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Effect of methacrylic acid beads on the sonic hedgehog signaling pathway and macrophage polarization in a subcutaneous injection mouse model

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ABSTRACT

Poly(methacrylic acid-*co*-methyl methacrylate) (MAA) beads promote a vascular regenerative response when used in diabetic wound healing. Previous studies reported that MAA beads modulated the expression of sonic hedgehog (*Shh*) and inflammation related genes in diabetic wounds. The aim of this work was to follow up on these observations in a subcutaneous injection model to study the host response in the absence of the confounding factors of diabetic wound healing. In this model, MAA beads improved vascularization in healthy mice of both sexes compared to control poly(methyl methacrylate) (MM) beads, with a stronger effect seen in males than females. MAA-induced vessels were perfusable, as evidenced from the CLARITY-processed images. In Shh-Cre-eGFP/Ptch1-LacZ non-diabetic transgenic mice, the increased vessel formation was accompanied by a higher density of cells expressing GFP (Shh) and β-Gal (patched 1, Ptch1) suggesting MAA enhanced the activation of the Shh pathway. Ptch1 is the Shh receptor and a target of the pathway. MAA beads also modulated the inflammatory cell infiltrate in CD1 mice: more neutrophils and more macrophages were noted with MAA relative to MM beads at days 1 and 7, respectively. In addition, MAA beads biased macrophages towards a MHCII−CD206+ ("M2") polarization state. This study suggests that the Shh pathway and an altered inflammatory response are two elements of the complex mechanism whereby MAA-based biomaterials effect vascular regeneration.

1. Introduction

Methacrylic acid (MAA)-based biomaterials have a vascular regenerative effect in the absence of exogenous cells or growth factors [reviewed in Refs. [1,2]]. These biomaterials were previously shown to promote vascularization [3–6], and improve myocutaneous graft survival [4] and diabetic wound healing [5]. Together with past work [6–9], the present study was aimed at further understanding the mechanism behind this effect. We presume that such biomaterials drive an "alternative foreign body response" that is distinct from the fibrosis associated with the classical foreign body response.

Gene expression analysis of diabetic wounds treated with poly(methacrylic acid-*co*-methyl methacrylate) (MAA) beads showed an over fourfold upregulation in the expression of the sonic hedgehog (*Shh*) gene [6], which has been implicated in adult vascularization [10,11]. The Shh pathway is also activated during inflammation [12] and has been shown to polarize macrophages towards an alternatively-activated, wound healing ("M2") phenotype [13]. Although, MAA-based biomaterials did not modulate the expression of classical angiogenic genes (e.g., vascular endothelial growth factor (*VEGF*)) [6–8], MAA beads modulated inflammation-associated genes (e.g., interleukin 1β (*IL1β*)) in diabetic wounds [6], in an air pouch model [9] and macrophage-like cells (dTHP1 cells) *in vitro* [7,8]. A phosphoproteomics study of dTHP1 cells treated for a few minutes with a MAA-based biomaterial distinguished a number of phosphorylated proteins involved in macrophage polarization (e.g., solute transporter monocarboxylate transporter 4) among the many phosphorylated proteins that were differentially regulated between a MAA-based and a control biomaterial [14]. Overall, these results led to the hypothesis that MAA-based biomaterials modulate the Shh signaling pathway and inflammatory

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cell responses, including macrophage polarization. Hence, a subcutaneous injection model was devised to investigate the effects of MAA beads on the host response without the confounding factors of diabetic wound healing. To investigate the activation of the Shh pathway specifically, MAA beads and control poly(methyl methacrylate) (MM) beads were injected subcutaneously in transgenic Shh-Cre-eGFP/Ptch1-LacZ mice. In these mice the expression of the reporters, GFP and β-Gal, was shown to be consistent with the pattern of Shh and Ptch1 mRNA expression, respectively [15,16]. The activation of the Shh signaling pathway was suggested as the densities of both GFP+ (Shh) and β-Gal+ (patched 1, Ptch1, the target of the pathway) cells were upregulated by treatment with MAA beads in this model. To investigate inflammatory cell responses, bead implants were analyzed by immunohistochemistry and flow cytometry for the number and polarization state of infiltrating inflammatory cells. MAA beads increased the density of neutrophils at day 1 and macrophages at day 7 and biased macrophages towards the MHCII−CD206+ state representative of the "M2" phenotype. We also compared the therapeutic effect of a biomaterial in both males and females illustrating a difference in response between sexes.

2. Methods

2.1. MAA and MM bead preparation

Poly(methacrylic acid-*co*-methyl methacrylate) (MAA-*co*-MMA or MAA) beads were composed of 45 mol% methacrylic acid (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), 1 mol% ethylene glycol dimethacrylate (Sigma-Aldrich Canada Ltd.) and 64 mol% methyl methacrylate (Sigma-Aldrich Canada Ltd.). MAA beads were synthesized by suspension polymerization as previously described [4] and were sieved to obtain beads in the diameter range of 150–250 μm. Methacrylic acid content of the synthesized beads was confirmed by titration. Control poly(methyl methacrylate) (MM) beads (same diameter) were obtained from Polysciences (Warrington, PA). Beads were washed in either 95% ethanol (MAA beads) or 1 N HCl (MM beads) repeatedly and then rinsed five times in LAL reagent water (MJS Biolynx Inc., Brockville, ON, Canada) prior to use *in vivo*. Analysis with a limulus amebocyte lysate (LAL) pyrochrome endotoxin test kit (Cape Cod Inc., Falmouth, MA) indicated that beads contained <0.25 EU/100 mg. Elemental surface composition analysis (ThermoFisher XPS, Surface-Interface Ontario, University of Toronto) showed minimal Si contamination (~0.07%) and that measured surface composition (atom%) was close to the theoretical expectation. MAA beads had a rough, porous surface, were negatively charged and did not degrade over time *in vivo*; MM beads were smooth [4,5].

For subcutaneous injections, a 1 mL syringe with an 18 gauge needle was loaded with either 5 mg MAA beads or 15 mg MM beads (or no beads, vehicle control) suspended in 250 μL of 50% w/v polyethylene glycol (PEG, avg. mol. wt. 1450, sterile-filtered; Sigma-Aldrich Canada Ltd.) in PBS. The 1:3 wt ratio (5 mg MAA: 15 mg MM) was used to account for MAA bead swelling upon hydration at physiological pH [4] to approximately equate implanted volumes. Vehicle control was used only for the flow cytometry study because the vehicle control implant area could not be defined reproducibly for vessel and cell density analyses.

2.2. Animals

All animal work was done with the approval of the University of Toronto Animal Care Committee. Animals were housed under sterile conditions in the University of Toronto's Department of Comparative Medicine. The experiments were done with CD1 mice (6–8 week old, males, Charles River Laboratories, MA) and Shh-Cre-eGFP/Ptch1-LacZ mice (10–12 week old, males and females). Shh-Cre-eGFP/Ptch1-LacZ heterozygous mice of CD1 background were bred in house by crossing CD1 females (Charles River Laboratories, MA or bred in house) with Shh-Cre-eGFP/Ptch1-LacZ heterozygous males. The original Shh-Cre-eGFP/Ptch1-LacZ male was donated by Professor Chi-chung Hui (Hospital for Sick Children, Toronto, ON, Canada) and created by crossing Shh-Cre-eGFP [15] with Ptch1-LacZ [16] mice. Shh-Cre-eGFP mice were created by inserting a *gfpcre* cassette at the ATG of Shh; expression of GFP protein was reported to colocalize with *Shh* mRNA [15]. Ptch1-LacZ mice were developed by inserting the *LacZ* gene into Ptch1; the expression of the reporter was consistent with the pattern of *Ptch1* transcription [16].

Mice were genotyped to detect the presence of Shh-Cre-eGFP and Ptch1-LacZ mutations. DNA from ear notches was extracted by alkaline lysis. Primers (Supplementary Information, Table 1) were synthesized by Sigma Genosys (Sigma-Aldrich Canada Ltd.) and prepared by resuspension in RNase/DNase free water. PCR reactions are detailed in the Supplementary Methods [80].

2.3. Subcutaneous injection

Mice were anesthetized with 0.5% w/v isofluorane prior to surgery and an analgesic (Ketoprofen, 5 mg/kg) was administered intraoperatively. The dorsal area of a mouse was shaved and the remaining hair was removed either by waxing (Nair wax strips) or by hair removal cream (Veet). The skin was sterilized with 70% ethanol and Betadine. An 18-gauge needle was used to inject MAA, control MM beads or vehicle (PEG). Two injections on either side of the dorsum were performed for each mouse. A small subcutaneous pocket was made with the needle on the side of the dorsum by moving the syringe from side to side, while deliberately attempting to nick small blood vessels to promote injury prior to injection (Fig. 1). Following surgery, mice were housed individually, fed chow and water ad libitum, and monitored for any signs of discomfort. At 1–7 days post-injection, the mice were sacrificed using CO_2, followed by cervical dislocation. The implants were removed surgically and processed for histology, imaging or flow cytometry.

2.4. Histology and immunohistochemistry

Immediately upon euthanizing mice, the bead implant and several mm of surrounding tissue was excised from the right side of the dorsum and fixed in formalin. Tissue samples were embedded in deep paraffin blocks, cut into sections, processed and stained with hematoxylin and eosin (H&E), Masson's trichrome, CD31, GFP, β-galactosidase (β-Gal), F4/80 and CD206 (Supplementary Information, Table 2). Histology slides were scanned (20×) using an Aperio ScanScope XT (Leica Microsystems, Concord, ON, Canada) by the Advanced Optical Microscopy Facility (AOMF, Toronto, ON, Canada).

The scanned slides were analyzed using Aperio ImageScope (Version 11) at 4 and 7 days post-implantation. For vessel counts, a region of interest (ROI) was defined by measuring a distance of 500 μm around each cluster of beads. CD31+ vessel-like structures (criterion being the presence of a lumen) were counted in the tissue within this defined region. The vessel density was calculated by dividing the total number of vessels by area of the ROI. For other counts (GFP, β-Gal, F4/80 and CD206), a distance of 200 μm around bead clusters was used to define the ROI. The cell density was calculated by dividing the total number of positive cells by the area of the ROI.