eagle's medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone) and 1% antibiotic-antimycotic (Life Technologies, Carlsbad, CA) in a 5% CO₂ and 37°C environment. When the myoblasts reached 90% confluence, the medium was replaced by high-glucose Dulbecco's modified Eagle's medium containing 1% fetal bovine serum for 5 days to allow the myoblasts to differentiate into myotubes. Myotubes then were treated with 1 μ g/mL lipopolysaccharides (LPS) (Sigma-Aldrich).

Enzyme-Linked Immunosorbent Assay

The concentrations of OPG and RANKL released by 1 mg/mL LPS-stimulated C2C12 myotubes were assessed with the commercial mouse Osteoprotegerin/TNFRSF11B and Mouse TRANCE/RANKL/TNFSF11 quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

All values are expressed as means $\pm$ SEM. OPG secretion by C2C12 myotubes were analyzed by one-way analysis of variance. All other data were analyzed by a three-way analysis of variance. All analyses of variance were followed by a Tukey test *a posteriori* (Instats Graph software version 3.1a; InStat, La Jolla, CA). $P < 0.05$ was considered statistically significant.

Results

Stimulated C2C12 Myotubes Secrete Osteoprotegerin

Skeletal muscle contains many cell types that potentially could produce OPG and/or RANKL, therefore we used C2C12 myotubes to determine whether muscle cells could secrete OPG *per se*. LPS was used to stimulate C2C12 myotubes because LPS is known to increase RANK expression and promote osteoclastogenesis *in vitro*.¹⁹ Soluble RANKL protein was undetectable in the culture media after LPS stimulation (data not

Figure 4 Schematic representation of the receptor-activator of NF-κB/receptor-activator of NF-κB/soluble decoy receptor osteoprotegerin (RANK/RANKL/OPG) pathway. In osteoclasts, RANK/RANKL interaction can activate different intracellular pathways, including TRAF6/NF-κB and Ca²⁺-calcineurin pathways. OPG is a soluble decoy receptor for RANKL and TRAIL, preventing osteoclastogenesis and/or cell apoptosis. Muscle cells can secrete OPG, and OPG-Fc treatment greatly improves muscle function and highlights the importance of cross-talk between skeletal muscle and bone, which may open new avenues for treatment of several forms of neuromuscular and muscular diseases. DR, death receptor; ER, endoplasmic reticulum; IP₃R, inositol triphosphate receptor; NFAT, nuclear factor of activated T cells; RyR1, ryanodine receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAF, tumor necrosis factor-receptor-associated factor; TRAF6, tumor necrosis factor-receptor-associated factor 6.

shown). However, LPS-stimulated myotubes produced and secreted OPG in a time-dependent manner, and OPG concentration in the media was almost threefold higher compared with control 24 hours after LPS stimulation (Figure 1).

OPG-Fc Treatment Greatly Increases Dystrophic Muscle Function

We next tested whether the pharmacologic blockade of RANK/RANKL with its molecular decoy OPG improves structural integrity and function of dystrophic muscles. Daily injections of 0.3 or 1 mg/kg per day OPG-Fc in *mdx* mice resulted in a dose-dependent gain of specific muscle force of 46% and 70% in SOL muscle, 114% and 233% in EDL muscle, and 27% and 59% in DIA muscle from *mdx* mice, compared with vehicle-injected *mdx* mice, respectively (Figure 2). No significant differences with respect to time-to-peak twitch tension, half-relaxation time, and muscle and body weight were observed after OPG-Fc treatment (Supplemental Table S1). Interestingly, OPG-Fc injection had no impact on muscle contractility in uninjured wild-type mice, suggesting that a pathologic process must be in place for OPG-Fc to be effective. Of note, contractile properties of developing SOL and EDL control muscles (5-week-old mice) are roughly 14 to 15 N/cm² at 25°C and 40% to 50% inferior to fully mature and adult muscles (12-week-old mice).^{20,21} These data show that muscle cells can secrete OPG and that OPG-Fc can restore muscle force in dystrophic *mdx* mice.

OPG-Fc Preserves Muscle Integrity and Reduces Leukocyte Accumulation in Dystrophic Muscles

Because OPG-Fc improves muscle force production drastically, we next tested whether OPG-Fc preserves muscle integrity and reduces inflammation in dystrophic muscles.

OPG-Fc treatment (1 mg/kg per day) for 10 days also resulted in a 52% and 44% reduction of neutrophil and macrophage numbers in SOL muscle (Supplemental Figure S1), a 68% and 47% reduction in EDL muscle (Figure 3, A–C), and a 30% and 18% decrease in DIA muscle (Supplemental Figure S2) from dystrophic mice relative to vehicle-injected dystrophic mice, respectively. Hematoxylin and eosin staining also showed a marked reduction in the damaged muscle area in the SOL muscle (Supplemental Figure S3A), the EDL muscle (Figure 3, D and E), and the DIA muscle (Supplemental Figure S3B) in OPG-Fc-injected *mdx* mice relative to phosphate-buffered saline-injected *mdx* mice. Thus, OPG-Fc treatment not only increases muscle force but also alleviates the structural muscle damage and inflammation observed in *mdx* mice.

Discussion

An intimate functional relationship exists between muscle and bone, and cumulative evidence indicates that anabolism or catabolism of these tissues occurs in synchrony.¹⁰ Consistently, mice deficient in myostatin have significantly more muscle mass and reciprocally present an increase in bone mineral density,²² whereas focal muscle paralysis has dramatic effects on bone resorption.²³ Here, we show for the first time that C2C12 myotubes, deprived of osteogenic conditions, can directly secrete significant amounts of OPG protein,²⁴ the bone antiresorptive agent. More importantly, we show that 10 days of treatment with OPG-Fc during the first and most important phase of muscle degeneration in dystrophic mice led to spectacular gain in muscle integrity and force, particularly in fast-twitch EDL muscle.

RANK, RANKL, and OPG mRNA expression have been noted previously in heart and skeletal muscles, but their roles on muscle physiopathology had not been investigated.^{25,26}

Our results show that OPG-Fc does not enhance the muscle force of healthy wild-type muscles, suggesting that a pathologic condition is required for OPG-Fc to be effective. One also could speculate that OPG-Fc may have an effect on other cell types, such as inflammatory cells, which potentially may affect muscle integrity. However, recent studies indicate that OPG-Fc is a pro-inflammatory molecule in several different biological tissues,^{27–29} implying that the reduction of neutrophil and macrophage accumulations in OPG-Fc-treated *mdx* muscles is a consequence of improved muscle integrity and not the cause. Nevertheless, the molecular mechanisms driving OPG-Fc protective effects on muscle function in dystrophic muscles remain to be clarified and may include one or several of the following pathways that are well characterized during osteoclastogenesis (Figure 4).

Because OPG-Fc reduces muscle weakness by controlling pathways that are dysregulated in dystrophic muscles, it is possible that OPG-Fc mediates its effects by regulating Ca^{2+} handling. Interestingly, increased Ca^{2+} levels and impaired Ca^{2+} flux are observed in dystrophic muscles even before the first signs of muscle degeneration.^{30,31} Overexpression of the transient receptor potential canonical 3 Ca^{2+} channel is sufficient to increase $[\text{Ca}^{2+}]_i$ and induce muscle degeneration similar to that observed in muscular dystrophy.³² By contrast, decreased Ca^{2+} influx by transgenic overexpression of a dominant-negative mutant of transient receptor potential canonical 6 in *mdx* or sarcoglycan-deficient (*Sgcd*^{−/−}) models of muscular dystrophy alleviates muscle degeneration.³³ Similarly, overexpression of the Ca^{2+} ATPase pump SERCA, which is responsible for Ca^{2+} reuptake into the sarcoplasmic reticulum, mitigates muscular dystrophy in dystrophin (*mdx/Dmd*) and sarcoglycan (*Sgcd*) null mice.³⁴ The presence of increased Ca^{2+} in dystrophic muscles also is associated with the activation of calpains (calcium-dependent cysteine proteases) and mitochondrial swelling.³⁵ The overexpression of calpastatin, a specific endogenous inhibitor of calpains, reduces muscle necrosis in dystrophic mice.³⁶ Overall, cumulative evidence indicates that dysregulation of $[\text{Ca}^{2+}]_i$ is related intimately to several forms of muscular dystrophy; however, a role for OPG-Fc in the regulation of calcium homeostasis has yet to be proven in skeletal muscles.

In bone, RANKL/RANK interaction activates cell signaling pathways via the recruitment of intracellular tumor necrosis factor–receptor–associated factor (TRAF) signal transducer proteins. The cytoplasmic domain of RANK can bind to TRAFs 2, 5, and 6, inducing the activation of NF- κ B, c-Jun NH(2)-terminal kinase, and mitogen-activated protein kinase pathways.³⁷ In particular, TRAF-6 has been identified as a key adaptor molecule downstream of RANK activation, and absolutely is required for functional bone resorption.³⁸ Muscle-specific TRAF-6 deletion reduces muscle atrophy and decreases the expression of ubiquitin-proteasome components in models of denervation or starvation.^{39–41} Interestingly, the level and activity of TRAF-6 is increased in dystrophic *mdx* mice and its inhibition improves muscle

structure, function, and mass.⁴² Furthermore, the NF- κ B pathway constantly is active in DMD patients,⁴³ and pharmacologic inhibition of NF- κ B in *mdx* mice reduces the inflammatory reaction and muscle necrosis associated with muscular dystrophy, ultimately resulting in increased muscle strength.⁴⁴ These results are very similar to our observations that OPG-Fc decreases muscle damage and inflammation and increases muscle function. It is highly plausible that OPG-Fc would reduce NF- κ B signaling in skeletal muscle in addition to regulating calcium handling. Indeed, NF- κ B and calcium signaling are interrelated tightly in dystrophic myofibers (eg, down-regulation of one pathway leads to down-regulation of the other).⁴⁵

Cumulative evidence supports the notion that myofiber death primarily occurs through necrosis in DMD patients and *mdx* mice and that the contribution of apoptosis in muscle wasting reportedly is negligible. However, caspases 3, 9, and 12 and BAX were shown to be activated in the *mdx* mouse, suggesting endoplasmic reticulum and mitochondrial stresses at early stages of muscular dystrophy.⁴⁶ More recently, muscle biopsy specimens showed an important increase of caspase-12 concentration in DMD patients and the genetic deletion of caspase-12 preserved muscle integrity and function of *mdx* mice after eccentric contraction-induced muscle damage.⁴⁷ TRAIL, mostly known for its anticancer properties, is potentially another cell signaling pathway through which OPG-Fc may interfere to preserve muscle function in *mdx* dystrophic mice. Indeed, TRAIL interacts with death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), inducing caspase cascade activation.⁴⁸ Although muscle cells express receptors DR4 and DR5,⁴⁹ a specific role for TRAIL-induced apoptosis has not been shown in DMD or Becker muscular dystrophy.⁵⁰ One cannot rule out the possibility that OPG-Fc, through its action as a decoy receptor for TRAIL, partially could reduce muscle degeneration in *mdx* mice.

In conclusion, we uncover a completely novel and critical role of OPG in controlling muscle function and integrity in a model of muscular dystrophy. Our data showing that OPG-Fc treatment protects SOL and DIA and to a greater extent EDL muscles are of paramount importance because defective fast-twitch fibers are recognized as one hallmark of several muscular and neuromuscular diseases.⁵¹ Although further studies certainly are required to decipher exactly how OPG, a known regulator of bone mass, improves muscle function, our discovery underscores the importance of cross-talk between muscle skeleton and bone and opens new avenues for the treatment of several forms of muscular and neuromuscular diseases in which osteoporosis often occurs synchronistically.

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experiments and data analysis; J.F., S.S.D., and N.A.D. wrote the manuscript; and all authors checked for scientific content and approved the final manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.01.006>.

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