Research article

Anabolic steroids reduce spinal cord injury-related bone loss in rats associated with increased Wnt signaling

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Background: Spinal cord injury (SCI) causes severe bone loss. At present, there is no practical treatment to delay or prevent bone loss in individuals with motor-complete SCI. Hypogonadism is common in men after SCI and may exacerbate bone loss. The anabolic steroid nandrolone reduces bone loss due to microgravity or nerve transection.

Objective: To determine whether nandrolone reduced bone loss after SCI and, if so, to explore the mechanisms of nandrolone action.

Methods: Male rats with complete transection of the spinal cord were administered nandrolone combined with a physiological replacement dose of testosterone, or vehicle, beginning on day 29 after SCI and continued for 28 days.

Results: SCI reduced distal femoral and proximal tibial bone mineral density (BMD) by 25 and 16%, respectively, at 56 days. This bone loss was attenuated by nandrolone. In *ex vivo* osteoclasts cultures, SCI increased mRNA levels for tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor; nandrolone-normalized expression levels of these transcripts. In *ex vivo* osteoblast cultures, SCI increased receptor activator of NF-kB ligand (RANKL) mRNA levels but did not alter osteoprotegerin (OPG) mRNA expression; nandrolone-increased expression of OPG and OPG/RANKL ratio. SCI reduced mRNA levels of Wnt signaling-related genes Wnt3a, low-density lipoprotein receptor-related protein 5 (LRP5), Fzd5, Tcf7, and ectodermal-neural cortex 1 (ENC1) in osteoblasts, whereas nandrolone increased expression of each of these genes.

Conclusions: The results demonstrate that nandrolone reduces bone loss after SCI. A potential mechanism is suggested by our findings wherein nandrolone modulates genes for differentiation and activity of osteoclasts and osteoblasts, at least in part, through the activation of Wnt signaling.

Keywords: Spinal cord injuries, Nandrolone, Androgens, Hypogonadism, Bone loss, Wnt signaling

Introduction

Spinal cord injury (SCI) causes unloading of skeletal regions immobilized by paralysis and extensive loss of sublesional bone.[1,2] The regions most affected by SCI are the distal femur and proximal tibia, where decreases in bone mineral density (BMD) may exceed 50%.[1,2] Such loss results largely from increased resorption of bone mediated through the increase of the numbers and activity of osteoclasts.[1,2] Bone resorption is stimulated by cells of the osteoblast lineage by the release of receptor activator of NF-kB ligand (RANKL), which stimulates differentiation and activity of osteoclasts.[3] RANKL is responsible for a great deal of the bone loss resulting from immobilization such as that occurring after SCI.[3] Cells of the osteoblast lineage also release osteoprotegerin (OPG), which inhibits RANKL.[3] Osteocytes, which are derived from osteoblasts after

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they become encased in bone, are the major source of RANKL in unloaded bone.[4]

The Wnt/β-catenin pathway is an important determinant of bone mass.[3,5] Canonical Wnt signaling occurs when Wnt signaling proteins such as Wnt3a bind to and activate a receptor complex composed of a frizzled receptor, of which there are 10, and either low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6.[3,5] Frizzled receptors then activate β-catenin which translocates to the nucleus.[3,5] Much of the signaling through β-catenin involves its binding to members of the TCF/LEF family of transcription factors, such as Tcf7 (also known as Tcf1).[3,5] Wnt signaling is coupled to mechanical loading of bone by alterations in osteocyte expression of the SOST gene, which encodes the Wnt inhibitor sclerostin.[6] Canonical Wnt signaling promotes osteoblastic differentiation of mesenchymal precursor cells through upregulation of Runx2.[7] Wnts stimulate osteoblasts to release OPG and to reduce expression of RANKL,[5,8] thereby reducing osteoclastogenesis and osteoclast activation.

Reduced levels of testosterone are common in men after SCI.[1,2] Hypogonadism results in high-turnover bone loss,[9,10] and thus may accelerate SCI-related bone loss. Interestingly, androgens, including nandrolone and testosterone, reduce bone loss after unweighting or nerve transaction.[11,12] Whether androgens prevent SCI-induced bone loss in animal models or humans is not known. Little is known about the molecular mechanisms by which androgens preserve bone in states of unloading or paralysis. Because androgens promote Wnt-dependent differentiation of cultured MC3T3 preosteoblasts,[13] one possibility is that androgens alter Wnt signaling in bone cells.

In this study, we tested whether nandrolone could reduce bone loss occurring in rats after transection of the mid-thoracic spinal cord. We also used *ex vivo* cultures of bone marrow cells to test the effects of nandrolone on expression levels of mRNA-encoding molecules necessary for osteoblast and osteoclast differentiation and activity, and of genes related to Wnt signaling.

Methods

Animals, spinal cord transaction, and drug administration

All animal studies were approved by the Institutional Animal Care and Use Committee at the James J. Peters VA Medical Center. As approximately 80% of individuals with SCI are male,[14] these studies were conducted using male rats. Because we found that administration of nandrolone during the second month after nerve transection reduced bone loss,[12] in the current study, administration of nandrolone was begun 29 days after SCI, and the animals were euthanized at day 56. Male Wistar rats aged 8 weeks were obtained from Taconic Farms (Hudson, NY, USA). One week later, the animals were anesthetized by inhalation of isoflurane and the spinal cord was transected at the interspace between the 9th and 10th vertebral bodies. Urine was voided at least three times daily; reflex voiding was not observed. Baytril was administered for the first 3–5 days postoperatively, and then as indicated for cloudy or bloody urine or for overt wound infection. Sham-transected (Sham-SCI) animals ($N = 7$) received an identical surgery, including a laminectomy, except that the spinal cord was not cut. Twenty-nine days after SCI rats were anesthetized with isoflurane, then randomly assigned to undergo implantation of Alzet pumps that infused either vehicle (SCI-vehicle; propylene glycol; Sigma, St Louis, MO, USA; $N = 8$) or nandrolone (SCI-Nandrolone; 0.75 mg/kg/week, Sigma; $N = 8$) plus testosterone (2.8 mg/kg/day; Spectrum Chemical Co., Gardena, CA, USA). Testosterone was administered together with nandrolone to control for effects of nandrolone to reduce circulating levels of testosterone due to feedback inhibition of central release of gonadotropins. The treatment of nandrolone plus testosterone is referred to henceforth in the text as "nandrolone."

Following spinal cord transection, the animals lost all voluntary movement of the knee and ankle joint, as well as most voluntary movement of the hip joint. There was no apparent difference among the groups in activity or movements of these joints or spasticity at any time during the 56 days after SCI. The animals were euthanized 56 days after SCI; at this time nandrolone had been administered for 28 days. Euthanasia was performed by transection of the aorta after anesthesia with isoflurane. The tibia, fibula, and femur were removed with the knee joint intact after carefully separating bone from muscle and connective tissue.

Bone densitometry

Areal BMD measurements were performed by using a small animal bone densitometer (Piximus, Fitchburg, WI, USA). The instrument was calibrated using a phantom following the manufacturer's recommendations. The precision for BMD measurements (coefficients of variation) is approximately 1.5% for the regions of interest (ROI). The femur and tibia were placed on a tray with the knee forming a 45° angle. Distal femur and proximal tibia were selected as ROI.

Ex vivo *osteoclastogenesis and osteoblastogenesis assays*

Procedures were adapted from those we have previously described.[15] To study osteoclast formation, bone marrow cells were isolated from the femora and tibiae in minimum essential alpha medium (α-MEM). Marrow cells were rinsed and resuspended in α-MEM then seeded into wells using an equal number of cells in each well, and were cultured for 2 days in α-MEM supplemented with human macrophage colony-stimulating factor (M-CSF; 5 ng/ml). The nonadherent cells were collected and purified by Ficoll-Plus (Amersham Pharmacia Biotech Inc., Arlington Height, IL, USA) then seeded into wells, again with an equal number of cells per plate, and incubated in α-MEM containing M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 4–6 days. To study osteoblast formation, equal numbers of bone marrow cells were seeded into wells and cultured in α-MEM supplemented with 15% preselected FCS (Hyclone, Logan, UT, USA) and ascorbic acid-2-phosphate (1 mM). Incubation was continued until day 28.

Quantitative polymerase chain reaction

Total RNA was extracted from cultured bone marrow cells and used to synthesize cDNA libraries that were used to measure mRNA levels by real-time quantitative polymerase chain reaction (qPCR) using TaqMan 2X PCR buffer. These measurements were performed using Assay on Demand probe sets obtained from Applied Biosystems (Forster City, CA, USA). qPCRs were performed in triplicate following the manufacturer's recommended procedures. The average of the crossing points for the replicates was used in subsequent calculations. Changes in mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method with levels of 18S ribosomal RNA serving as the internal control.

Statistics

The data are expressed as mean ± SEM. The number of animals (N) is provided in the legend of each figure. The statistical significance of differences among means was tested using one-way analysis of variance and a Newman–Keuls test *post hoc* to determine the significance of differences between individual pairs of means. A P value of 0.05 was used as the cut-off for significance of differences. Statistical calculations were performed using Prism 4.0c (Graphpad Software, La Jolla, CA, USA).

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Results

At 56 days after SCI, BMD for the SCI-vehicle group at the distal femur and proximal tibia were reduced ($P < 0.05$) compared with the Sham-SCI group by 25 and 16%, respectively (Figs. 1A and B). BMD in these regions was increased in the SCI-nandrolone group ($P < 0.05$) compared with the SCI-vehicle group by approximately 30%, although the values for BMD in the SCI-nandrolone group remained below the values observed for the Sham-SCI group (Figs. 1A and B).

Using total RNA from *ex vivo* cultures of bone marrow cell-derived osteoclasts, the expression of mRNA transcripts encoding two markers of osteoclast differentiation, tartrate-resistant acid phosphatase (TRAP) and the calcitonin receptor was examined. At 56 days after SCI, an elevation (two-fold; $P < 0.05$) of each transcript was observed compared with the Sham-SCI group (Fig. 2A). The levels of these mRNAs were reduced ($P < 0.01$) for the SCI-nandrolone group compared with the SCI-vehicle group to values that were not significantly different from the Sham-SCI group (Fig. 2A).

The mRNA levels for the osteoblast differentiation marker Runx2, and for the osteoblast-derived bone proteins osteocalcin and bone sialoprotein (BSP) were also assessed in *ex vivo* cultures of bone marrow-derived osteoblasts. The levels of all three mRNAs were markedly reduced ($P < 0.001$) for the SCI-vehicle group compared with the Sham-SCI group (Fig. 2B). The levels for Runx2 and osteocalcin were increased ($P < 0.05$) by 30 and 250%, respectively, for the SCI-nandrolone group compared with the SCI-vehicle group, although these

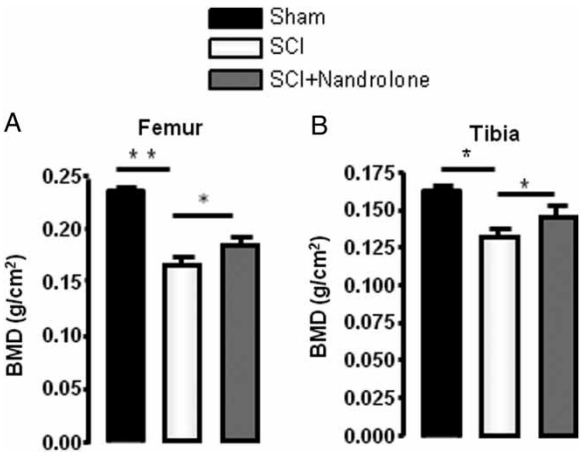

Figure 1 Effect of nandrolone on areal BMD of (A) the distal femur and (B) proximal tibia at 56 days after SCI. $N = 7$–8 per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls test *post hoc*. *$P < 0.05$ and **$P < 0.01$ versus the indicated group.

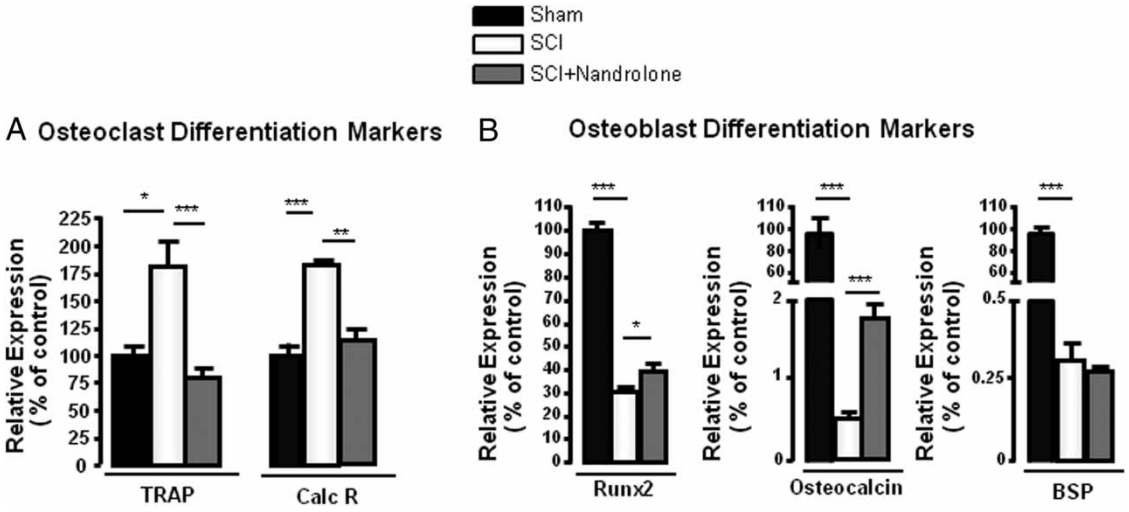

Figure 2 mRNA levels of differentiation markers in *ex vivo* cultured osteoclasts and osteoblasts derived from bone marrow cells from femur and tibia. (A) Levels in osteoclasts of mRNAs for TRAP and calcitonin receptor (Calc R). (B) Levels in osteoblasts of mRNAs for Runx2, osteocalcin, and BSP. Results are expressed as mean ± SEM for three to four animals per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls test *post hoc*. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the indicated group.

levels remained greatly reduced compared with the Sham-SCI group (Fig. 2B).

The levels for RANKL mRNA in *ex vivo* cultures of bone marrow-derived osteoblasts were markedly ($P < 0.001$) increased for the SCI-vehicle group compared with the Sham-SCI group; mRNA levels for OPG were not significantly different between these groups. Thus, SCI led to a marked decrease ($P < 0.05$) in the OPG/RANKL ratio (Fig. 3). Expression of OPG was increased ($P < 0.05$) in the SCI-nandrolone group compared with the SCI-vehicle group, without significant changes in RANKL mRNA levels (Figs. 3A and B), resulting in an increase ($P < 0.05$) in the OPG/RANKL ratio (Fig. 3C).

To evaluate changes in Wnt signaling, mRNA levels for Wnt3a, LRP5, Fzd5, and the Wnt-responsive gene ectodermal-neural cortex 1 (ENC1)[16] were examined. Expression of one transcription factor from the TCF/LEF family, Tcf7, was also examined. The levels for all of these transcripts were greatly reduced ($P < 0.05$) in the SCI-vehicle group compared with the Sham-SCI group (Figs. 4A–E). The levels of each of these mRNAs were increased ($P < 0.05$) for the SCI-nandrolone group compared with the SCI-vehicle group (Figs. 4A–E). More importantly, the expression level of Wnt3a for the SCI-nandrolone group was greater than that of the Sham-SCI group. The effects of nandrolone on expression levels of Tcf7, LRP5, and Fzd5 were

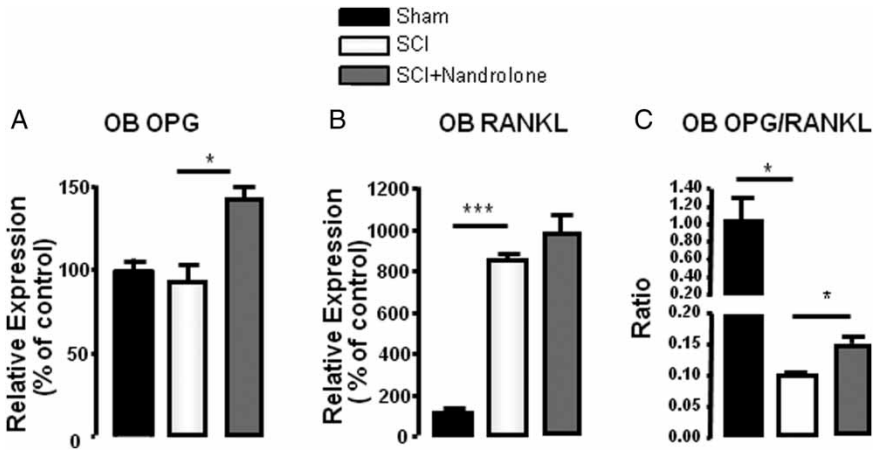

Figure 3 mRNA levels for OPG, RANKL and OPG to RANKL ratio in *ex vivo*-cultured osteoblasts. (A–C) Results are expressed as mean ± SEM of determinations for three to four animals per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls *post hoc* test. *$P < 0.05$, ***$P < 0.001$ versus the indicated group. OB, osteoblast.

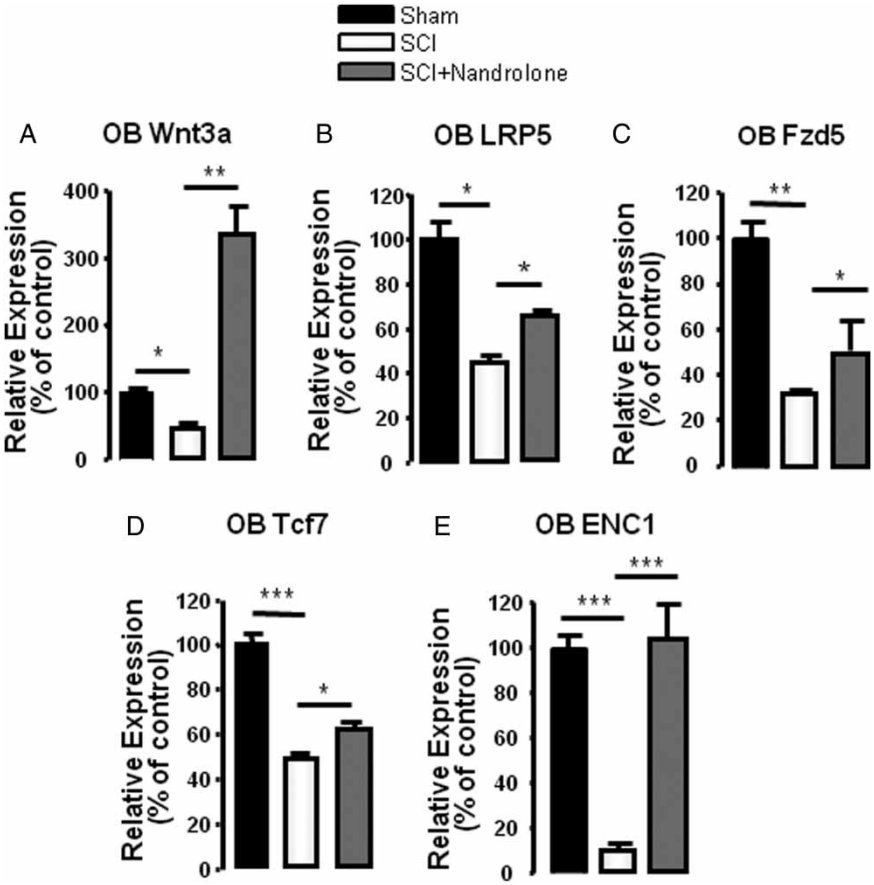

Figure 4 Effects of nandrolone on Wnt signaling. (A–E) Expression of Wnt3a, LRP5, Fzd5, Tcf7, or ENC1 mRNAs in *ex vivo* cultures of osteoblasts. Results are expressed as mean ± SEM of determinations for three to four animals per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls test *post hoc*. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the indicated group.

less dramatic; the levels for the SCI-nandrolone group remained lower than those for the Sham-SCI group (Figs. 4B–D), while the levels of ENC1 were same in the SCI-Sham and SCI-vehicle groups (Fig. 4E).

Discussion

Bone loss and alterations in gene expression after SCI in rats

Substantial loss of bone mineral content was found for the distal femur and proximal tibia of rats at 56 days after SCI; these are the two skeletal regions most greatly affected by SCIs in humans.[1,2] We found increased expression of two osteoclast markers in *ex vivo* osteoclast cultures from SCI rats, suggesting activation of these cells. We also observed marked increase in expression of RANKL, which stimulates osteoclast formation from marrow progenitor cells,[3] in *ex vivo* cultures of osteoblasts from SCI rats, consistent with increased RANKL expression by osteocytes in other models of bone unloading.[4]

Wnt signaling, a critical determinant of bone mass, exerts anabolic effects on osteoblasts, and both reduces release of RANKL and increases that of OPG, thereby reducing osteoclastogenesis and osteoclast activation.[5,8] It is thus notable that following SCI, *ex vivo* cultures of osteoblasts, demonstrated reduced expression of several genes for Wnt signaling, specifically Wnt3a, Tcf7, Fzd5, and LRP5. These changes in expression of Wnt-signaling genes were accompanied by reduced expression of the Wnt-responsive genes ENC1[16] and Runx2.[5,8] Such changes would be anticipated to exacerbate the known effects of unloading of bone to upregulate the expression by osteoblasts and osteocytes of sclerostin and DKK1, two Wnt inhibitors.[3] These findings are consistent with those reported after a microarray analysis of osteoblasts.[17] Thus, collectively, these alterations raise the possibility that following SCI, there is a reduction in Wnt signaling in osteoblasts in sublesional bone after SCI.

Effect of nandrolone on bone loss after SCI

Our study demonstrated for the first time that nandrolone reduced bone loss after SCI. Additional evidence

of the relevance of androgens to bone loss after skeletal unloading is provided by findings that deletion of the androgen receptor increased bone loss and levels of markers of bone resorption in a mouse model of hind limb suspension[18] and that nandrolone reduced bone loss after nerve transection or microgravity.[11,12] This finding is particularly important in the setting of SCI because of the prevalence of hypogonadism in men with SCI, particularly early after injury.[1,2] It should be noted that in our study nandrolone did not restore normal bone mass after SCI. Whether this reflects an inability to completely prevent bone loss after SCI as opposed to lack of effectiveness in reversing bone loss that occurred during the first 28 days after injury cannot be determined from our findings. An interesting question is whether greater protection against bone loss would result if administration of nandrolone was begun at the time of SCI.

Molecular mechanisms of nandrolone action after SCI

Cell culture studies suggest that androgens increase canonical Wnt signaling in cells of the osteoblast lineage and, thereby, promote osteoblastic differentiation.[13] Several findings from this study indicated that nandrolone increased expression of Wnt signaling after SCI. In *ex vivo* osteoblast cultures, nandrolone increased expression of the Wnt-signaling genes Wnt3a, Fzd5, LRP5, and Tcf7. Nandrolone also increased the expression of three Wnt/β-catenin-responsive genes (Runx2, OPG, and ENC1), all of which were downregulated by SCI. Runx2 is an early differentiation factor in osteoblastogenesis of mesenchymal progenitor cells,[3] and ENC1 has also been proposed to promote such differentiation (John Whitehead, personal communication). These findings indicate that nandrolone increased Wnt signaling in *ex vivo*-cultured osteoblasts and suggest that nandrolone increases Wnt signaling in unloaded bone after SCI. We expect that gene expression changes in osteocytes may closely resemble those in *ex vivo*-cultured osteoblasts, and this will be an interesting area for future study.

In *ex vivo* cultures of osteoclasts at 56 days after SCI, nandrolone reduced expression of TRAP and calcitonin receptor, suggesting inhibitory effects on osteoclastogenesis and/or activity. Such effects of androgens on osteoclastogenesis and/or activity may be, at least in part, result from androgen-mediated activation of Wnt signaling and subsequent increase in OPG expression relative to that of RANKL. This notion is supported by the evidence that testosterone increased OPG expression in mouse 3T3-E1 cells.[19]

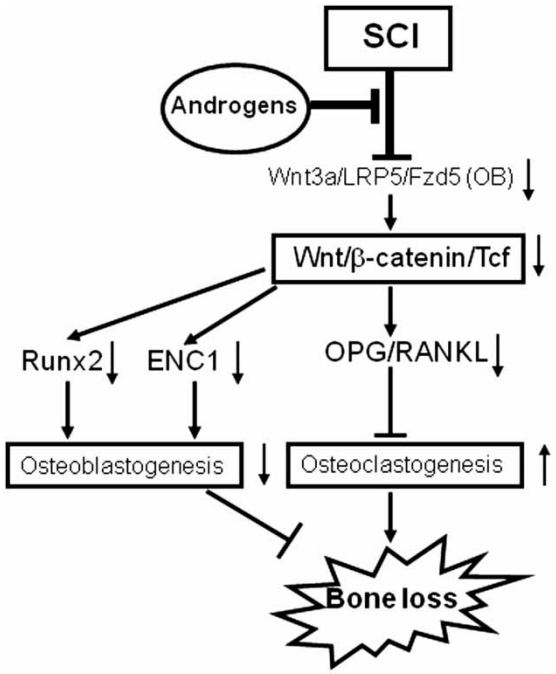

Figure 5 A scheme illustrating potential mechanisms by which nandrolone reduces bone loss after SCI. Small arrows indicate the direction of change after SCI. The findings of our study suggest that nandrolone increases levels of Wnt3a, LRP5, and Fzd5, and activates Wnt-signaling pathway, resulting in an increase in OPG/RANKL ratio, thereby inhibiting osteoclast differentiation. These effects of nandrolone are accompanied by increases in expression of RunX2 and ENC1, which may act by promoting osteoblast differentiation. These effects lead to a protection against bone loss after SCI.

Conclusions and clinical significance

The major finding of this study is that nandrolone reduced bone loss in a rat model of hind limb paralysis caused by a complete SCI. This finding is of potential clinical relevance because bone loss in individuals with motor complete SCI is rapid, severe, and refractory to bisphosphonates.[2] The possibility that a therapy with androgens might slow such loss is thus exciting. Our findings suggest a potential mechanism by which androgens reduce bone loss after SCI through the activation of Wnt signaling, leading to increased OPG expression and reduced osteoclastogenesis and osteoclast activity, as well as, potentially, increased osteoblastogenesis and osteoblast activity (Fig. 5).

Acknowledgements

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