Mechanisms of the Angiogenic Effect of Poly(Methacrylic Acid-co-Methyl Methacrylate) Beads

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Statement of Purpose: Diabetic complications can include debilitating chronic wounds which, due to lack of effective clinical treatments, often diminish quality of life, increase morbidity and mortality and lead to loss of limbs. Impaired angiogenesis has been identified as a major contributor to poor wound healing in people with diabetes, causing the wounds to remain in an unresolved state of pathological inflammation, and severely delaying the healing process. Previous work by our group has demonstrated the pro-angiogenic properties of a synthetic co-polymer in wound healing models. Poly(methacrylic acid-co-methyl methacrylate) (MAA) beads were shown to significantly improve wound healing of skin graft models in rats[1] and full-thickness wound models in diabetic mice[2] by increasing angiogenesis in the grafts and granulation tissue. However, the mechanism of MAA-mediated angiogenesis remains unknown. The goal of this project is to identify molecular and cellular mechanisms of the angiogenic effect of MAA beads.

Methods: *Bead Preparation*: MAA beads (150 – 250 μm diameter, 45 mol% methacrylic acid) were donated by Rimon Therapeutics Ltd (Toronto, Canada). Poly(methyl methacrylate) (PMMA) was used as a "non-bioactive" control material[1,2]. PMMA beads (150 – 250 μm diameter, 100% methyl methacrylate) were obtained from Polysciences (Warrington, Pa.). Beads were tested for endotoxin using LAL Pyrochrome Endotoxin Kit (Associates of Cape Cod, Falmouth, MA) to ensure endotoxin levels were less than 0.25 EU/ml (100mg beads/ml). *Wounding Assay*: Two full-thickness dermal wounds (7.5 mm x 7.5 mm) were created on the backs of 8-10 week old male $Lepr^{db/db}$ mice (Jackson Laboratories, Bar Harbor, ME). Either 7 mg of MAA beads or 7 mg of PMMA beads were applied topically to the wound beds, or the wounds were untreated (control). All wounds were left undressed and scabs were allowed to form. *Total RNA preparation*: Mice were sacrificed on post-operative day 4 and 7. Total RNA was isolated from the wound tissue using TRIzol Reagent (Invitrogen, Burlington, ON) followed by Qaigen RNeasy Mini kit Clean-up (Qiagen, Mississauga, ON), according to the respective manufacturers' protocols. *Microarray*: Total RNA was submitted to The Center of Applied Genomics (TCAG, The Hospital for Sick Children, Toronto, Canada), who first tested to quality of the RNA using Agilent 2100 Bioanalyzer (Agilent Technologies) before performing an Affymetrix Mouse Gene 1.0 ST microarray, using Ambion® WT Expression Kit for sample preparation. The arrays were scanned with an Affymetrix GeneChip Scanner 3000 and images were processed using Affymetrix Expression Console software. Gene expression differences between treatments were assessed using Partek Genomics Suite (Partek Inc., St Louis, MO). All array data were normalized using the Robust Multi-array Average (RMA) method. Differential expression of genes was assessed using ANOVA (Partek Gene Expression workflow) for each time point. Genes with a p-value ≤ 0.05 and a fold-change ≥ 1.5 were considered differentially expressed. Gene ontology (Functional Annotation Clustering) and pathway analyses were conducted for differentially expressed genes using Database for Annotation, Visualization and Integrated Discovery[3].

Results: Differentially expressed genes among the three wound treatments (MAA, PMMA and Control) were detected for day 4 (n = 4 per treatment) and day 7 (n = 3 per treatment), Figure 1.

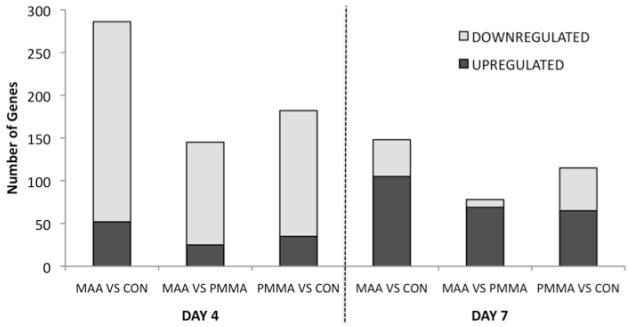

Figure 1. Differentially expressed genes at day 4 and 7.

On day 4, wounds treated with MAA beads had decreased expression of mRNA for inflammation and immune response-related genes relative to PMMA-treated wounds (*CD48, Mtcp-1, CysLTR1, Chi3l3, CD209, CCl12*) and untreated wounds (*CD48, Mtcp-1, CysLTR1, CXCL15, MARCO*). Genes involved in the p53 apoptosis pathway (*Perp, Lgals7, Trep63*) were also downregulated in MAA samples compared to control (untreated) samples. In contrast, an inhibitor of FAS-induced apoptosis (*FAIM*) was downregulated in MAA compared to PMMA and control. This may indicate that MAA beads affect reduced recruitment or altered activity of T-cells, NK cells and macrophages at the wound site, and reduced apoptosis via p53 signaling. The most notable annotations from Day 7 were the upregulation of muscle-related genes (*Ttn, Mylk2, Jph1*) in MAA-treated wounds relative to PMMA-treated and untreated wounds, which may be interpreted as an increase in myofibroblast presence and suggests an advanced wound healing state compared to PMMA-treated or untreated wounds.

Conclusions: The microarray analysis of mRNA expression in diabetic wounds treated with MAA beads suggests a reduced or altered inflammatory response and faster progression of wound healing compared to PMMA-treated or untreated wounds. Future studies will include PCR validation of array data and examining the effect of MAA beads on individual cell populations (e.g. macrophage, endothelial) *in vitro*.

References: (1. Eckhaus, AA. Plast Reconstr Surg, 2008, 122:1361-1370. 2. Martin DC. J Biomed Mater Res A. 2009, Jul 7. [Epub ahead of print]. 3. Dennis G. Genome Biol. 2003, 4:R60.)