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Research paper

Effect of heparin on the biological properties and molecular signature of human mesenchymal stem cells

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ABSTRACT

Chronic use of heparin as an anti-coagulant for the treatment of thrombosis or embolism invokes many adverse systemic events including thrombocytopenia, vascular reactions and osteoporosis. Here, we addressed whether adverse effects might also be directed to mesenchymal stem cells that reside in the bone marrow compartment. Harvested human bone marrow-derived mesenchymal stem cells (hMSCs) were exposed to varying doses of heparin and their responses profiled. At low doses (<200 ng/ml), serial passaging with heparin exerted a variable effect on hMSC proliferation and multipotentiality across multiple donors, while at higher doses (≥100 μg/ml), heparin supplementation inhibited cell growth and increased both senescence and cell size. Gene expression profiling using cDNA arrays and RNA-seq analysis revealed pleiotropic effects of low-dose heparin on signaling pathways essential to hMSC growth and differentiation (including the TGFβ/BMP superfamily, FGFs, and Wnts). Cells serially passaged in low-dose heparin possess a donor-dependent gene signature that reflects their altered phenotype. Our data indicate that heparin supplementation during the culturing of hMSCs can alter their biological properties, even at low doses. This warrants caution in the application of heparin as a culture supplement for the *ex vivo* expansion of hMSCs. It also highlights the need for careful evaluation of the bone marrow compartment in patients receiving chronic heparin treatment.

1. Introduction

Heparin, a highly sulfated heparan glycosaminoglycan variant produced and stored primarily by mast cells (Ronnberg and Pejler, 2012), possesses the highest net negative charge density of all known biological molecules (Alter et al., 1987). Its negative charge binds to positively charged, heparin-binding domains (HBDs) present in a large number of extracellular proteins. This group of proteins includes fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs), bone morphogenetic proteins (BMPs) and large extracellular structural molecules such as fibronectin and laminin, as well as its main clinical target antithrombin III (Naimy et al., 2008). Both unfractionated (UFH) and low molecular weight heparin (LMWH) have been widely used as anticoagulants to enable surgery and dialysis, as well as to treat pathological conditions such as thrombosis and embolism.

The ability of heparin to interact with many proteins renders it a potential therapeutic agent beyond its use as an anti-thrombotic (Lane and Adams, 1993). Heparin's high affinity for protein has resulted in

Abbreviations: hMSCs, human bone marrow-derived mesenchymal stem cells; RNA-seq, high throughput next generation RNA sequencing; FGF, fibroblast growth factor; HBD, heparin-binding domain; VEGFs, vascular endothelial factors; UFH, unfractionated heparin; LMWH, low-molecular-weight heparin; hESC, human embryonic stem cell; HS, heparan sulfate; ECM, extracellular matrix; FCS, fetal calf serum; BMMNCs, bone marrow mononuclear cells; BrdU, 5-bromo-2-deoxyuridine; SVD, singular value decomposition; ALP, alkaline phosphatase; BSPII, bone sialoprotein II; C/EBP, CCAAT/enhancer binding protein; ALBP, adipocyte lipid binding protein; CST3, Cystatin-3; ITGA, integrin; CCNG2, cyclin G2; INHB, inhibin; RPKM, reads per kilobasepair per million mapped reads.

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its application in cell culture to enhance the desirable activity of critical extracellular biomolecules used as supplements for the expansion of human stem cells. For example, heparin has been reported to promote both Wnt and FGF signaling in human embryonic stem cells (hESCs), thereby increasing their proliferation (Furue et al., 2008; Sasaki et al., 2008). Similarly, heparin has been shown to enhance Wnt-induced differentiation signals in osteogenic cells (Ling et al., 2010a), further highlighting its diverse effects. Tissue culture surfaces coated with glycosaminoglycans such as heparin support greater proliferation of MSCs (Uygun et al., 2009; Ratanavaraporn and Tabata, 2012). Heparin-functionalized hydrogels and heparinized nanoparticles have also been developed to support the viability and differentiation of hMSCs (Na et al., 2007; Benoit et al., 2007).

The widespread use of heparin in both clinical and research practice makes a detailed study of both its mechanism of action and long-term effects in cell culture highly advisable. Heparin non-selectively facilitates the binding of a wide spectrum of proteins to high-affinity receptors on cells, particularly within the endothelium (Ferrara, 1999; Folkman and Shing, 1992; Gengrinovitch et al., 1995). Factors such as $VEGF_{165}$ and FGF-2 normally associate with heparan sugars on cell surfaces to form ligand:sugar:receptor complexes that induce proliferative signals (Kim et al., 2011; Turnbull et al., 2001). Furthermore, $VEGF_{165}$ affinity-selected sugar has been shown to exert pro-angiogenic effects on endothelial cells (Wang et al., 2014). In contrast, short heparin fragments (~5.0-kDa) purified from porcine intestinal mucosa can suppress $VEGF_{165}$-mediated angiogenesis when delivered subcutaneously (Norrby and Ostergaard, 1997). Also, heparin derivatives as well as heparan sulfate (HS) isolated from bone marrow stromal cells and BMP-2 affinity-selected HS have been shown to increase the osteogenic potential of BMP-2 (Ratanavaraporn and Tabata, 2012; Bramono et al., 2012; Murali et al., 2013). Genes encoding the synthesis of ECM proteins can also be regulated by heparin (Hitraya et al., 1995). Heparin has been shown to activate metalloproteinase-2, leading to remodeling of the ECM (Tyagi et al., 1997). It can act as a heparanase inhibitor and is known to affect the *in vitro* tubular morphogenesis of microvessels (Sweeney et al., 1998). In certain fibroblasts, inflammatory cells, and tumor cells (most prominently), heparanase activity is enhanced, where the expression of heparanase mRNA is known to correlate with increased metastatic potential (Hulett et al., 1999). Furthermore, type 1 diabetes has been shown to be a heparanase-dependent disease (Dudakovic et al., 2014). These broad biological effects of heparin and heparin-degrading enzymes are consistent with the multiplicity of proteins that interact with its hyper-sulfated sugar chains and maintain tissue homeostasis.

In most tissues, heparin-binding proteins are usually controlled by physiologically relevant and tissue-specific HS on the cell surface. There are notable differences in the structure between heparin and HS; most importantly heparin contains 3-O-sulfation and lacks discrete protein-binding domains (Langeslay et al., 2011). Excess heparin with its greater negative-charge density can out-compete physiologically relevant HS-protein interactions and thus disrupt a number of biological processes associated with tissue development and repair that require proper maintenance of stem cell pools. Also, safety concerns ascribed to heparin's binding promiscuity are evident from patients presenting with heparin-induced thrombocytopenia (Bambrah et al., 2012), osteoporosis (Mazziotti et al., 2010; Sackler and Liu, 1973) and vascular reactions (Bounameaux et al., 1987; Gollub and Ulin, 1962). Indeed, heparin has been shown to enhance osteoclastic bone resorption through an interaction with osteoprotegerin (OPG) (Irie et al., 2007), whilst other HS variants have been shown to exert anti-osteoclastic effects (Ling et al., 2010b). Mastocytosis, a disorder characterized by increased numbers of mast cells that produce excessive heparin, is associated with osteoporosis, which again indicates the generally adverse effect of heparin on skeletal tissue (Benucci et al., 2009). Even though chronic heparin use is associated with unwanted clinical events, it is widely used as a stem cell culture supplement without a clear understanding of its effects on stem cell phenotypes.

Adult stem cells are a key driver of natural tissue replenishment, and are among the small number of cells that can both undergo proliferation and differentiate into the various lineages needed to repair or regenerate damaged tissue (Prockop and Oh, 2012; Samsonraj et al., 2015). Heparin supplementation in medium has been reported to promote hMSC proliferation (Mimura et al., 2011). Heparin-functionalized hydrogels have been formulated in such a way that they are able to retain combinations of FGFs and ECM proteins and so support the growth, adhesion or differentiation of hMSCs (Na et al., 2007; Benoit et al., 2007; Benoit and Anseth, 2005; Bhakta et al., 2012). However, we lack precise knowledge of the biological effects of heparin on hMSCs.

This study set out to determine whether heparin, over a range of doses, could change the intrinsic properties of hMSCs *in vitro*. It proved capable of altering the molecular profiles of hMSCs, even at low doses, and affected their potential for growth and differentiation in a donor-dependent manner. Our findings suggest that caution should be exercised whenever stem cells are serially passaged in heparin-supplemented media.

2. Materials and methods

Throughout the study, control data generated in hMSCs grown in maintenance conditions (Helledie et al., 2012) was compared to heparin-treated hMSCs in Figs. 1A, B, 2 and 3. Experimentation with heparin was performed in parallel with studies using HS (Helledie et al., 2012). Data obtained with heparin and HS were analyzed separately to address distinct findings on different biological properties of hMSCs that emerged from the two datasets.

2.1. Cell culture

Human MSCs were either directly purchased from Lonza or isolated in our laboratory from human bone marrow mononuclear cells from young healthy male donors aged 20–30 years provided by Lonza (Donors 1 to 3) as previously described (Samsonraj et al., 2015; Rider et al., 2008). Cells were expanded and maintained in complete Dulbecco's Modified Eagle's Medium using our standard protocols (Helledie et al., 2012) and we reported the number of passages under various treatment conditions (experimental passage number). Unless otherwise indicated, hMSCs were treated with 160 ng/ml of heparin (Sigma-Aldrich), a glycosaminoglycan dosage that is within the range used in other related stem cell studies (Mimura et al., 2011; Helledie et al., 2012).

2.2. Cell proliferation and cumulative growth analysis

Human MSCs were plated in triplicate at 5000 cells/cm^2 in the presence or absence of heparin as indicated and proliferation determined by monitoring viable cell number using the GUAVA PCA-96 benchtop flow cytometer as per manufacturer's instructions (Millipore). Briefly, cells were dislodged by trypsinization and stained with GUAVA Flex dye. Cell suspensions were counted using the GUAVA Viacount program.

To monitor the cumulative growth, cells were plated at the same density (5000 cells/cm^2) with or without heparin as indicated and sub-cultured upon reaching 70–80% confluency. The cells were re-plated under the same condition at each passage and the viable cells were counted using GUAVA system as described above.

2.3. Image cytometry

Human MSCs were seeded into chamber slides at 3000 cells/cm^2 and allowed to attach overnight. Cells were then treated with or without 500 μg/ml heparin for 3 days. Cells were fixed with 4% paraformaldehyde and stained with Rhodamine-conjugated phalloidin and DAPI (Life Technologies). Samples were imaged with an Olympus IX83 inverted microscope with a Cool Pix HQ2 camera and MetaMorph software, using slide