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Secreted Wnt Signaling Inhibitors in Disuse-Induced Bone Loss

PRINCIPAL INVESTIGATOR:
Dr. Alexander Robling, Ph.D.

CONTRACTING ORGANIZATION:
Indiana University, Indianapolis
Indianapolis, IN  46202

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To investigate whether antagonizing secreted inhibitors of Wnt signaling is a viable approach for combating the bone loss that normally accompanies neuromuscular paralysis, we have conducted several paralysis experiments in mice that address the potential role of sclerostin inhibition in preventing paralysis-induced bone loss. In the first series, adult female mice were subjected to unilateral Botox-induced muscle paralysis of the lower limb via Botox (20 U/kg) injection into the right lower limb musculature. We performed this treatment in 2 types of mice: Wild type (Sost+/+) and Sost knock-out mice (Sost-/-). Despite the equal loss in muscle mass induced by Botox, Sost+/+ mice lost a significant percentage of their initial lower-limb aBMD and BMC over the experimental period, whereas bone mass in Sost-/- mice actually increased significantly (~2-4%; p<0.05) over the same period. Similar effects were seen for BV/TV, trabecular thickness, and trabecular bone mineral content (Tb.BMC) in the distal femur. We also investigated the osteopenic effects of Botox-induced muscle paralysis in WT mice that were given neutralizing antibody to sclerostin. Botox injection into the right limb musculature of vehicle-treated mice resulted in an 8.4% decrease (p<0.001) in femoral aBMD, whereas mice given Scl-AbIII after Botox injection exhibited an 8.0% increase in femoral aBMD in the paralyzed limb. In summary, these data suggest that sclerostin inhibition is a useful approach for overcoming the bone loss that normally occurs with disuse. We are also investigating the efficacy of Dkk1 neutralization (and genetic deletion) for preventing disuse induced bone loss. Those studies are still underway.
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INTRODUCTION

Osteoporosis (porous bone disease) is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures. Disuse osteoporosis occurs when the normal loading environment experienced by bone cells is reduced or removed. A decreased mechanical loading environment (e.g., as occurs in soldiers after spinal cord injury or muscle paralysis) leads to rapid bone loss via enhanced local osteoclastic bone resorption, concomitant with a suppression of bone formation. We proposed to determine whether local, secreted regulators of Wnt/Lrp signaling (Sost, Dkk1) modulate bone loss in response to mechanical disuse. Furthermore, we proposed to test whether these molecules can be manipulated to prevent bone loss that normally accompanies disuse. To accomplish this goal, we proposed to induce disuse (using Botox-induced paralysis of the quadriceps, hamstrings, and soleus) in one hindlimb of a series of mice with mutations in Wnt modulators (Sost<sup>−/−</sup>, Dkk1<sup>−/−</sup>) and in wild-type mice that are also treated with neutralizing antibody to Dkk1 or Sost. These experiments have the potential to reveal new treatment strategies for overcoming the disuse-associated bone loss that accompanies spinal cord injury, and other battlefield-related injuries resulting in neuromuscular impairment.

BODY

**Sclerostin knockout mice are protected from the bone-wasting effects of muscle paralysis.**

Adult male Sost<sup>+/+</sup> and Sost<sup>−/−</sup> mice were raised to 25 wks of age, at which point they were subjected to unilateral Botox-induced muscle paralysis of the lower limb via botulinum toxin (Botox; 20 U/kg) injection into the quadriceps, hamstrings, triceps surae, and leg extensor compartment of the right lower limb. The left lower limb was injected identically with saline (internal control). The mice underwent unilateral muscle paralysis for three weeks. Impaired limb function was apparent 2 days after Botox treatment, revealed by a failure of the foot to cling to a support when the mouse was inverted. Prior to Botox treatment, the mice were given whole-body scans via high-resolution DEXA using a pixiMUS mouse densitometer. Mice lost a slight significant (~13-15%, P<0.05) percentage of their body mass over the experimental period (Fig 1, left), but Sost genotype had no effect on their weight loss. Likewise, Botox treatment caused significant muscle atrophy in both genotypes (~47-49% loss; p<0.001), as assessed by quadriceps weight at sacrifice from Botox-treated vs. saline treated limbs (Fig. 1, right). Despite the equal loss in muscle mass, Sost<sup>+/+</sup> mice lost a significant percentage of their initial lower-limb aBMD and BMC over the experimental period, whereas bone mass in Sost<sup>−/−</sup> mice actually increased significantly (~2-4%; p<0.05) over the same period (Fig 2). This trend in the Sost<sup>−/−</sup> mice was also observed in the saline treated limb, suggesting that the normal increase in bone mass in adult male Sost<sup>−/−</sup> mice was not hampered by disuse. We next examined
bone loss in the distal femoral metaphysis by comparing saline-treated to Botox-treated femora after sacrifice (Fig. 3). Bone volume fraction (BV/TV), trabecular thickness, and trabecular bone mineral content (Tb.BMC) were significantly decreased (~18-28%; p<0.01) by Botox treatment in Sost+/+ mice, but those same trabecular properties were unchanged by Botox treatment in Sost−/− mice. Next, we extracted mRNA from whole tibias that were snap-frozen in liquid N₂ immediately after sacrifice. We performed real-time PCR reactions for genes associated with osteoclastic resorption regulation (Fig. 4). Disuse resulted in no change in M-csf in either genotype. Osteoprotegerin was reduced significantly by Botox treatment in both genotypes, whereas RankL expression was significantly enhanced in the paralyzed limbs of Sost+/+ but not Sost−/− mice.

**Sclerostin-neutralizing antibody protects the normal (wild-type) skeleton from disuse-induced bone loss.**

Adult female Swiss-Webster mice were subjected to unilateral botox-induced muscle paralysis of the lower limb via botulinum toxin (Botox; 20 U/kg) injection into the quadriceps, hamstrings, triceps surae, and leg extensor compartment of the right lower limb. The left lower limb was injected identically with saline (internal control). The mice underwent unilateral muscular paralysis for three weeks in the presence or absence of twice-weekly dosing with a sclerostin neutralizing antibody (Scl-AbIII; 25 mg/kg) injection. Prior to Botox treatment, the mice were scanned through the proximal and midshaft tibia using high-resolution pQCT (Stratec SA+), and a whole-body DEXA scan was collected using high resolution DEXA (pixiMUS) prior to disuse onset, and again at sacrifice. Mice receiving Scl-AbIII exhibited a slight (5%) but significant increase in body weight over the 3-wk treatment period, whereas mice receiving vehicle control injections had no change in weight (Fig. 5). Saline injection into the left lower limb musculature resulted in slight but significant decrease in femoral aBMD (-2.6%, p<0.01) over the 3-wk treatment period, whereas Botox injection into the right limb musculature of these same animals resulted in an 8.4% decrease (p<0.001) in femoral aBMD (Fig. 6). Mice given Scl-AbIII during the wk period following Botox injection exhibited an 8.0% increase in femoral aBMD in the paralyzed limb. This increase was significantly different from the changes occurring in the saline-injected limbs of mice not receiving antibody (p<0.001), suggesting that Scl-AbIII not only protected these
paralyzed limbs from bone loss, but actually increased bone mass over non-paralyzed, saline-injected limbs. Similar effects were noted for femoral BMC. We were unable to detect any Botox-related changes in the distal femur metaphyseal trabecular bone using μCT scanning, and therefore the Scl-AbIII effects on disuse were not amenable to evaluation using this technique. However, we did take baseline and post-treatment pQCT slices through the proximal tibia, which is a trabecular-rich site, and found a significant Botox effect (~3.5% loss), which was nullified by Scl-AbIII treatment (Fig. 7). In summary, these data suggest that sclerostin inhibition is a useful approach for overcoming the bone loss that normally occurs with disuse/paralysis.

**Genetic manipulation of Dkk1 in the bone-wasting effects of muscle paralysis.**

We began by looking at the effects of disuse in mice that lack Dkk1. Homozygous knockout of Dkk1 produces an embryonic lethal phenotype, so it is not possible to conduct muscle paralysis experiments in a true Dkk1 knockout. For that reason, we had proposed to look at the effects of muscle paralysis in mice that are heterozygous mutants (Dkk1+/-). Unfortunately, our Dkk1+/- colony had great difficulty breeding over the winter, to the point where we lost the mutant allele. We typically experience a large drop-off in breeding capacity in our colonies over the winter, but not to the point of losing an allele. The complete inability (or refusal) of the Dkk1+/- mice to breed was completely unexpected. Having no mice with which to study the effects of genetic deletion or reduced expression of Dkk1 on mechanical disuse, we decided to take this opportunity to refine our experiments. We acquired a floxed loss-of-function mouse model for Dkk1 generated by Christof Niers’ group in Finland. This mouse has loxP sites flanking exons 1 and 2 of the Dkk1 coding region.1 Rather than reimport the Dkk1+/- mouse from our colleagues, we decided that a better strategy would be to acquire the Dkk1 flox (+) mouse from Finland (Figure 1). We have revised our ACURO animal protocol to include that mouse in the DOD work. We have bred the Dkk1 flox mouse to the Dmp1-Cre, then interbred these mice to generate offspring that are homozygous for the Dkk1 flox allele and are positive or negative for Cre. This strategy allowed us to delete Dkk1 from osteocytes (the mechanosensors) and late stage osteoblasts. These oldest cohort of these mice is now (as of June 5, 2013) 12 wks of age. We are waiting until they are 16 wks of age, as described in the original protocol, before we begin Botox experiments, in order to model the adult skeleton in disuse. This will allow complete recombination in the bone and produce a bone-selective knockout of Dkk1. In the end, this will be a much better approach than a loinsufficient Dkk1 mouse model, which was our original intent.

**Role of Dkk1-neutralizing antibody in the bone-wasting effects of muscle paralysis**

Adult male Swiss-Webster mice were purchased and subjected to unilateral Botox-induced muscle paralysis of the lower limb via botulinum toxin (Botox; 20 U/kg) injection into the quadriceps, hamstrings, triceps surae, and leg extensor compartment of the right lower limb. The left lower limb was injected identically with saline (internal control). The mice underwent unilateral muscle paralysis for three weeks. Impaired limb function was
apparent 2 days after Botox treatment, as revealed by a failure of the foot to cling to a support when the mouse was inverted. Prior to Botox treatment, the mice were given whole-body scans via high-resolution DEXA using a pixiMUS mouse densitometer. Mice in the control group (non-Botoxed) gained ~5% of their initial BMC in the tibia, over the experimental period (Figure 9A). Conversely, mice in the paralysis group lost ~10% of their initial tibial BMC. Dkk1 neutralizing antibody treatment significantly altered the bone response to paralysis, lowering the loss to ~3%. While this difference reached significance when compared to the saline-injected Botox-treated mice, it was significantly different from (less than) zero, indicating that significant bone loss occurred in the paralyzed limb despite Dkk1 inhibition. A much less clear result was found when the distal femur metaphysis was analyzed. We scanned the distal femur using microCT, and found no real effect of either antibody (which was very surprising) or disuse (Figure 9B).

Upon further probing of the raw data, we found that the amount of variation among the different mice within a group was extremely large. Figure 10 shows the BV/TV from the control group (no antibody, no Botox). Upon inspection, it is evident that BV/TV ranges from 8% to 43%. Give this degree of variation, it would be nearly impossible to find any between-group differences that reached statistical significance. It is unclear why the batch of mice that we used had such great variation, but there are two solutions to the problem. One is to account for the variation prior to the experiment by taking a pre-treatment microCT.

We did not have the technology for in vivo microCT when the experiment was conducted, but we now have access to such an instrument (Scanco In Viva 40) through Theresa Guise’s lab at our institution. The second remedy is to use an inbred strain of mouse. We have taken both approaches, and we have found that, based on the pQCT analysis in the proximal tibia, it appears that they lose bone with Botox treatment, but that the antibody is not very effective in preventing the disuse induced bone loss (Fig. 11). These results are in agreement with some recent data that the Amgen group has published regarding Dkk1 expression as a function of aging. They found (JBMR 2011 26:2610-21) that Dkk1 expression is markedly reduced in the aged rodent skeleton, and that Dkk1 antibody might not be effective in the adult because there is very little Dkk1 for the antibody to inhibit. If that result is correct, Dkk1 inhibitors might not be a viable therapeutic for disuse-induced bone loss among soldier-age (adult) individuals. Our experiments employing bone-specific genetic deletion of Dkk1 will be definitive in terms of whether this mechanism will be therapeutic for disuse.

**KEY RESEARCH ACCOMPLISHMENTS**

- We determined that genetic deletion of the Sost gene protected mice from the bone-wasting effects of...
We determined that Sost deletion prevents the paralysis-induced upregulation of RankL expression, which normally accompanies disuse.

We determined that adult-onset sclerostin inhibition (via neutralizing antibody) prevented the bone-wasting effects of muscle paralysis in normal (wild-type) mice.

We determined that anti-sclerostin therapy might actually induce increased bone formation in the presence of mechanical disuse.

Our preclinical studies indicate that sclerostin inhibition might be a useful therapy for soldiers that have experienced muscle paralysis, in order to maintain bone mass in the rehabilitating limb.

Our results suggest that Dkk1 inhibition might be less efficacious in protecting limbs from bone loss that accompanies muscle paralysis, though our genetic study (to be completed this summer) will make this result more clear.

REPORTABLE OUTCOMES

Our data regarding the efficacy of sclerostin inhibition in preventing disuse induced bone loss is encouraging, and suggests a potential application to military personnel returning from the battlefield with muscle paralysis. These data have been presented at a number of scientific conferences, including the American Society for Bone and Mineral Research meetings, and the American College of Rheumatology. Other smaller conferences have also been host to these data, including Genetics and Evolution of the Skeleton Research Initiative” (GESRI) held at UCSF. The second is a Gordon Conference on Bones and Teeth, held in Les Diableretes, Switzerland. In these talks, I have and will continue to attribute my funding source for the data to the DOD’s CDMRP.

None of the data have yet been published. I plan to begin writing up the Sost genetic study, the sclerostin antibody study, and both the Dkk1 genetics study and antibody study all as one large and thorough paper, and the target date for submission is October 2014.

CONCLUSIONS

Our experiments, to date, support the hypothesis that sclerostin inhibition can significantly reduce, or perhaps even eliminate, the bone-wasting effects of mechanical disuse that occur following neuro-muscular paralysis. Amgen, Inc. currently has a humanized monoclonal antibody to sclerostin (romosozumab) that is currently in phase III clinical trials for postmenopausal osteoporosis. Once that compound makes it to market, there is significant precedent to try that compound in veterans returning from the battlefield that are undergoing disuse osteoporosis. The efficacy of Dkk1 inhibition is less clear, mainly because the genetic models to properly study this mechanism have only recently come on line.
“So what?”

These pre-clinical results are important because many soldiers returning from Afghanistan present with spinal cord injuries (SCI) or other peripheral nerve injuries that induce paralysis. Disuse osteoporosis is a common sequelae of spinal cord injury (SCI) an peripheral nerve damage. Bone mineral content (BMC) can decrease by as much as 70% following SCI. This bone loss results in increased bone fragility and a subsequent increase in the risk for low-trauma fractures. Disuse osteoporosis is a particularly debilitating disease for soldiers in whom recovery of neuromuscular function is possible, because hard-fought gains in neuromuscular rehabilitation can be lost if the underlying bony structure has deteriorated to the point where muscle activity induces fractures. If this situation arises, neuromuscular training must cease to allow the fracture to heal. Our experiments suggest that targeting sclerostin might be a therapeutic approach to accompany neuromuscular rehabilitation, because it appears to preserve the bone mass and structure in paralyzed limbs, ultimately providing a foundation for neuromuscular recovery.

PERSONNEL SUPPORTED BY THE AWARD
Alexander Robling
Stuart Warden
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REFERENCES
A large, high-impact paper containing all of the positive Sost/sclerostin results, and also including the negative results of the Dkk1 experiments (should the negative result hold up in the further analysis of the antibody study and in the genetic study) is being assembled now. We plan to submit this work to J Biol Chem.

Abstracts:
Downregulation of Sost/sclerostin Expression is Required for the Osteogenic Response to Mechanical Loading (ASBMR)
* Xiaolin Tu, Indiana University School of Medicine, USA, Yumie Rhee, Department of Internal Medicine, College of Medicine, Yonsei University, SOUTH KOREA, Racheal Lee, Indiana University, USA, Jeffrey Benson, Indiana University, USA, Keith Condon, Indiana University, USA, Nicoletta Bivi, Eli Lilly & Co., USA, Lilian Plotkin, Indiana University School of Medicine, USA, Charles Turner, Indiana University, Purdue University Indianapolis, Alexander Robling, Indiana University, USA, Teresita Bellido, Indiana University School of Medicine, USA; Oral Poster Session, Presentation Number: FR0053

Lrp5 G171V and A214V Knock-in Mice are Protected from the Osteopenic Effects of Sost Overexpression (ASBMR)
* Alexander Robling, Indiana University, USA, MATTHEW Warman, Children's Hospital Boston, USA, Teresita Bellido, Indiana University School of Medicine, USA, Paul Niziolek, Indiana University School of Medicine, USA; Oral Presentations, Presentation Number: 1227

Lrp5-deficient Mice are Responsive to the Osteo-anabolic Action of Sclerostin Antibody (ASBMR)
* Alexander Robling, Indiana University, USA, Matthew Warman, Howard Hughes Medical Institute, Department
Sclerostin Antibody Protects the Skeleton from Disuse-induced Bone Loss (ASBMR)
* Alexander Robling, Indiana University, USA, Stuart Warden, Indiana University, USA, Chris Paszty, Amgen, Inc., USA, Charles Turner, Indiana University, Purdue University Indianapolis; Oral Presentations, Presentation Number: 1039

APPENDICES
N/A

SUPPORTING DATA
N/A