Expression of Sonic Hedgehog in Non-Diabetic Wounds Treated with Poly(Methacrylic Acid-co-Methyl Methacrylate)

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Statement of Purpose: This work focuses on the cellular and molecular mechanisms of angiogenesis in the presence of a bioactive biomaterial - poly(methacrylic acid-co-methyl methacrylate) (polyMAA-co-MMA or MAA). It was previously shown that direct application of MAA beads promoted angiogenesis and subsequent wound healing in diabetic mice (Martin DC. J Biomed Mater Res A. 2010;93:484-492); however, the molecular and cellular mechanisms of angiogenesis in the presence of MAA beads are largely unknown. Knowledge of these mechanisms can potentially be used to advance the development of wound dressings and the design of other tissue constructs where vascularization is important. Preliminary gene expression studies in diabetic wounds treated with MAA beads revealed the upregulation of a potent pleiotropic gene - Sonic hedgehog (Shh) (Fitzpatrick LE. Biomaterials.2012;33(21):5297-307), which is vital in adult angiogenesis (Pola R. Nat Med. 2001;7:706-711) and wound healing (Asai J. Circulation. 2006; 113:2413-2424). Hence, we investigated the role of the Shh pathway as a potential mechanism responsible for angiogenesis in the presence of MAA.

Methods: Large (1.5x1.5 cm) full thickness wounds were created on the dorsum of mice and 20 mg of MAA beads (150-250 µm in diameter, 45 mol% methacrylic acid) or control poly(methyl methacrylate) beads (PMMA, 150-250 µm in diameter, 100 mol% methyl methacrylate) were applied evenly over the wounds, or the wounds were left untreated in non-diabetic Shh-eGFP-Cre/Ptch1-lacZ CD1 mice. In a subcutaneous injection model, MAA (5 mg) or PMMA (15 mg) beads in 200 µL of PEG solution were injected subcutaneously into two sites on the either side of the mid-dorsum of non-diabetic Shh-eGFP-Cre/Ptch1-lacZ CD1 mice (n=6). In both models the tissue was explanted 4 and 7 days post-surgery and processed for histological and differential gene expression (qRT-PCR) studies.

Results: MAA beads were previously shown to promote angiogenesis in diabetic mice (Martin DC. J Biomed Mater Res A. 2010;93:484-492). Here, we demonstrated that MAA treatment also improved angiogenesis in healthy non-diabetic mice ($p<0.05$) [Figure 1A]. The transgenic animal model in which Shh and Ptch1 (Shh receptor) were co-expressed with two reporter genes, GFP and lacZ respectively, was used to detect and quantify cells expressing these proteins. Shh+ cells (GFP+ cells) were quantified within the healthy tissue surrounding the wound bed and in the granulation tissue. The number of Shh+ cells increased in the tissue surrounding the wound at day 4 ($p<0.05$) [Figure 1B]. Morphological examination of Shh+ cells suggested that potential source of Shh *in vivo* were follicle, immune, fibroblast-like cells and cells associated with adipose tissue and neuromas. Differential gene expression analysis of wound tissue demonstrated that MAA treatment increased expression of Shh, Ptch1 and Gli2 in comparison to both controls ($p<0.05$) and Gli3 in comparison to PMMA ($p<0.1$) at day 7. Ptch1 is a product of Shh pathway (Stanton BZ. Mo Biosyst. 2010; 6:44-54); thus, its upregulation confirmed the pathway activation. Gli2 is implicated in embryonic folliculogenesis (Mill P. Genes & Development. 2003;17:282-294) and Gli3 is the main transcription factor that regulates Shh associated angiogenesis (Renault M. Circ Res. 2009;105:818-826).

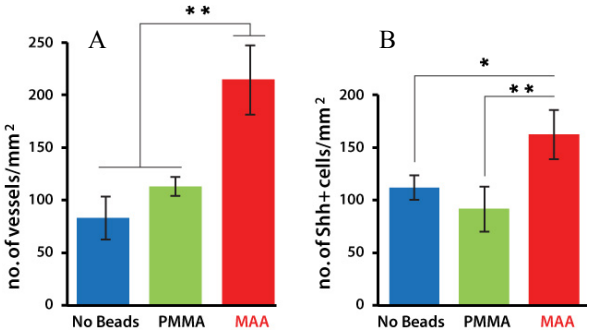

Figure 1. MAA increased average vessel density in granulation tissue (A) and Shh+ cells in the healthy tissue surrounding at the wound bed (B) at day 4 (±SEM, n=5) **$p<0.05$, *$p<0.1$

In the wound healing model MAA beads become entrapped in the scab, which falls off prior to the studied time points; hence, identification of cells that closely associate with the beads is not possible. As a result, a new model is used in which MAA or PMMA beads are injected subcutaneously under the skin of the non-diabetic Shh-eGFP-Cre/Ptch1-LacZ mice allowing histological evaluation and characterization of the cells, which are directly affected by the MAA beads and in response express Shh and Ptch1 proteins [Figure 2].

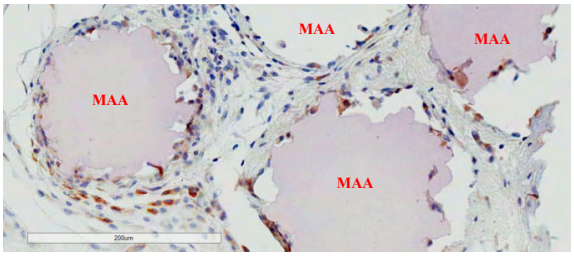

Figure 2. Expression of Shh (brown stain) in presence of MAA beads (bar = 200 µm)

Conclusions: In the wound healing study, in non-diabetic animals, MAA treatment increased vessel density and expression of Shh, Ptch1, Gli2 and Gli3 in the wound tissue, and Shh+ cell density in the tissue surrounding the wound bed suggesting activation of the Shh signaling pathway and its involvement in MAA-mediated angiogenesis. Subcutaneous injection studies are on-going to further characterize the *in vivo* source of Shh.