Plasmin is Essential for Preventing Formation of Heterotopic Ossification After Skeletal Muscle Trauma

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Disclosures:

Introduction: Traumatic heterotopic ossification (HO) refers to a spectrum of disease resulting in the development of bone in abnormal areas. The etiology of HO is not fully understood, however it is thought to result from chronic inflammation leading to ultimately to the development of bone forming cells outside of the bone environment. Despite the use of anti-inflammatory and anti-osteoblastic medications, HO continues to inflict significant morbidity in these patient populations causing chronic pain and musculoskeletal dysfunction. It is now understood that impaired coagulation system within tissues is a principle cause of chronic inflammation and tissue dysfunction in many chronic diseases. Our novel hypothesis is that aberrant coagulation is, in part, the etiology of HO. The rationale for our hypothesis stems from the observation that muscle or bone damage, traumatic brain and spinal cord injury, severe burns all cause changes in coagulation system. From these principles we propose that dysregulation of coagulation system may result in HO. To test this hypothesis we utilized mice heterozygous mutation for Plasminogen, the zymogen of the protease plasmin, which is essential for fibrinolytic system. Of note, in this study, we also demonstrated a novel validated model to create HO formation without using BMPs in vivo study.

Methods: All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Vanderbilt University Medical Center. We used well established muscle injury model by injecting 40 uL of 10 uM Cardiotxin (CTX; Latoxan, Valence, France) into calf muscle on wild type (WT, 20 legs total), plasminogen heterozygous (Plg+/-: 20 legs) mice which is kindly given by Dr. Jay Degen (Cincinatti, Ohio). As a control, we injected same amount of PBS to the contralateral side. We took lateral x-ray (Faxitron, IL) of lower legs on 1, 3, 5, 7, 10, 14, 21 and 28 days post injection (DPI) and sacrificed them on each day for analysis. Samples at 28 DPI were scanned by μCT (Scanco40) to detect ossification. We stained each section with H&E solution clearly showed no ossification in Plg+/- mice whereas in WT mice, even at 10 DPI, abundant non regenerated muscle fibers were observed (Figure1A). Plg+/- mice which was confirmed by undecalcified sections stained with von Kossa solution (Figure1B). To confirm the healing process of injured muscle, we stained time course sections from 1 DPI up to 10 DPI with H&E, von Kossa and TRAP staining. For the purpose of preventing HO formation, we also treated Plg+/- mice with antisense oligonucleotide of α2 anti-plasmin (α2AP ASO; provided by ISIS pharmaceutical) weekly to knock down α2AP level 3 weeks before injection (16 legs). As a control, we used scrambled ASO on Plg+/- mice (10 legs). To assess the association of fibrin (ogen) of forming HO, we used immunohistochemical staining for fibrin. In vitro study, we extracted muscle tissues from one leg that was injected cardiotxin 3 days before extraction and the other leg that was injected PBS of Plg+/- mice. Tissues were minced and digested with Collagenase D solution (Roche). After tissues were poured through cell strainer, they were seeded into 75 flasks for the culture. After passage 1, both PBS injected cells and CTX injected cells were seeded into 24 well dish at the concentration of 20,000/ well and cultured with or without osteogenic media (beta glycerophosphate and ascorbic acid) for 14 days. Each well was then fixed with 10% formalin and stained with ALP staining, von Kossa staining and alizarin red staining.

Results: In serial X-rays, WT mice showed no apparent heterotopic ossification. However, Plg+/- mice displayed HO formation at the injected site (WT: 0 leg/20 legs total; Plg+/-: 18 legs/20 legs total; Figure1-A). uCT analysis also confirmed the ossification in the calf muscle in Plg+/- mice (Figure1-B). Axial section of injected area stained with H&E at 28 DPI also showed trabecular bone and bone marrow in Plg+/- mice whereas in WT there were no evidence of ossification at the injected site (Figure1-C). To determine the healing process of injured muscle in both WT and Plg+/- mice, we stained time course sections from 1 DPI up to 10 DPI by H&E. WT and Plg+/- mice showed no significant difference in first 3 days in histological examination. In WT we observed complete muscle regeneration which can be confirmed the existence of nucleai in the muscle fiber and most of the injured muscle healed by 10 DPI whereas in Plg+/- mice, even at 10 DPI, abundant non regenerated muscle fibers were observed (Figure2-A). We also observed damaged muscle mineralization in Plg+/- mice which was confirmed by undecalcified sections stained with von Kossa solution (Figure2-C). TRAP staining also confirmed the existence of osteoclasts at the mineralization area. By using fluorescent immunohistochemistry, we observed fibrin remnant at the injury site (Figure2-B) however we did not observe fibrin accumulation at the ossification site at 28 DPI. From these results, we speculated that coagulation system or fibrinolytic system may play an important role to regulate muscle HO. We then manipulate the fibrinolytic system by treating Plg+/- mice with α2AP ASO to prevent HO formation. In serial X-rays, no HO was observed till 28 DPI (0 leg/16 legs; Figure3-A). Sections stained with H&E solution clearly showed no ossification in Plg+/- mice with α2AP ASO (Figure3-B). To confirm osteogenic capability of cells in the damaged muscle which is injected with CTX, we analyzed the osteogenic differentiation of cells injected by PBS and cells injected by CTX. ALP, Alizarin Red and von Kossa staining clearly showed the superior osteogenic capability in CTX injected cells.

Discussion: This is the first study to demonstrate that the proteolytic activity of the coagulation protein plasmin is protective against developing heterotopic ossification following skeletal muscle injury. Recent work has demonstrated that inhibition of plasminogen binding to its receptor on cell surface leads to impaired muscle regeneration (Ref1). However, this work was
conducted in mice with a complete loss of plasminogen whereas here we observed impaired muscle healing and development of 
HO in mice only partially deficient in plasminogen. In addition we clearly show that by reducing plasmin’s principle inhibitor, 
α2anti-plasmin, we were able to prevent HO formation. Together, these data reveal that plasmin protease activity dose 
dependently protects against the formation of HO. The principle in vivo target of plasmin is thought to be fibrin resulting in clot 
degradation. Indeed, we observed fibrin deposition within areas of developing HO. However, the biologic role of coagulation 
proteases is not limited to intravascular events and coagulation. For example, plasmin is capable of digesting multiple bone 
related proteins. It activates and degrades interstitial and extracellular matrix proteins such as betaglycan and collagen-1, 
activates precursor growth factors (TGFβ) and proteases (MMP-9) and solubilize bioactive molecules (VEGF). Future studies are 
required to determine which proteolytic target(s) of plasmin are responsible for these observations.
Significance: Clinically, the development of HO is associated with conditions known to alter the coagulation system’s ability to 
activate the coagulation protease plasmin, such as head injury, burn and spinal cord injury. The findings in this paper are the first 
to demonstrate that plasmin protease activity is protective against HO suggesting that restoring its activity could potentially 
prevent, or reverse, HO clinically.
Acknowledgments: N\A
References: Ref1: Angels Diaz-Ramos et al. Requirement of Plasminogen Binding to Its Cell-Surface Receptor α-Enolase for 
Efficient Regeneration of Normal and Dystrophic Skeletal Muscle. Vol. 7 Issue 12, Dec 2012 PLOS ONE