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Shh pathway in wounds in non-diabetic Shh-Cre-eGFP/Ptch1-LacZ mice treated with MAA beads

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ABSTRACT

Previously, poly(methacrylic acid-*co*-methyl methacrylate) (MAA) beads were shown to improve vessel formation with a concomitant increase in the expression of the sonic hedgehog (*Shh*) gene, a pleiotropic factor implicated in vascularization. The aim of this study was to follow up on this observation in the absence of the confounding factors of diabetes in non-diabetic Shh-Cre-eGFP/Ptch1-LacZ mice; in this mouse, expression of GFP and β-Gal is consistent with the transcription patterns of Shh and its receptor patched 1 (Ptch1), respectively. In agreement with studies in diabetic males, MAA beads improved vascularization in large (15 mm × 15 mm) wounds in non-diabetic males at day 7. Shh pathway activation was suggested, as the numbers of GFP+ (Shh) and β-Gal+ (Ptch1, a target of the pathway) cells increased in the granulation tissue. Shh signaling pathway modulation was also suggested in the healthy skin surrounding the wound bed, as evidenced by an increase in the number of GFP+ and β-Gal+ cells in males at day 4. Gene expression analysis of the wounds confirmed increase in *Ptch1* and showed the upregulation of a downstream transcription factor *Gli3*, involved in the vascular effect of the Shh pathway, implicating the pathway in the effect of MAA beads. The efficacy of MAA beads was also investigated in females; MAA beads modulated the Shh pathway within granulation tissue similarly as in males, but had no enhancement effect on the healthy skin and on vascularization. We believe that understanding the molecular and cellular mechanisms of MAA-based biomaterials and testing the efficacy of therapeutics in both sexes will inform the development of novel therapeutic biomaterials.

1. Introduction

In diabetes, the wound healing process is impaired and is characterized by abnormal inflammation, vascularization and altered cell phenotypes [reviewed in Refs. [1–4]]. Earlier studies in diabetic animals demonstrated the therapeutic effect of methacrylic acid (MAA)-based biomaterials in wound healing [5,6]. Poly(methacrylic acid-*co*-methyl methacrylate) (MAA) beads increased vessel formation and subsequently improved wound repair (e.g., degree of wound closure and epithelial gap) [5]. Recent studies shifted focus to understanding the cellular and molecular mechanisms by which these MAA-based biomaterials achieve these effects [6–9] to advance fundamental knowledge of biomaterial-host interactions and ultimately facilitate the design of novel therapeutic biomaterials.

In the earlier study, the expression of sonic hedgehog (*Shh*) gene was increased fourfold in diabetic wounds treated with MAA beads compared to controls at day 4 post-wounding [8]. While better known for its involvement in embryonic development, there is now much evidence that Shh is important in adult wound healing [10–13], vascularization [14–21] and inflammation [13,22]. Inhibition of the Shh pathway disrupted multiple parameters in wound healing such as wound closure, granulation tissue formation, vascularization and follicular regeneration [10]. The Shh pathway is impaired in diabetes [12] and exogenous addition of *Shh* has been shown to accelerate diabetic wound healing by increasing cellular infiltration, collagen deposition and microvascular remodeling [11]. Similarly, inhibition of the endogenous Shh pathway impaired vascularization as measured by decreased blood flow and capillary density in an ischemic limb model [16], while exogenous *Shh* improved blood flow and limb salvage [17]. Here, transgenic Shh-Cre-eGFP/Ptch1-LacZ mice (CD1 background) were used to

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investigate the cells in which promoters for Shh and its receptor patched 1 (Ptch1) were activated. GFP expression was previously shown to co-localize with *Shh* mRNA [23], while the expression of the β-Gal reporter was consistent with the pattern of *Ptch1* transcription [24]. This animal is not diabetic, simplifying the model compared to previously used diabetic mice while focusing on the Shh pathway. The effect of MAA beads was also studied in both females and males to understand the sex differences in response to this therapeutic biomaterial.

2. Methods

2.1. Biomaterial preparation

Chemicals for MAA synthesis were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Poly(methacrylic acid-*co*-methyl methacrylate) (polyMAA-*co*-MMA or MAA) beads (150–250 μm in diameter) were synthesized by suspension polymerization as previously described [25]. Beads were composed of 45 mol% methacrylic acid, 1 mol% ethylene glycol dimethacrylate and 64 mol% methyl methacrylate. Titration confirmed methacrylic acid composition and X-ray Photoelectron Spectroscopy (XPS) (Thermo Scientific Theta Probe, Surface Interface Ontario, University of Toronto) showed that measured surface composition (atom %) was close to the theoretical numbers. Control poly(methyl methacrylate) (MM) beads (150–250 μm) were obtained from Polysciences (Warrington, PA, USA). Beads were washed and sterilized in 95% ethanol (MAA) or 1 N HCl (MM) repeatedly and then rinsed in LAL reagent water (MJS Biolynx Inc., Brockville, ON, Canada). Beads contained <0.25 EU/100 mg of endotoxin as determined with a LAL pyrochrome test kit (Cape Cod Inc., Falmouth, MA). Elemental surface composition analysis showed minimal Si contamination (~0.07%) (Thermo Scientific Theta Probe).

2.2. Animals

All animal work was done in accordance with the approval of the University of Toronto Animal Care Committee. Mice were housed under sterile conditions in the Division of Comparative Medicine animal facility at the University of Toronto. All wound healing experiments were done in mice of CD1 background with or without modifications in Shh and/or Ptch1 genes; specifically, "CD1 mice" (no transgenic modifications), "Shh-Cre-eGFP/Ptch1-LacZ mice" (CD1 mice with modifications in Shh and Ptch1 genes) and "Ptch1-LacZ mice" (CD1 mice with modifications in Ptch1 gene only) were used. The studies were done in 9–11.5 week old (young adults; a 12 week mouse is comparable to ~20-year old adult human [26]) heterozygous Shh-Cre-eGFP/Ptch1-LacZ males (39.4 ± 4.6 g) and females (31.1 ± 3.1 g) that were bred in-house by crossing CD1 females with Shh-Cre-eGFP/Ptch1-LacZ heterozygous males. The effect of MAA on vessel density was confirmed in CD1 mice (9–11 week old, males, bred in-house) and Ptch1-LacZ mice (10–11 week old, males, bred in-house). The original Shh-Cre-eGFP/Ptch1-LacZ male was donated by Dr. C-c Hui (Hospital for Sick Children, Toronto, ON, Canada) and created by crossing Shh-Cre-eGFP [23] with Ptch1-LacZ [24] mice. Shh-Cre-eGFP mutation was created by inserting the *gfpcre* cassette at the ATG of the *Shh* allele; in situ hybridization showed that GFP expression co-localized with Shh mRNA [23]. Ptch1-LacZ mice were created by inserting the *LacZ* gene into the *Ptch1* allele; the expression of the reporter was shown to be consistent with the pattern of Ptch1 transcription [24]. Mice were genotyped to identify Shh-Cre-eGFP/Ptch1-LacZ, Ptch1-LacZ and CD1 mice (Supplementary information, Methods).

The logistics of in-house breeding required performing multiple separate surgeries (5 or more) to obtain the required number of animals ($n \geq 7$ for males, $n = 10$ for females). Both sexes were used, as previous studies indicated sex differences in response to therapeutic treatments [reviewed in Refs. [27,28]].

2.3. Wound healing surgery

Mice were anesthetized with 0.5% w/v isofluorane and an analgesic buprenorphine (0.1 mg/kg) was administered intraoperatively. The dorsal area of each mouse was shaved and subsequently waxed (Nair wax strips). The skin was sterilized with 70% ethanol and Betadine. A mid-dorsal 15 mm × 15 mm square wound was inflicted by excising the epidermis and dermis including the panniculus carnosus. One of three treatments was applied: 20 mg of MAA beads, 20 mg of MM beads (biomaterial control) or left untreated (denoted as "No bead", no treatment control); the rationale for this dose is discussed below. The wounds were left undressed allowing a scab to form. Following the surgery, mice were housed individually and monitored for signs of discomfort. At 4 or 7 days post-wounding, the mice were euthanized using CO_2, followed by a cervical dislocation. The wound bed with several mm of tissue surrounding the wound was excised and split in half. One half of the tissue was fixed in formalin for histology analyses and the other was preserved in RNAlater Stabilization Solution (Thermo Fisher Scientific, Waltham, MA) for gene expression studies.

2.4. Histology and immunohistochemistry

For histological analysis, tissue samples were embedded in paraffin blocks, cut into serial sections, processed and stained with hematoxylin and eosin, Masson's trichrome, CD31 (SC-1506, 1/2500; Santa Cruz Biotechnology, Dallas, TX), GFP (ab6556, 1/3000; abcam, Cambridge, MA) and β-galactosidase (β-Gal) (ab9361, 1/600; abcam). Histology slides were scanned at 20× magnification with the Aperio ScanScope XT (Leica Microsystems, Concord, ON, Canada) by the Advanced Optical Microscopy Facility (AOMF, Toronto, ON, Canada). The scanned images were analyzed using Aperio ImageScope (Version 11).

2.5. Vessel and cell counts

For days 4 and 7 post-wounding, ten equally spaced windows within the granulation tissue (160,000 μm^2 each) were assessed for each sample. $CD31^+$ vessel-like structures that exhibited a lumen were counted and the granulation area was quantified for each of the 10 windows. The vessel density was calculated by dividing the total number of vessels by the total granulation area.

The scanned images of GFP and β-Gal were analyzed to determine the density of GFP+ and β-Gal+ cells both within the newly formed granulation tissue and the tissue immediately surrounding the wound bed ("healthy skin"). Within the granulation tissue, eight windows (160,000 μm^2 each) were assessed for each sample. The windows were distributed uniformly throughout the wound bed, providing a representative expression of the GFP+ and β-Gal+ cells. The total GFP+ or β-Gal+ cell density was calculated by dividing the total number of GFP+ and β-Gal+ cells by the total granulation tissue area. In the skin surrounding the wound bed, four 160,000 μm^2 windows (two windows on each side of the wound) were assessed. Within the skin, cell density was calculated by dividing the total number of GFP+ or β-Gal+ cells over the total area of the skin. Cell density within hair follicles was first calculated for individual windows and then averaged over four windows to account for the high variability among the follicles. Additionally, the proportion of high intensity GFP+ cells was also assessed by distinguishing between cells expressing GFP at high and low intensity.