Role of Transient Muscle Paralysis on the Development of Heterotopic Ossification

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Abstract: Heterotopic ossification (HO), the formation of bone in soft tissues, is a complication associated with brain and spinal cord injury, joint arthroplasty, musculoskeletal trauma, and amputation. As current HO treatments are ineffective, the development of novel therapies to prevent HO has potential to improve patient quality of life and reduce healthcare costs. Recently, we observed that blockade of neuromuscular function by botulinum toxin A (BTxA) in a murine bone trauma model inhibited 90% of the injury-induced bone formation. Based on this result, and the established literature on neuronal involvement in HO pathogenesis, we hypothesized that neuromuscular blockade would inhibit HO. To test this hypothesis, we implemented a model for HO, achieved via an intramuscular injection of bone morphogenic protein (BMP) impregnated in a carrier matrix (Matrigel). BMP2 or BMP4 (2.5µg/50µl) was injected into the right calf and HO formation was measured via high resolution microCT imaging (Scanco vivaCT 40; 21µm voxel) at days 0, 10, 17, and 21. MicroCT scanning identified pronounced HO with BMP2- and BMP4- Matrigel injections beginning at day 10, which became progressively more mineralized by day 21. Following model implementation we designed a follow up study to test our primary hypothesis and demonstrated that paralysis of the calf muscle with BTxA reduced BMP-induced HO at day 10 and 17. These pilot data provide preliminary evidence that neuromuscular signaling may be important in the induction and mineralization of HO.

1. INTRODUCTION

Heterotopic ossification (HO), the formation of new osseous (ectopic) bone in soft tissues, is a complication associated with brain and spinal cord injury, joint arthroplasty, musculoskeletal trauma, and amputation. While HO develops, joint motion becomes restricted. Annually, 1.7 million cases of HO are reported. Of those cases, 10 to 20% are considered clinically significant requiring surgery [3,8].

Currently, there is no standard of care to prevent HO. Treatments used in the clinic all vary in efficacy, side effects, and cost. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to decrease HO induction, yet 12.5% of patients continue to develop clinically significant HO. Long term NSAID usage can cause skeletal complications and gastrointestinal bleeding. Radiation therapy may provide a more targeted treatment method; however, it is limited in use and excess radiation causes known side effects. Finally, surgical treatment is the most costly and invasive option; but postoperative complications occur at a much greater frequency in this patient population [8].

As current HO treatments are minimally effective, the development of novel therapies to prevent HO has potential to improve patient quality of life and reduce healthcare costs. In preliminary studies, we observed that when blocking neuromuscular function by botulinum toxin A (BTxA) in a murine bone trauma model, 90% of the injury-induced bone formation was inhibited. No effect was observed on bone healing at the defect site or formation of bone on the endocortical surface had occurred. BTxA (registered Botox®), a neurotoxin, induces dose-dependent muscle paralysis by inhibiting the release of acetylcholine into the neuromuscular junction, resulting in a transient and localized muscle paralysis at the injection site [7].

Given the pathophysiology of HO is poorly understood, there is speculation in the literature that an association exists between neurogenic signals and HO induction [1,8]. Specifically, De Paz et al. have posited that HO in patients with injuries to the central nervous system is related to dysfunction of neuromuscular signaling pathways [1]. Notably, Kan et al. provided evidence showing transgenic mice over expressing BMP4 at the neuromuscular junction under control of the neuron-specific enolase promoter develop a fibrodysplasia ossificans progressiva (FOP)-like phenotype [4]. FOP is a rare hereditary tissue disease of HO [2].
Based on our preliminary results, and the established literature on neuronal involvement in HO pathogenesis, we have hypothesized that neuromuscular blockade will inhibit the formation of heterotopic bone. This hypothesis was explored via two specific aims: 1) HO model development; and 2) Effect of BTxA on development and mineralization of HO.

2. MATERIALS & METHODS

2.1 BMP-Matrigel Preparation
Matrigel basement membrane matrix high concentration (BD Biosciences) was stored at between -20°C and -70°C and thawed before usage at 4°C on ice. The 10μg BMP-2 or BMP-4 was reconstituted with 25μl 4mM HCl. The reconstituted BMP-2 (R&D Systems Recombinant Human) or BMP-4 (R&D Systems Recombinant Mouse) was added to 175μl Matrigel for a final volume of 200μl to yield a concentration of 2.5μg/50μl [5]. The control suspension was formulated the same, except 25μl of 4mM HCl was added to the Matrigel. Efficient delivery required the use of a Hamilton syringe with 25G needle.

2.2 In vivo HO model
All animal procedures were performed using protocols and procedures approved by the Institutional Animal Care and Use Committee of the University of Washington. Ten female C57BL/6 mice (16wk) were injected intramuscularly with bone morphogenic protein 2 (BMP2-Matrigel; n=3), bone morphogenic protein 4 (BMP4-Matrigel; n=3), or matrigel vehicle (n=4) at 2.5μg/50μl in the mid-belly of the right calf under isoflurane anesthesia on day 0 of experimentation [5]. High resolution microCT imaging (Scanco vivaCT 40; 21μm voxel) of the tibial diaphysis and surrounding soft tissue followed at days 0, 10, 17, and 21. Primary outcome measures assessed mineralized nodule volume denoted by bone volume/ tissue volume (BV/TV) and bone mineral density (BMD) to determine the magnitude and maturation of HO. Three-dimensional computerized images of the region injected were reconstructed to identify HO nodules. The volume (mm³) of the mineralized tissue was calculated by the software program given with the micro-CT system. Bone density was determined with a calibration phantom; density greater than 260 mg/ml hydroxyapatite was considered mineralized tissue [6]. Statistical mean and standard deviation were calculated. All mice were group housed and allowed free ambulation during the experiment.

2.3 In vivo transient muscle paralysis effect on HO
Six mice received an injection of BTxA (Botox®, Allergan; 2U/100g, 20μl final volume; n=4) or equal volume saline (n=2) one day prior to BMP2- or BMP4- Matrigel injections (n=3 each). High resolution microCT imaging followed in the same region of interest as before at day 10 and 17. Bone volume (BV) and bone volume/ tissue volume (BV/TV) of the mineralized HO nodule was calculated. All calculations and statistics were measured the same as in the aforementioned procedure.

3. RESULTS

3.1 Model Development: BMP2 and BMP4 Induce Heterotopic Bone in Calf Muscle
MicroCT scanning identified pronounced HO with BMP2-Matrigel and BMP4-Matrigel injections at day 10, which became progressively more mineralized by day 21 in the region of the tibial diaphysis and surrounding tissue (Figure 1). No visible mineralized tissue was identified or calculated with Matrigel control. Two mice in the BMP4-Matrigel group did not receive an injection volume. Mean BV/TV at day 10 was 0.01 ± 0.01 and 0.01 for BMP2-Matrigel (n=3) and BMP4-Matrigel (n=1), respectively. By day 17 and day 21, the mean BV/TV increased to 0.07 ± 5.72E-4 and 0.08 ± 5.26E-3 for BMP2-Matrigel; BV/TV increased to 0.06 and 0.07 for BMP4-Matrigel (Figure 2A). BV/TV appeared greater in mice
Figure 1. MicroCT scanning identified pronounced HO with (A) BMP2-Matrigel and (B) BMP4-Matrigel injections at day 10, which became progressively more mineralized by day 21.

Figure 2. Quantification of mineralized nodule in calf muscle- (A) bone volume/total volume (BV/TV) and (B) bone density. BV/TV appeared greater in mice injected with BMP2 (n=3) vs. BMP4 (n=1) at days 17 and 21. Maturation of mineralized tissue increased in both from day 10 to 21.
injected with BMP2 vs. BMP4. Maturation of mineralized tissue (i.e. density greater than 260 mg HA/ml) increased in both from day 10 to 21 (Figure 2B). Mineralized nodule density for BMP2-Matrigel (n=3) injections were 376 ± 38, 493 ± 40, and 535 ± 26; for BMP4-Matrigel (n=1) injections were 403, 552, and 553 at days 10, 17, and 21, respectively.

3.2 Effect of BTxA on Development and Mineralization of HO

Preliminary results by microCT scanning identified reduced induction of HO at day 10 and 17 with BTxA treatment (n=4) one day prior to BMP2- or BMP4- Matrigel injections compared to a saline control (n=1) (Figure 3).

The second saline control received no BMP injection volume. For this particular study, BMP2 and BMP4 are denoted BMP as the main objective was determining the effects of BTxA on HO formation and not differences in BMP2 or BMP4. BV of mineralized HO nodule (mm³) in BTxA treated mice were 0.11 ± 0.20 at day 10 and 1.04 ± 0.68 at day 17; the saline treated mouse was 0.81 and 2.48 at day 10 and 17, respectively (Table 1, Figure 4). BV/TV of HO nodule calculated 0.01 ± 0.01 and 0.07 ± 0.02 for day 10 and 17 with BTxA treatment compared to 0.03 and 0.11 at day 10 and 17, respectively, with saline control (Table 1, Figure 4).

**Figure 3.** MicroCT scanning identified reduced HO with BTxA (Botox) vs. saline treated mice injected with BMP2- or BMP4- Matrigel at day 10 and 17.
Table 1. BV and BV/TV of mineralized HO nodule (see Figure 4).

<table>
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<tr>
<th>Animal ID</th>
<th>Treatment</th>
<th>BV (mm³) Day 10</th>
<th>BV (mm³) Day 17</th>
<th>BV (mm³) Day 10</th>
<th>BV (mm³) Day 17</th>
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Figure 4. BV and BV/TV of mineralized HO nodule in BTxA vs. saline treated mice injected with BMP2- or BMP4-Matrigel.

4. DISCUSSION

We implemented a well-described model for HO, achieved via an intramuscular injection of bone morphogenic protein (BMP2 or BMP4) impregnated in a carrier matrix (Matrigel) [5]. Temporal formation of heterotopic bone measured in vivo via microCT clearly identified the formation and progression of HO. Qualitatively, BMP2-Matrigel injections appeared more potent than BMP4-Matrigel injections at initiating HO. However, the study was not sufficiently powered to detect BMP differences in the induction of HO. Regardless, the establishment of this model enabled testing of our primary hypothesis.

Based upon the preliminary results, we have observed that paralysis of the calf muscle with BTxA reduced induction of HO at day 10 and 17 following BMP2- or BMP4-Matrigel injections vs. saline treatment. Significantly, we recognize that the study was not sufficiently powered to demonstrate statistical significance but these pilot data provide evidence that neuromuscular signaling is important in the induction and mineralization of HO.
Future direction for this study will be to take histological samples of the implanted nodules and surrounding muscle. Samples will be processed for determination of osteoblastic activity, percent mineralization, and neovascularization of the nodule using bone histology. The rationale behind these tests is to provide further evidence to the importance of neuromuscular signaling in HO pathology. If confirmed in future studies, this approach holds significant potential to be more effective, more targeted, less costly, and less likely to induce side effects compared to current therapeutic regimens.

In summary, we established a repeatable in vivo model of HO. BMP2 appeared more potent than BMP4 at initiating HO. Preliminary data suggest that BTxA mitigates the formation of ectopic bone in our HO model showing the importance of neuromuscular signaling in the induction of HO. However, more data and animals are necessary to confirm a central role of neuromuscular signaling in HO pathogenesis.

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