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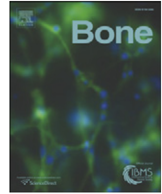

Full Length Article

Altered interaction and distribution of glycosaminoglycans and growth factors in mucopolysaccharidosis type I bone disease

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ABSTRACT

The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders characterized by deficient degradation and subsequent accumulation of glycosaminoglycans (GAGs). Progressive bone and joint disease are a major cause of morbidity, and current therapeutic strategies have limited effect on these symptoms. By elucidating pathophysiological mechanisms underlying bone disease, new therapeutic targets may be identified. Longitudinal growth is regulated by interaction between GAGs and growth factors. Because GAGs accumulate in the MPSs, we hypothesized that altered interaction between growth factors and GAGs contribute to the pathogenesis of MPS bone disease. In this study, binding between GAGs from MPS I chondrocytes and fibroblast growth factor 2 (FGF2) was not significantly different from binding of FGF2 to GAGs from control chondrocytes. FGF2 signaling, however, was increased in MPS I chondrocytes after incubation with FGF2, as compared to control chondrocytes. Using bone cultures, we demonstrated decreased growth of WT mouse bones after incubation with FGF2, but no effect on MPS I bone growth. However, MPS I bones showed decreased growth in the presence of GAGs from MPS I chondrocytes. Finally, we demonstrate altered GAG distribution in MPS I chondrocytes, and altered GAG, FGF2 and Indian hedgehog distribution in growth plates from MPS I mice. In summary, our results suggest that altered interaction and distribution of growth factors and accumulated GAGs may contribute to the pathogenesis of MPS bone disease. In the future, targeting growth factor regulation or the interaction between in growth factors and GAGs might be a promising therapeutic strategy for MPS bone disease.
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1. Introduction

The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders, each characterized by the deficiency of a single lysosomal enzyme required for glycosaminoglycan (GAG) degradation. This leads to accumulation of the GAGs heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate (KS) in lysosomes and in the extracellular matrix (ECM). Accumulation of these products leads to progressive cellular dysfunction, resulting in central nervous system disease, multi-organ failure and reduced lifespan [1]. In addition, skeletal dysplasia, generally referred to as dysostosis multiplex, is a striking feature of most of the MPSs and a major cause of morbidity. Patients present with shortened and thickened long bones, progressive loss of range of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in spinal cord compression [1,2]. This musculoskeletal disease frequently leads to orthopedic surgeries such as cervical decompression, femoral osteotomy and hip replacements. These procedures carry significant risks due to airway compromise, cardiac disease and cervical instability, often present in the MPSs [2,3].

Current available therapies for the MPSs are enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT) [1]. Potential future therapeutic options include gene therapy, substrate reduction therapy and anti-inflammatory therapy [4]. Current therapies, however, have limited effects on bone disease which may be at least partially due to the fact that treatment is usually started after the onset of irreversible bone lesions, which may already be present before birth [5]. In addition, the effect of both HSCT and ERT is limited due to the inability of the relatively large lysosomal enzymes to traffic through the poorly vascularized cartilage to target cells [6]. Also, cartilage cells are derived from mesenchymal stem cells, which are not transplanted in sufficient amounts by HSCT [7]. Finally, the pathophysiological

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cascades initiated by accumulated GAGs are still poorly understood, limiting the development of new therapeutic strategies.

Longitudinal growth of long bones results from the complex developmental process of endochondral bone formation. During this process, mesenchymal cells differentiate into chondrocytes, which proliferate and produce extracellular matrix (ECM). Chondrocytes undergo the process of hypertrophy, attract blood vessels and stimulate perichondral cells to differentiate into osteoblasts, followed by mineralization. These processes are dependent on the production and distribution of multiple growth factors, such as Indian hedgehog (Ihh), Fibroblast Growth Factors (FGFs) and Bone Morphogenic Proteins (BMPs) [8]. Proteoglycans, complexes of protein-attached GAGs, are a major class of ECM molecules, and play a pivotal role in regulating growth factor signaling [9]. Upon release from producing cells, growth factors may be transported across several cell diameters via interaction with GAGs on the cell surface or in the ECM, which creates a gradient of growth factors through the developing growth plate. At the cellular membrane, HS proteoglycans are required as co-receptors for proper interaction of growth factors with their receptors [10,11]. Accumulation of GAGs may have a direct effect on the size and spacing of chondrocytes, but also trigger secondary pathophysiological processes in the extracellular matrix, such as inflammatory responses. These processes may lead to the development of abnormally shaped bones, or, by mechanisms yet unknown, influence the previous mentioned steps of endochondral bone formation [12–14].

Because the MPSs are characterized by the accumulation of non-degraded or partially degraded GAGs, we hypothesized that altered interaction between growth factors and GAGs may contribute to alterations in extracellular matrix modeling, endochondral bone formation and ultimately, MPS bone disease. We used MPS I chondrocytes and MPS I mouse bones, in which HS and DS accumulate, as a model for MPS bone disease, to study binding between GAGs and FGF2, FGF2 signaling, the influence of FGF2 on bone growth, and GAG and growth factor distribution.

2. Materials and methods

2.1. Chemicals and materials

Minimal Essential Medium (MEM), L-glutamine and Non Essential Amino Acids (NEAA) were from Life Technologies. Diethylaminoethyl (DEAE) sepharose fast was from Pharmacia and 3 kD centrifugal filters from Millipore. Proteinase K (*Tritirachium album*) and heparin were from Sigma. FGF2 protein for ELISA experiments and western blot experiments were from e-Biosciences. Anti-FGF2 antibody for ELISA experiments were from Novus Biologicals (catalog number NB100-78224). GAG binding plates were from Iduron. Streptavidin-HRP was from R&D systems. Tetramethyl Benzidine Liquid Substrate (TMB) was from MP Biomedicals. P-p44/42 MAPK (phosphorylated ERK, pERK, catalog number 43,713) antibody was obtained from Cell Signaling Technologies and total MAPK-1 (ERK, catalog number m5670) and β-actin antibodies were from Sigma Aldrich. All secondary antibodies were from Westburg B.V. BGJb medium was from Life Technologies. Penicillin, streptomycin and amphotericin were from Lonza. FGF2 protein for bone growth experiments was from either Novus Biologicals or e-Biosciences. Anti-FGF2 antibody for immunohistochemistry was from Abcam (catalog number ab8880). Anti-Ihh antibody was from LifeSpan Biosciences (catalog number C40514). Primers were from Sigma Aldrich. Goat serum, Rabbit Immunoglobulin and Envision System HRP Kit were from Dako. The VSV-tagged (Vesicular Stomatitis Virus tag) antibodies for specific HS (HS4C3, EV3C3, HS4E4, NS4F5, LKIV69), DS (LKN1, GD3A12), CS domains (IO3H10, GD3G7) and anti-VSV antibody were prepared as described [15–22]. Brightvision DPVB-AP kit was from Immunologic and SIGMA Fast 3,3-diaminobenzidine (DAB) tablets and DPX mounting medium were from Sigma. Ammoniumacetate, sodiumchloride, triton-X-100, tween-20, Nonfat dried milk powder, phosphate buffered saline (PBS), sulfuric acid, paraformaldehyde, eosin and hematoxylin were of analytical grade.

2.2. Experiments with human cell lines

2.2.1. Cell culture

Informed consent for the use of fibroblasts was obtained from all patients or parents. Chrondrogenic differentiation of fibroblasts was performed essentially as described earlier [23], with minor modifications for FGF2 signaling experiments. Instead of DMEM, MEM supplemented with 1% L-glutamine and 1% NEAA was used. In addition, 4 days before the completion of chondrogenic differentiation (3 weeks after plating), the medium was replaced by serum-free medium. For all cell experiments, MPS I Hurler (severe phenotype) cell lines were used.

2.2.2. Isolation of GAGs

All steps were carried out at room temperature (RT) unless otherwise stated. Columns containing 3 mL of DEAE resin were washed with wash buffer containing 20 mM ammonium acetate pH 6, 0.11 mM sodium chloride and 1 g/L Triton-X-100. Next, culture medium of healthy control and MPS I chondrocytes was loaded on a column, washed with wash buffer and eluted with 20 mM ammonium acetate pH 6 and 1 mM sodium chloride. The elution fractions were applied onto a 3 kD filter and centrifuged at 14,000 *g* for 30 min. The filter was washed twice with MilliQ by centrifuging at 14,000 *g* for 3 min. Finally, to elute the GAGs from the filter, 150 μl MilliQ was applied to the filter, and the filter was inverted and centrifuged at 1000 *g* for 2 min. The GAG content of the elution fractions was analyzed with HPLC–MS/MS, as previously described [23]. Before use in bone growth experiments, isolated GAGs were incubated with 2.5 mg/ml (final concentration) proteinase K for 15 min at 37 °C, to remove remaining protein. The reaction was stopped by the addition of a 10% volume of 200 mM w/v phenylmethanesulfonyl fluoride (PMSF) in propanol and samples were boiled for 3 min and another 10% of the total volume of the sample of PMSF solution was added.

2.2.3. Growth factor ELISA

GAG binding plates were coated overnight at RT with 0.4 μg/ml GAGs isolated from the medium of healthy control or MPS I chondrocytes, diluted in standard assay buffer (SAB) consisting of 50 mM sodium acetate, 100 mM sodium chloride and 0.2% Tween-20. Between each of the following steps, the plate was washed three times with SAB and all subsequent steps were carried out at 37 °C unless otherwise stated. Plates were blocked with 1% w/v nonfat dried milk powder and 0.1% v/v Tween-20 in PBS and subsequent dilutions were made in blocking solution. Plates were incubated with 1 μg/ml of FGF2 for 2 h, followed by incubation with anti-FGF2 antibody (diluted 1:500) for 1 h. Next, the plates were incubated with streptavidin-HRP (diluted 1:200). Color development was performed with TMB at RT and the reaction was stopped by adding 1.8 M sulfuric acid. Absorbance at 450 nm was measured and concentrations were calculated in the arbitrary unit % binding capacity, relative to a standard curve of heparin.

2.2.4. Immunohistochemistry

Chondrocytes were grown on coverslips. All steps of the immunohistochemical procedures were carried out at RT unless otherwise stated and coverslips were washed three times with PBS for 5 min between each step. Fibroblasts were fixed in 4% w/v paraformaldehyde in PBS for 15 min. Subsequently, coverslips were incubated for 3 min with 3% v/v H_2O_2 to eliminate endogenous peroxidase activity. Coverslips were blocked with 3% v/v goat serum for 1 h and incubated overnight at 4 °C with antibodies against specific HS, DS and CS domains, diluted 1:10 in block solution. To check the specificity of the staining, negative controls (slides incubated with 5% v/v goat serum instead of the primary antibody) were included in each experiment. Next, coverslips were incubated with a mouse anti-VSV antibody (1:10 dilution) for 1 h, with

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