Heparanase Modulates Chondrogenic Factor Signaling And Is Upregulated In Ectopic Cartilage

Julianne Huegel, Motomi Enomoto-Iwamoto, PhD, Federica Sgariglia, Tricia Bhatti, Eiki Koyama, Maurizio Pacifici.
Children's Hospital of Philadelphia, Philadelphia, PA, USA.

Disclosures:

Introduction: Development of skeletal elements initiates with formation of mesenchymal condensations at prescribed times and locations. These condensations undergo endochondral ossification during which the mesenchymal cells differentiate into chondrocytes, produce cartilaginous structures, become organized into growth plates, and are eventually replaced by endochondral bone and marrow.

Heparan sulfate proteoglycans (HSPGs) and their heparan sulfate (HS) chains influence key events that occur during endochondral ossification. Members of the bone morphogenetic protein (BMP), hedgehog, Wnt, and fibroblast growth factor families of signaling factors all bind to HS, allowing the chains to influence their distribution, bioavailability, and providing tissue-specific functionality to the factors. The importance of HS chains and HSPGs in skeletogenesis is reiterated by the fact that there are a number of skeletal phenotypes related to mutations in HS-synthesizing enzymes or core proteins. Patients with heterozygous mutations in the HS synthesizing enzymes EXT1 or EXT2 develop the disease Hereditary Multiple Exostoses. HME is characterized by the formation of ectopic cartilage outgrowths called exostoses that form adjacent to the growth plates in postnatal growing skeletal elements. We showed previously that loss of HS coincides with increased chondrogenic factor signaling, possibly facilitating or triggering exostosis formation.

Another interesting aspect of the biology of HS chains and HSPGs is that the chains can be modified extracellularly by the action of enzymes such as heparanase (HPSE), the only human enzyme with HS-degradation capability. Indeed, increased expression of HPSE has been described in human exostosis tissue. Responsible for cleaving HS chains into small fragments, HPSE has pro-proliferative activity and is implicated in a range of cancers by assisting in structural remodeling of the extracellular matrix during cellular invasion and release of growth factors. Additionally, increased levels of HPSE are present at the chondro-osseous junction in developing bones, suggesting that HPSE plays a role in late chondrocyte differentiation. It also suggests that regulated degradation of HS chains could promote factor release and signaling during other key points of the bone formation process. However, the exact roles of HPSE in skeletogenesis and skeletal growth as well as exostosis formation are incompletely understood. The present study was carried out to investigate what roles HPSE plays in cartilage development and maintenance and test whether excess HPSE expression is linked to skeletal pathologies and exostosis formation in particular.

Methods: E11 mouse embryo limb bud cells were seeded in micromass cultures and treated with bacterial heparitinase or BMP2. RNA was isolated with Trizol reagent, reverse transcribed, and amplified using GoTaq DNA polymerase. Cultures were also collected in lysis buffer, separated by electrophoresis in 4-12% gradient gels, electrotransferred to nitrocellulose membranes, and probed with antibodies to phospho-Smad1/5/8 and HPSE detected using peroxidase-conjugated antibodies and chemiluminescent substrate. Paraffin sections of osteochondromas removed from consenting HME patients as well as rib cartilage removed during autopsyffin were incubated with HPSE antibodies (Abcam) at 1:200 dilution in 3% NGS in PBS overnight. Staining was performed with DAB and slides were counterstained with Fast Green.

Results: HPSE induces cell proliferation and migration in a number of cancer types. Thus, we hypothesized that exogenously-provided enzyme would increase the rate of cell division and migration in chondrogenic ATDC5 cells and primary mouse limb bud cells plated in high-density micromasses, the latter being a common model of chondrogenesis. We measured these parameters by analyzing DNA content, tracking scratch healing, and measuring micromass diameter over time. Addition of heparitinase did increase cell proliferation and migration compared to control cultures. Exostoses in HME patients often contain little to no HS, in fact much lower than what would be expected from their heterozygous EXT mutations and suggesting a role for HPSE. To investigate whether HPSE expression would actually increase as the overall HS levels drop, limb bud cells were treated with exogenous heparitinase or the small molecule HS inhibitor Surfen. Indeed HPSE expression at both mRNA and protein levels was significantly increased over levels in untreated cells (Fig 1).

We have previously shown that BMP2 signaling is up-regulated in vitro by Surfen treatment and in vivo in Ext1 knockout mice; this effect contributes to ectopic cartilage formation. To analyze the relationship between BMP2 and HPSE expression, limb bud cultures were treated with recombinant human BMP2. Indeed, BMP treatment did stimulate HSPE expression (and also chondrogenesis), suggesting that the enzyme is part of the pro-chondrogenic effect of BMP. To analyze BMP signaling activity, we measured Smad1/5/8 phosphorylation by Western blot. pSmad levels were increased by BMP treatment as well as heparitinase treatment, supporting the idea that degradation of HS stimulates chondrogenic signaling pathways.

An interesting characteristic of the exostoses is their growth plate-like organization. Exostoses display regions of resting,
proliferative, and hypertrophic chondrocytes adjacent to a bone marrow cavity and bordered by perichondrium. We evaluated HPSE expression in both normal human growth plates as well as human exostosis tissue. Immunohistochemical analysis of paraffin sections revealed specific staining patterns in normal growth plates (Fig 2A,B). HPSE was prominent in hyperthrophic chondrocytes, the chondro-osseous junction, and the perichondrium, with little to no staining in resting or proliferative chondrocytes. In contrast, exostosis cartilage from HME patients showed strong HPSE staining in all chondrocytes, regardless of maturation stage and location within the tissue (Fig 2C). Additionally, large hypertrophic cells often displayed particularly intense staining in their peri- and extra-cellular matrix (Fig 2D).

**Discussion:** Our data show that interference with HS function or levels by Surfen or bacterial heparitinase treatment readily increases endogenous HPSE expression in chondrogenic cell cultures. In addition, heparitinase treatment stimulates BMP signaling and chondrogenic differentiation. We interpret the results to signify that an important inverse regulatory loop exists by which a drop in HS would trigger an increase in HPSE, leading to very steep decreases in overall HS levels. The increase in endogenous HPSE could thus have a significant pathogenic role in HME by eliciting HS degradation, growth factor release and ectopic cartilage formation. This interpretation is in line with previous reports suggesting that exostoses in HME patients show far less HS content than expected. Our data also indicate that HPSE may also have physiologic roles in the hypertrophic zone of growth plate and along the chondro-osseous border where it could also be involved in growth factor release and/or matrix remodeling. In conclusion, HPSE may represent a therapeutic target to modulate normal and abnormal skeletal tissue formation.

**Significance:** Skeletal development and growth are fundamental processes that, when defective, can cause major pathologies in the craniofacial, trunk and limb skeleton and can lead to impairment of body function and quality of life. This work shows that the enzyme HPSE is up-regulated when HS levels or function drop and that HPSE may be a contributing factor in exostosis development. Targeting this enzyme could offer therapeutic opportunities for HME.

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**References:**
Fig 1. Treatment of chondrogenic limb bud cells plated in micromass with an HS inhibitor increases heparanase expression at the mRNA (A) and protein (B) levels.
Fig 2. Immunohistochemical localization of heparanase in a normal human growth plate (A, B) and in exostosis (C, D). Scale bars, 300 μm in A, 75 μm in B.)