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Heparin affects human bone marrow stromal cell fate: Promoting osteogenic and reducing adipogenic differentiation and conversion

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ABSTRACT

Heparins are broadly used for the prevention and treatment of thrombosis and embolism. Yet, osteoporosis is considered to be a severe side effect in up to one third of all patients on long-term treatment. However, the mechanisms underlying this clinical problem are only partially understood. To investigate if heparin affects differentiation of skeletal precursors, we examined the effects of heparin on the osteogenic and adipogenic lineage commitment and differentiation of primary human bone marrow stromal cells (hBMSCs). Due to the known inverse relationship between adipogenesis and osteogenesis and the capacity of pre-differentiated cells to convert into the respective other lineage, we also determined heparin effects on osteogenic conversion and adipogenic differentiation/conversion. Interestingly, heparin did not only significantly increase mRNA expression and enzyme activity of the osteogenic marker alkaline phosphatase (ALP), but it also promoted mineralization during osteogenic differentiation and conversion. Furthermore, the mRNA expression of the osteogenic marker bone morphogenic protein 4 (BMP4) was enhanced. In addition, heparin administration partly prevented adipogenic differentiation and conversion demonstrated by reduced lipid droplet formation along with a decreased expression of adipogenic markers. Moreover, luciferase reporter assays, inhibitor experiments and gene expression analyses revealed that heparin had putative permissive effects on osteogenic signaling via the BMP pathway and reduced the mRNA expression of the Wnt pathway inhibitors dickkopf 1 (DKK1) and sclerostin (SOST). Taken together, our data show a rather supportive than inhibitory effect of heparin on osteogenic hBMSC differentiation and conversion in vitro. Further studies will have to investigate the net effects of heparin administration on bone formation versus bone resorption in vivo to unravel the molecular mechanisms of heparin-associated osteoporosis and reconcile conflicting experimental data with clinical observations.

Introduction

Heparins are best known as effective anticoagulants commonly used for the inhibition of blood clotting and in the prevention and treatment of a broad spectrum of thrombosis and embolism. The major anticoagulant effect results from interaction with antithrombin III, thereby enhancing its inactivating effect on several enzymes involved in blood coagulation [1]. In addition, heparin interacts with a multitude of other proteins, including growth factors, chemokines, cytokines, enzymes, extracellular matrix proteins, lipoproteins, and hormone receptors that are associated with numerous biological processes like development, signaling, cancer, extracellular matrix assembly, host–pathogen interaction, and angiogenesis [2–7]. Being a negatively charged, highly sulfated and polydispersed glucosaminoglycan, heparin exhibits a high affinity to bind to a wide range of positively charged proteins, which accounts for its main clinical limitations [8].

Besides its beneficial effects regarding anticoagulant and antithrombotic treatment, the long-term use of heparin has been widely associated with the development of osteopenia and even osteoporosis due to

Abbreviations: ALP, alkaline phosphatase; ALPL, alkaline phosphatase bone, liver, kidney; BMD, bone mineral density; BMP, bone morphogenetic protein; BMPR, BMP receptor; BRE, BMP responsive element; co-SMAD, common-mediator SMAD; DKK1, dickkopf Wnt signaling pathway inhibitor 1; FABP4, fatty acid binding protein 4; FGF, fibroblast growth factor; FGFR, FGF receptor; IGFBP2, insulin growth factor binding protein 2; hBMSCs, human bone marrow stromal cells; KRM1, kringle containing transmembrane protein 1; LPL, lipoprotein lipase; OC, osteocalcin; OPG, osteoprotegerin; OPN, osteopontin; PPARγ2, peroxisome proliferator-activated receptor gamma 2; R-SMAD, receptor-regulated SMAD; RANK, receptor activator of nuclear factor κ B; RANKL, RANK ligand; ROR2, receptor tyrosine kinase-like orphan receptor 2; RUNX2, runt-related transcription factor 2; SBE, Smad binding element; SFRP1, secreted frizzled-related protein 1; SMAD, mothers against decapentaplegic homolog; SOST, sclerostin; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor β; TZP, tinzaparin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; WNT5A, wingless-type MMTV integration site family member 5A.

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decreased bone mineral density (BMD) in up to one third of treated patients. Additionally, fracture rates in those patients were increased up to 15% (reviewed in [9]). Nevertheless, some clinical studies rather attributed the occurrence of osteoporosis to pre-existing adverse conditions of the patients, e.g. immobilization, breast feeding, or pregnancy itself [10,11].

Additionally, there have been several in vitro and animal studies on the effects of heparin on osteogenesis, showing controversial results concerning osteogenic outcome depending on the animal species or cell culture system as well as on the concentrations, durations, and types of heparin fractions used. According to Turan et al., injections of 1000 IU/kg of heparin per day led to improper lamellar bone structure and largely uncalcified bone matrix in rabbits [12]. Moreover, heparin caused bone loss in a rat model of heparin-induced osteoporosis by increasing osteoclast number and activity as well as osteoclast formation in vitro [13–15]. Hausser and Brenner reported that heparin had a dose-dependent effect on osteoblast-like Saos-2 cells, being inhibitory at higher concentrations ($\geq$5 μg/ml) but stimulatory at lower concentrations (5–500 ng/ml) [16]. Kanzaki and colleagues demonstrated that heparin had dual effects on bone morphogenetic protein (BMP) 2-induced osteogenic activity in MC3T3-E1 cells, on the one hand reducing osteogenic outcome after a shorter exposure time while on the other hand supporting it after a longer period [17]. Takada et al. showed that sulfated polysaccharides like heparin enhanced the biological activities of BMPs [18]. Moreover, heparin was reported to enhance BMP-induced osteoblast differentiation in vitro and in vivo by protecting BMP2 from degradation and inhibiting BMP antagonists [19].

To date, there have been only very few studies on human bone marrow stromal cells (hBMSCs), examining the effects of heparin with regard to differentiation. Honda and colleagues identified an osteogenic cocktail containing BMP2 in combination with heparin to accelerate in vitro mineralization of human and murine mesenchymal stem cells (MSC) by using a feedback system control and a differential evolution algorithm [20]. Lanfer et al. demonstrated multilineage differentiation of MSC (chondrogenic, adipogenic, and osteogenic) in aligned collagen matrices incorporating heparin [21]. The low molecular weight heparin tinzaparin (TZP) was reported to negatively affect hMSC proliferation but not osteogenic nor chondrogenic differentiation [22]. In addition, bone marrow-derived stromal cells grown on a heparin-treated surfaceshowed increased alkaline phosphatase (ALP) activity and mineralization [23].

As multipotential hBMSCs are the progenitor cells of osteoblasts as well as other mesenchymal lineages like adipocytes, they could provide a more reliable in vitro system compared to the use of immortalized cell lines and thereby aid in understanding the mechanisms of heparin action on the cellular and molecular level. Meanwhile, exploring the effect of heparin on hBMSC lineage decision and possible lineage switches after commitment (termed 'conversion' in the current study) is of high interest for two main reasons. Firstly, evidence suggests that osteoporosis is at least in part caused by increased adipogenic differentiation at the expense of osteogenesis leading to the common clinical observation of fat accumulation in bone marrow referred to as fatty degeneration [24,25]. To the best of our knowledge, there has been only one study testing the effects of heparins on osteogenesis as well as adipogenesis in parallel, showing a decrease in osteoblast and an increase in adipocyte cell number [26]. Secondly, besides undifferentiated stromal cells, the bone marrow also contains various stages of progenitor cells of several differentiation lineages, which might also be affected by heparin. Since we and others have previously shown that pre-differentiated adipogenic as well as osteogenic cells retain the ability of conversion into the respective other cell lineage, the current study also assessed the effects of heparin after such conversion events [27–31]. As mentioned above, evidence suggests an inverse relationship between adipogenesis and osteogenesis; hence the adipogenic commitment of undifferentiated hBMSCs as well as the adipogenic conversion of osteogenically pre-differentiated cells may account for the development of osteoporosis leading to the commonly observed manifestation of fatty degeneration in the bone marrow.

In summary, the mechanisms underlying the heparin-induced development of osteopenia and osteoporosis are still a matter of scientific debate. To date, literature on primary hBMSCs, the precursors of osteoblastic cells in the bone marrow, and on the effects of heparin on hBMSC lineage decisions towards osteogenesis and adipogenesis are scarce.

Therefore, the aim of our study was to explore the impact of heparin on both osteogenic and adipogenic differentiation and conversion of primary human BMSC. For this purpose, we used classical in vitro multipotency testing followed by a set of quantifying assays, marker gene expression analyses, and reporter assays. Our study clearly depicts that heparin treatment results in a significant increase in osteogenic differentiation and conversion as well as a significant reduction in adipogenic outcome. Interestingly, this suggests that, at least in vitro, osteogenic commitment and differentiation of skeletal precursors are not compromised by heparin treatment. Future studies will have to focus on whether key players in bone formation and remodeling, e.g. endothelial cells, or bone resorption by osteoclasts are altered in vivo, in order to explain the clinical phenomenon of heparin-induced osteoporosis.

Materials and methods

Chemicals

Cell culture reagents were purchased from Life Technologies (Darmstadt, Germany). Fetal calf serum (FCS) and heparin were obtained from Biochrom GmbH (Berlin, Germany). Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). Isobutylmethylxanthine was obtained from Applichem (Darmstadt, Germany). For alkaline phosphatase detection, CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) ready-to-use solution was purchased from Roche (Mannheim, Germany). Quant-iT™ PicoGreen dsDNA Reagent was supplied by Life Technologies. The QuantiChrom™ calcium assay kit (DICA-500) was purchased from Biotrend (Cologne, Germany). For determining protein content according to Bradford, Roti®-Quant 5× solution was purchased from Roth (Karlsruhe, Germany). The inhibitors Dorsomorphin and KRN633 were obtained from MedChem Express (Stockholm, Sweden). For reporter assays, recombinant human BMP4 was purchased from PeproTech Germany (Hamburg, Germany) and reporter lysis buffer (5×) was purchased from Promega GmbH (Mannheim, Germany).

Cell culture

Human bone marrow stromal cells (hBMSCs) were isolated from the trabecular bone of femoral heads as described previously [29,32] using a modified protocol originally published by Haynesworth and colleagues [33]. Patients were undergoing hip replacement surgery due to age-related or hip dysplasia-related attrition, but were otherwise healthy and did not receive medications with relation to bone metabolism. Experiments were performed upon approval by the Local Ethics Committee of the University of Würzburg and informed consent from each donor (7 male and 1 female patient aged 41–76 years). After isolation, hBMSCs were grown in 1:1 Dulbecco's modified eagle's medium: Ham's F-12 nutrient mixture (DMEM/Ham's F12) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml ascorbic acid phosphate. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and medium was changed every 3–4 days. Upon confluence, hBMSCs were passaged 1:3 using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in Dulbecco's phosphate buffered saline (PBS). For the analysis of alkaline phosphatase activity, cells were seeded into 12-well plates with 24×10^4 cells/well.