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The role of insulin growth factor-1 on the vascular regenerative effect of MAA coated disks and macrophage-endothelial cell crosstalk

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ABSTRACT

The IGF-1 signaling pathway and IGF-1-dependent macrophage/endothelial cell crosstalk was found to be critical features of the vascular regenerative effect displayed by implanted methacrylic acid –co-isodecyl acrylate (MAA-co-IDA; 40% MAA) coated disks in CD1 mice. Inhibition of IGF-1 signaling using AG1024 an IGF1-R tyrosine kinase inhibitor abrogated vessel formation 14 days after disk implantation in a subcutaneous pocket. Explanted tissue had increased arginase 1 expression and reduced iNOS expression consistent with the greater shift from "M1" ("pro-inflammatory") macrophages to "M2" ("pro-angiogenic") macrophages for MAA coated disks relative to control MM (methyl methacrylate-co-IDA) disks; the latter did not generate a vascular response and the polarization shift was muted with AG1024. In vitro, medium conditioned by macrophages (both human dTHP1 cells and mouse bone marrow derived macrophages) had elevated IGF-1 mRNA and protein levels, while the cells had reduced IGF1-R but elevated IGFBP-3 mRNA levels. These cells also had reduced iNOS and elevated Arg1 expression, consistent with the *in vivo* polarization results, including the inhibitory effects of AG1024. On the other hand, HUVEC exposed to dTHP1 conditioned medium migrated and proliferated faster suggesting that the primary target of the macrophage released IGF-1 was endothelial cells. Although further investigation is warranted, IGF-1 appears to be a key feature underpinning the observed vascularization. Why MAA based materials have this effect remains to be defined, however.

1. Introduction

Tissue engineering has grown quickly in response to the needs for tissue and organ replacement, although the need for a vasculature is a limiting issue. Growth factor (e.g. VEGF) or cell (e.g. endothelial) delivery strategies are being explored to this end [1,2] but another approach is suggested by the discovery of vascular regenerating materials based on methacrylic acid (MAA [3–6]).

MAA based biomaterials have been found to promote vascularization [3–5] and wound healing [4,6] in diabetic and healthy animals. MAA-based materials have been prepared in different forms such as beads [3], scaffolds [7], films [8] and injectable hydrogels [9]; all have the same vascular regenerative effect. The mechanism behind vessel formation in this context is unclear, since there are no exogenous growth factors or cells that are being delivered with the biomaterial. Rather host cells including endothelial cells and macrophages are recruited to the biomaterial and drive what we have termed an alternative foreign body response. Earlier reports have focused on activation of the sonic hedgehog pathway [10], polarization of macrophages towards the CD206+ (M2) phenotype [11] and the adsorption of complement proteins on MAA beads [12] as contributing elements of the vascular response.

An early microarray study identified increased expression of Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) in dTHP1 cells cultured with MAA beads [5], while phosphoproteomic analysis also identified the insulin signaling pathway (insulin receptor substrate 2, Raf, Akt1) as a feature of the interaction of dTHP1 cells with MAA coated coverslips [4]. There is significant homology between insulin growth factor receptor 1 (IGF1-R) and the insulin receptor [13,14].

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Insulin-like growth factor −1 (IGF-1) is a peptide hormone, which is essential for normal pre- and postnatal growth and development [15,16]. IGF-1 was shown to play an important role in regeneration of nerves and skeletal muscle [17–19]. IGF-1 mediates cell proliferation, differentiation and survival upon binding its receptor and has a significant role in the physiology of endothelial cells by promoting migration and tube formation [14]. Notably, M2 macrophages produce IGF-1 [20–22], which can help tissue regeneration [21] and have a role in M2 macrophage activation [23]. IGFBP-3, a product of the IGF pathway, acts like a decoy by binding with high affinity to IGF-1 and IGF-2 [31–33]. IGFBP-3 is the major binding protein for IGF-I, which circulates throughout the body and has multiple functions, many of which are associated with IGF regulation, such as transport of IGF in plasma, the control and regulation of their efflux from the vascular space and their clearance, the tissue-specificity of their function, and the control of their interaction with their receptor [24,25].

Here we focused on IGF-1 and IGF1-R. We used the IGF-1 pathway inhibitor AG1024 to examine the effect of MAA on vessel formation in CD1 mice using MAA (and control) coated silicone disks. We also studied the effect of MAA stimulated macrophages on endothelial cells *in vitro*.

2. Materials and methods

2.1. Cells

Human leukemia monocytic cells THP1 (ATCC TIB-202, Manassas, VA) were grown in RPMI 1640 medium (Gibco, US) supplemented with 10% fetal bovine serum (FBS, Gibco, US), 25 mM HEPES, 2 mM L-glutamine and 1% penicillin/streptomycin at 37 °C and 5%CO_2. Cells were differentiated into adherent macrophage-like cells (dTHP1) with 100 nM Phorbol Myristate Acetate (PMA, Sigma, Oakville, Canada)) for 24 h and PMA was washed away with PBS and then replaced with fresh medium without PMA.

Human umbilical vein endothelial cells (HUVEC) (Lonza, Mississauga, Canada) were grown in EBM2 medium supplemented with an EGM2 singlequot kit at 37 °C and 5%CO_2. HUVEC were passaged with trypsin and used at passage 4–6.

To generate mouse bone marrow-derived macrophages (BMDM), bone marrow cells from femurs and tibias from CD1 mice were harvested and cultured, following a standard protocol [27]. BMDM were suspended in RPMI1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin and 100 ng MCSF (Macrophage Colony-Stimulating Factor, eBioscience, San Diego, CA, US) and differentiated for 7 days. On day 3 non-adherent cells were aspirated, the medium was replaced and cells remained in culture with MCSF for 4 more days.

2.2. Macrophage polarization

BMDM (in RPMI1640 with 10% FBS, 1% penicillin/streptomycin and 100 ng MCSF) were seeded (10^6 cells/per 6-well plate) for overnight adhesion. Macrophages were polarized for 24 h with interferon-γ (INF-γ) (100U/ml, Abcam Inc, Toronro, ON, Canada) for M1 phenotype (labelled as M1(INF-γ)), interleukin-4 (IL-4) (20 ng/ml, Abcam Inc, Toronro, ON, Canada) for M2 phenotype (labelled as M2(IL-4)). After polarization, BMDM were resuspended in complete RPMI without polarizing cytokine. Non-polarized cells were referred to as M0 macrophages. Polarization was confirmed by qPCR analysis of gene expression of established polarization markers (see below).

2.3. MAA and MM co-polymer synthesis

As described earlier [8], methacrylic acid (MAA, Sigma, Oakville, Canada) or methyl methacrylate (MM, Sigma, Oakville, ON) were copolymerized with IDA (isodecyl acrylate, Sigma, Oakville, Canada), using benzoyl peroxide (1 wt%); co-polymers (containing 40 mol % MAA or MM) are designated as MAA or MM, respectively. To produce 40% MAA, for example, 0.025 mol of MAA and 0.037 mol of IDA containing 4×10^{-4} mol of dissolved benzoyl peroxide were reacted in 12 ml of endotoxin free water at 70 °C for 90 min with stirring. The polymer was then purified by precipitation from tetrahydrofuran (THF) into water-methanol and dried over several days at room temperature. The reaction and purity of copolymers were monitored by NMR and titration [8].

The purified copolymers were dissolved in THF (50 mg/ml) and cast onto 22-mm-diameter glass coverslips or 3–4 mm diameter silicone disks (punched from 0.010″ thick non-sterile Long-Term Implantable Silicone Sheeting, Pillar Surgical Inc., La Jolla, CA, USA). Before experiments, the polymer coated coverslips or disks were washed twice in 70% ethanol (for 20 min, each wash) and twice in PBS (20 min, each wash) to remove endotoxin and for "sterilization". Coverslips or disks were kept in medium overnight. Equivalent endotoxin levels of polymer were measured by using Limulus Amebocyte Lysate Pyrochrome test kit (Cape Cod Inc., Falmouth, MA, US) and level of toxins was determined to be 0.048 EU/ml for MM polymer and 0.02 EU/ml for MAA polymer, as noted previously [8]. The Food and Drug Administration guideline recommends an endotoxin level of under 0.50 EU/ml. Polymer surface composition was checked by X-ray photoelectron spectroscopy (XPS) analysis, as before [8,28].

2.4. Conditioned medium preparation and cells treatment

Serum starved (overnight) dTHP1 cells or polarized BMDM were cultured on porous transwell inserts (0.4 μm pore size, Fisher Scientific, ON, Canada) and exposed to 40% MM or 40% MAA by placing co-polymer coated coverslips with polymer atop cells for 24 h or 72 h. The transwell enabled cells to be supplied with nutrients from below, while covered with a glass coverslip. After 24 h the conditioned medium was collected for HUVEC treatment or ELISA analysis. After 72 h incubation, the coverslip and the medium were removed, the cells were quickly rinsed with PBS and lysed for RNA isolation. dTHP1 or BMDM treated with uncoated coverslips were defined as non-treated cells (designated as NT).

2.5. Coated disk implantation

7-9 week old, male CD1 mice (Jackson Laboratories, Bar Harbor, ME) were used with the approval of the University of Toronto Animal Care Committee. Mice were anaesthetized with isoflurane, the dorsal surface was shaved, followed by treatment with hair removal cream (Veet) and the surgical site was sterilized with Betadine and 70% Ethanol. An analgesic - ketoprofen (5 mg/kg) - was given subcutaneously prior to surgery. Co-polymer coated silicone disks were implanted into bilateral subcutaneous pockets (one disk/pocket; two disks/animal) created by blunt dissection with forceps with the same material placed in both pockets. One disk was used for histology and another for gene expression analysis. The incisions were closed with staples and left undressed; thereafter, the mice were caged individually. Mice were sacrificed using CO_2 asphyxiation, followed by cervical dislocation, on day 7 and 14 post-treatment.

In addition to MAA or MM coated disks, uncoated silicone