MPS I: Early diagnosis, and treatment of bone disease

Kingma, S.D.K.

Citation for published version (APA):
Chapter
Altered interaction and distribution of glycosaminoglycans and growth factors in Mucopolysaccharidosis type I bone disease

Sandra D.K. Kingma 1,2, Tom Wagemans1,2, Lodewijk IJlst 2, Antonius L.J.J. Bronckers 3, Toin H. van Kuppevelt 4, Vincent Everts 3, Frits A. Wijburg 1, Naomi van Vlies 1,2

1 Department of Pediatrics and Amsterdam Lysosome Center ‘Sphinx’, 2 Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Centre, University of Amsterdam, 3 Department of Oral Cell Biology ACTA, University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Amsterdam, 4 Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands.

Manuscript in preparation
ABSTRACT

The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders characterized by deficient enzymatic degradation and subsequent accumulation of glycosaminoglycans (GAGs). Progressive bone and joint disease are a major cause of morbidity in the MPSs, and current therapeutic strategies have limited effect on these symptoms. By elucidating the pathophysiological mechanisms underlying bone disease, new therapeutic targets may be identified. Longitudinal growth is regulated by the interaction between GAGs and various growth factors. Because GAGs accumulate in the MPSs, we hypothesized that altered interaction between growth factors and GAGs contributes to the pathogenesis of MPS bone disease. In this study, binding between GAGs from MPS I chondrocytes to 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 a bone culture system, we demonstrated decreased growth of WT mouse bones after incubation with FGF2, but no effect on growth of MPS I bones. However, MPS I bones showed decreased growth in the presence of GAGs from MPS I chondrocytes. Finally, we demonstrate alterations in GAG distribution in MPS I chondrocytes, and alterations in 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 contributes to the pathogenesis of MPS bone disease. In the future, targeting growth factor regulation or the interaction between certain growth factors and GAGs might be a promising therapeutic strategy for MPS bone disease.
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. 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 may show progressive loss of range of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in spinal cord compression. This musculoskeletal disease frequently leads to orthopaedic 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.

Current available therapies for the MPSs are enzyme replacement therapy (ERT) and haematopoietic stem cell transplantation (HSCT). Potential future therapeutic options include gene therapy, substrate reduction therapy and anti-inflammatory therapy. 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. 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. Also, cartilage cells are derived from mesenchymal stem cells, which are not transplanted in sufficient amounts by HSCT. Finally, the pathophysiological 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). Proteoglycans, complexes of protein-attached GAGs, are a major class of ECM molecules, and play a pivotal role in regulating growth factor signaling. 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. 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 contributes to 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.

**MATERIALS AND METHODS**

**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 3kD 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 43713) 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 tagged) antibodies for specific HS (HS4C3, EV3C3, HS4E4, NS4F5, LKIV69), DS (LKN1, GD3A12), CS domains (IO3H10, GD3G7) and anti-VSV antibody were a kind gift from dr. van Kuppevelt (Radboud University Medical Center, Nijmegen, The Netherlands). 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.

**Experiments with human cell lines**

*Cell culture*

Informed consent for the use of fibroblasts was obtained from all patients or parents.
Altered interaction and distribution of GAGs and growth factors in MPS I

Chondrogenic differentiation of fibroblasts was performed essentially as described earlier 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.

Isolation of GAGs

All steps were carried out at room temperature (RT) unless otherwise stated. Columns containing 3mL of DEAE resin were washed with wash buffer containing 20mM ammonium acetate pH6, 0.11mM sodium chloride and 1g/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 20mM ammonium acetate pH6 and 1mM sodium chloride. The elution fractions were applied onto a 3kD filter and centrifuged at 14,000g for 30 minutes. The filter was washed twice with MilliQ by centrifuging at 14,000g for 3 minutes. Finally, to elute the GAGs from the filter, 150µl MilliQ was applied to the filter, and the filter was inverted and centrifuged at 1,000g for 2 minutes. The GAG content of the elution fractions was analyzed with HPLC-MS/MS, as previously described. Before use in bone growth experiments, isolated GAGs were incubated with 2.5mg/ml (final concentration) proteinase K for 15 minutes at 37°C, to remove remaining protein. The reaction was stopped by the addition of a 10% volume of 200mM w/v phenylmethanesulfonfyl fluoride (PMSF) in propanol and samples were boiled for 3 minutes and another 10% of the total volume of the sample of PMSF solution was added.

Growth factor ELISA

GAG binding plates were coated overnight at RT with 0.4µg/ml GAGs isolated from the medium of healthy control or MPS I chondrocytes, diluted in standard assay buffer (SAB) consisting of 50mM sodium acetate, 100mM 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 1µg/ml of FGF2 for 2 hours, followed by incubation with anti-FGF2 antibody (diluted 1:500) for 1 hour. 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 1.8M sulfuric acid. Absorbance at 450nm was measured and concentrations were calculated in the arbitrary unit of percent binding capacity of HS, relative to a standard curve of heparin.
CHAPTER 5

Immunochemistry
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 minutes between each step. Fibroblasts were fixed in 4% w/v paraformaldehyde in PBS for 15 minutes. Subsequently, coverslips were incubated for 3 minutes with 3% v/v H$_2$O$_2$ to eliminate endogenous peroxidase activity. Coverslips were blocked with 3% v/v goat serum for 1 hour 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 hour, with post-antibody block (BrightVision Kit) for 15 minutes and subsequently with HRP goat anti-mouse/rabbit (Brightvision Kit) for 30 minutes. Staining was visualized with DAB substrate (SIGMA-Fast). Finally, coverslips were stored in PBS at 4°C for at least 1 day, dehydrated, and mounted in DPX.

FGF2 signaling
To examine FGF2 signaling in control and MPS I cells, chondrocytes were incubated for 10 minutes with 3.75ng/ml FGF at 37°C. At this concentration phosphorylation of the second messenger phosphorylated ERK (pERK) is induced to 50% of the maximal level in control chondrocytes (results not shown). Next, cells were harvested and levels of total and phosphorylated ERK were analyzed by western blot analysis as described earlier 20, with the following antibody dilutions: pERK 1:2,000, ERK 1:10,000, actin 1:10,000, IRDye 800 goat anti-rabbit 1:10,000, IRDye 680 donkey anti-mouse; 1:5,000.

Experiments with mouse bones
Animal experiments
MPS I mice (B6.129-Idua$^{tm1Clk}$/J 21) were purchased from Jackson Laboratory and maintained as a heterozygote line on an inbred C57BL/6J background at the Academic Medical Centre, Amsterdam, The Netherlands. The mice were housed at 21 ± 1°C, 40–50% humidity, on a 12 hours light-dark cycle, with ad libitum access to regular food pellets and water. Genotypes were identified by PCR using a protocol provided by Jackson Laboratory, using the primers: 5’-GGAACGTTCAGACTGGTAATGGAACCAG-3’ (common forward), 5’-CATTGAAATAGGGGTATCCTTTGAAACTC-3’ (WT reverse) and 5’-GGATTGGGAAGACAATAGCAGGCATGCT-3’ (MPS I reverse). At the age of 6 days, 3 weeks or 11 weeks, mice were anesthetized with an intraperitoneal injection of 100mg/kg pentobarbital and euthanized by exsanguination via cardiac puncture. All experimental procedures involving animals were approved by the institutional review board for animal experiments at the Academic Medical Centre, University of Amsterdam (Amsterdam, The Netherlands).
CHAPTER 5 Altered interaction and distribution of GAGs and growth factors in MPS I

**Bone growth experiments**

Metatarsalia of 6 day old female WT and MPS I mice were collected and placed in BGJb medium containing 0.1% w/v BSA and 100 U/ml penicillin, 100μg/ml streptomycin and 250ng/ml amphotericin in a humidified atmosphere containing 5% CO₂ at 37°C. Pictures of bones were made at day 0 and 5, were anonymized for the researcher and bone length was determined using Adobe Illustrator. To enable comparisons, bone growth of a metatarsal was always compared with bone growth of the corresponding metatarsal from the other paw of the same animal. 2ng/ml FGF2 was compared with untreated, 2ng/ml FGF2 + 5ng/ml healthy control or MPS I GAGs isolated from chondrocytes was compared with 2ng/ml FGF2, and 2ng/ml FGF2 + 5ng/ml MPS I GAGs was compared with 2ng/ml FGF2 + 5ng/ml healthy control GAGs. Factor correction was used to remove the between-session variation, as described by Ruijter et al. 22.

**Immunohistochemistry**

Femurs of 3 and 11 week old male WT and MPS I mice were collected and placed in 5% w/v paraformaldehyde in PBS. After 1 day, femurs were decalcified in a buffer containing 41.3g/L disodium-ethylenediaminetetraacetic acid and 0.8% v/v formalin, for approximately 6 weeks. Next, bones were embedded in paraffin by routine procedures and 4.5µm transversal sections were cut. After dewaxing and dehydration, sections were treated with 1% w/v proteinase K for 7 minutes at 37°C. GAGs in bones were stained as described for chondrocytes, with slight modifications. The antibody dilutions were as follows: LKN1 1:5, HS4C3 1:10, EV3C3/HS4E4/NS4F5 1:20, LKIV69/IO3H10/GD3A12 1:40, GD3G7 1:80 and a routine counterstain with hematoxylin was performed. For the FGF2 and Ihh immunohistochemistry, the same protocol was used with a few modifications. The H₂O₂ step was performed with peroxidase block from the Envision kit and blocking with a 1:3 dilution of goat serum. After incubation with the primary antibody against FGF2 in a 1:200 dilution or Ihh in a 1:50 dilution, sections were incubated for 1 hour with the labeled polymer HRP goat anti-rabbit (Envision kit), followed by visualization using DAB substrate (Envision kit). To check the specificity of the staining, negative controls (slides incubated with matched non-immune IgG instead of the primary antibody) were included in each experiment.

To study morphology, a routine hematoxylin/eosin (HE) staining was performed on femurs of 3 and 11 week old mice.

**Statistical analysis**

Statistical analysis was performed using SPSS Statistics software. Data were analyzed using two-tailed Mann-Whitney-U tests for nonparametric analysis. Significance was assumed where p values were less than 0.05.
CHAPTER 5

RESULTS

FGF2 binding of MPS I GAGs

To study the binding capacity of GAGs to FGF2, a GAG binding plate was coated with GAGs isolated from the medium of healthy control chondrocytes and GAGs from the medium of MPS I chondrocytes. As expected, medium of MPS I chondrocytes contained 2.5 fold more GAGs, compared to medium of control chondrocytes (results not shown). When equal quantities of GAGs were coated to the plates, there was a trend, which was not significant ($p=0.13$) towards lower binding capacity of MPS I GAGs, as compared to healthy control GAGs (fig. 1).

Figure 1. Binding of FGF2 to GAGs isolated from the medium of healthy control and MPS I chondrocytes, expressed as % of the binding capacity of the same amount of coated heparin. All values are mean ± standard deviation of 3 different cell lines. All analyses were repeated at least once in independent cell cultures, with similar results. Each sample was analyzed in triplicate.

Figure 2. Immunostaining of healthy control (A) and MPS I chondrocytes (B) with the anti-HS antibody LKIV69, healthy control (C) and MPS I chondrocytes (D) with the anti-HS antibody HS4C3, and healthy control (E) and MPS I chondrocytes (F) with the anti-DS antibody GD3A12. For each group, the experiment was performed in 3 different cell lines, 1 representative figure per group is shown.
GAG distribution in MPS I chondrocytes

Staining with antibodies against specific HS, DS and CS domains was performed to study GAGs content and distribution in the ECM of healthy control and MPS I chondrocytes. Using the anti-HS antibody LKIV69 which binds to 2-0-, and N-sulfated HS domains 18, an even distribution of GAGs in the ECM of healthy control chondrocytes was observed (fig. 2A). In MPS I chondrocytes, this antibody showed a more focal GAG distribution (fig. 2B). Similar changes were observed using the anti-HS antibody HS4C3 (fig. 2C, D), which binds to 3-0-sulfated glucosamine domains 19, and the anti-DS antibody GD3A12 (fig. 2E, F) which binds to disaccharides composed of iduronic acid and 4-0-sulfated galactosamine, and possibly also to 2-0-sulfated DS domains 17. No differences between cell types were observed with the other antibodies (results not shown).

FGF2 signaling in MPS I chondrocytes

To study differences in FGF2 signaling, healthy control and MPS I chondrocytes were incubated with FGF2 and protein levels of the second messenger pERK were measured,
which were normalized for β-actin and total ERK levels in each sample. FGF2 incubation led to a mild but significant ($p<0.05$) increase in pERK protein levels in MPS I chondrocytes (fig. 3), as compared to healthy control chondrocytes.

**Ex vivo growth in MPS I bones**

To study the effect of FGF2 on bone growth, metatarsalia from WT and MPS I mice were cultured and growth was measured. The coefficient of variation (%CV) of bone growth measurements was 0.3% (results not shown). After 5 days of incubation, bone growth slowed to a plateau phase (results not shown), therefore, bones were incubated for 5 days in subsequent experiments. Growth of MPS I bones (16±6%) was significantly decreased ($p<0.01$), as compared to WT bones (22±3.5%, results not shown).

After FGF2 incubation, growth was significantly different between WT and MPS I bones ($p<0.01$, fig. 4A). As expected, FGF2 incubation led to decreased growth of WT bones ($p<0.01$), but FGF2 incubation had no effect on growth of MPS I bones (fig. 4A).

Next, the additional effect of GAGs isolated from the medium of healthy control and MPS I chondrocytes was analyzed. When incubation with FGF2 and MPS I GAGs was compared to incubation with FGF2 and healthy control GAGs, there was a significant difference in growth between WT and MPS I bones ($p<0.01$, fig. 4B). WT bones showed increased growth when incubated with MPS I GAGs, as compared to healthy control GAGs ($p<0.05$), however, MPS I bones showed decreased growth in the presence of MPS I GAGs ($p<0.05$), as compared to healthy control GAGs.

To confirm this, incubation with FGF2 was compared to incubation with FGF2 and control GAGs, or FGF2 and MPS I GAGs in a separate experiment. Incubation with control GAGs, in addition to FGF2, did not significantly alter growth of healthy control or MPS I bones (results not shown). Incubation with MPS I GAGs, however, significantly decreased growth of MPS I bones ($p<0.01$), as compared to incubation with only FGF2, but did not affect WT bone growth (results not shown).

**Structural abnormalities in MPS I growth plates**

Femur length of 11 week old MPS I mice was 4% decreased ($p<0.05$), as compared to WT mice (results not shown). As earlier reported, growth plates of MPS I mice exhibit several structural abnormalities, as compared to growth plates of WT mice (fig. 5). In growth plates of 3 week old WT mice, large proliferating zones and smaller hypertrophic zones of chondrocytes were observed (fig. 5A). These zones were of equal size in MPS I growth plates (fig. 5B). With a higher magnification, long rows of proliferating chondrocytes and a heterogeneous aspect of the hypertrophic chondrocytes were observed in WT growth
Altered interaction and distribution of GAGs and growth factors in MPS I

plates (fig. 5C). In MPS I growth plates, however, the rows of proliferating chondrocytes were shorter and more chaotically distributed (fig. 5D). In addition, the large hypertrophic zone was densely packed with chondrocytes with a square and swollen aspect (fig. 5D). In growth plates of 11 week old MPS I mice, the hypertrophic zones still showed an increased amount of large hypertrophic chondrocytes (fig. 5F), as compared to WT growth plates (fig. 5E).

GAG distribution in MPS I growth plates

To study whether growth plates of MPS I mice exhibit alterations in GAG distribution, as was observed for MPS I chondrocytes (fig. 2), staining with antibodies against specific HS, DS and CS domains was performed. Using the anti-HS antibody LKIV69 (fig. 1A, B), which binds to 2-O-, and N-sulfated HS domains \(^{18}\), brown GAG staining was observed in the ECM of growth plates of 3 week old WT mice (fig. 6A), but not in the ECM of MPS I growth plates (fig. 6B). In addition, the use of the anti-HS antibody HS4C3 revealed a higher abundance of 3-O-sulfated glucosamine domains \(^{19}\) in growth plates of 11 week old MPS I mice (fig. 6D), as compared to WT growth plates (fig. 6C).
Growth factor distribution in MPS I growth plates

DAB staining using an anti-FGF2 antibody was performed to analyze the distribution of FGF2 in growth plates of 3 week old WT and MPS I mice. FGF2 staining was clearly present in chondrocytes and in the ECM of the proliferating and hypertrophic zone of the WT growth plate (fig. 7A). In the proliferating zone of MPS I growth plates, however, a low abundance of FGF2 and a clearly visible blue nuclear counterstaining was observed (fig. 7B).

To study whether MPS I growth plates also exhibited alterations in other growth factors, DAB staining using an anti-Ihh antibody was performed. In the WT growth plate, most Ihh staining was present in the hypertrophic and prehypertrophic zone, with clearly visible nuclear counterstaining in the proliferating zone (fig. 7C). In the MPS I growth plate, however, most of the Ihh staining was present in the proliferating and prehypertrophic zone (fig. 7D).
DISCUSSION

Skeletal disease is one of the most prevalent and incapacitating disease manifestations in patients suffering from the MPSs and frequently results in the need for multiple surgical interventions. Current therapeutic strategies have a limited effect on the progression of MPS bone disease. The pathophysiological processes initiated by accumulated GAGs are still poorly understood, which limits the development of new therapeutic strategies. In this study, we demonstrate alterations in the interaction and distribution of GAGs and growth factors in chondrocytes from MPS I patients and growth plates of MPS I mice. This suggests that these pathophysiological processes contribute to MPS bone disease and we hypothesize that growth factor regulation may be a future therapeutic target.

Figure 7. Immunostaining of WT (A) and MPS I (B) growth plates using an anti-FGF2 antibody and staining of WT (C) and MPS I (D) growth plates with an anti-Ihh antibody in 3 week old mice. For FGF2 staining, the arrows show brown DAB staining in nuclei of proliferating chondrocytes of WT (A) and absence of brown DAB staining, but clearer blue counterstaining in the nuclei of proliferating chondrocytes in MPS I (B) growth plates. For Ihh staining, the arrows show clear blue counterstaining of nuclei of proliferating chondrocytes in WT (C) and brown DAB staining in nuclei of proliferating chondrocytes in the MPS I growth plate (D). For each group, the experiment was performed in bones from 4 different mice, 1 representative figure per group is shown. P is the proliferating zone and H the hypertrophic zone of chondrocytes.
The influence of GAGs on growth factor regulation and signaling is complex and depends on the growth factor itself, on the binding capacity of GAGs for growth factors, and on the quantity, location (ECM, cell membrane, intracellular) structure (sulfation) and distribution of GAGs. Increased GAG levels in the ECM, for instance, may enhance the distribution of certain growth factors to their target cells, or on the other hand, may cause a delay in the distribution of other growth factors.

Because previous studies showed that GAGs with abnormal sulfation patterns accumulate in MPS cells and tissues, we investigated whether this results in altered interaction and distribution of GAGs and growth factors, as this may contribute to MPS I bone disease.

Pan et al. showed that binding of FGF2 to GAGs from the culture medium of human multipotent stem cells was decreased in MPS I. The medium of MPS I cells, however, probably contained an increased amount of GAGs, as compared to the medium of healthy control cells, which may have influenced the results. Using the same amounts of GAGs that were isolated from the conditioned medium of chondrocytes, we observed a trend, although not significant, towards lower FGF2 binding capacity of MPS I GAGs, as compared to healthy control GAGs. A small decrease in growth factor binding capacity of GAGs may, especially in the presence of increased quantities of these GAGs and abnormal GAG structure or distribution, significantly alter growth factor function. For instance, a decrease in growth factor binding may alter growth factor distribution, and may also change the rate of inactivation of growth factors, as it has been shown that growth factors bound by GAGs are protected against inactivation.

Next, to investigate whether MPS I chondrocytes exhibit abnormalities in GAG content or distribution, chondrocytes were stained using antibodies against specific HS, DS and CS domains. We observed altered distribution of 2-O-, 3-O-, and N-sulfated HS-domains and DS-disaccharides composed of iduronic acid and 4-O-sulfated galactosamine residues in the ECM of MPS I chondrocytes.

As alterations in GAG distribution may cause alterations in growth factor signaling, FGF2 signaling was studied by analyzing the second messenger pERK. After FGF2 incubation, pERK levels were increased in MPS I chondrocytes, as compared to control chondrocytes. Possible causes of this effect include GAG accumulation in the ECM of MPS I chondrocytes, which may, despite the small decrease in binding capacity of MPS I GAGs, lead to increased binding of FGF2, protection from inactivation, and increased distribution to target cells. We cannot exclude that MPS I cells are more sensitive to the effects of FGF2, and MPS I chondrocytes show no differences in gene-expression of FGF-receptors (unpublished results).
To study the effect of FGF2 on bone growth, metatarsals from WT and MPS I mice were incubated with FGF2. FGF2 treatment led to decreased growth of WT bones, which was expected, because FGF2 is an anti-proliferative growth factor in growth plates. With the concentration used in our experiments, FGF2 did not affect growth of MPS I mouse bones. An explanation may be that, due to the altered distribution of GAGs in MPS I bones, FGF2 is not distributed effectively and cannot induce its anti-proliferative effect. Also, due to accumulation of GAGs in the ECM, FGF2 may be bound and delayed the ECM. On the other hand, decreased binding of FGF2 to MPS I GAGs may result in increased degradation of FGF2, and a loss of its anti-proliferative effect.

Next, we studied the additional effect of GAGs isolated from the medium of healthy control or MPS I chondrocytes. Incubation of MPS I bones with medium containing MPS I GAGs, in the presence of FGF2, led to decreased bone growth, as compared to medium that contained control GAGs. In contrast, incubation of WT bones with MPS I GAGs, in the presence of FGF2, led to increased bone growth, or more correctly, decreased growth inhibition, as compared to incubation with control GAGs. Taken together, these results show that WT and MPS I bones react differently on treatment with FGF2 and externally added GAGs. The observed differences in growth patterns, however, are difficult to explain based on only alterations in FGF2 binding, signaling or distribution. Our results, therefore, suggest that externally added GAGs may also impact other growth factors that are present in the bone itself, or growth factors that are excreted in the medium by bone cells. Earlier reports on the effects of incubation of MPS I multipotent stem cells with growth factors showed decreased BMP4 signaling and a decreased effect of FGF2 treatment. These results are in contrast to our observation of increased FGF2 signaling in MPS I chondrocytes, but are in agreement with our observed decrease in FGF2-induced growth inhibition of MPS I mouse bones. Bone growth and growth factor signaling depend on many factors, such as growth factor activity and distribution, GAG distribution in the ECM and on the cell membrane and growth factor receptor expression. Likely, these factors, which were not all analyzed in our study, contribute to the differences in the effect of FGF2 that are observed in different studies.

We confirmed our results on altered distribution of 2-O-, 3-O-, and N-sulfated HS-domains in MPS I chondrocytes in MPS I mouse bones. Because these GAG domains are known to interact with growth factors, growth factor distribution was studied. The distribution of FGF2 in the developing growth plate of MPS I mice was altered, as compared to WT growth plates. Also, distribution of another essential growth factor, Ihh, suggests that alterations in GAG quantity, structure and distribution may cause alterations in the function and distribution of multiple growth factors, which may significantly contribute to the pathophysiological mechanisms underlying MPS bone disease.
CHAPTER 5

Previous studies have shown GAG accumulation in the ECM of MPS cells 31, but distribution of different GAG domains and growth factors, have, to our knowledge not been described earlier. Our results, however, are in agreement with the results of Heppner et al. 32 on abnormalities in gene expression of ECM proteins in MPS I mouse bones, and their suggestion that ECM disruption significantly contributes to the pathogenesis of MPS I bone disease.

There are a number of promising future therapeutic strategies for MPS bone disease, such as anti-inflammatory therapy to prevent the secondary inflammation processes induced by accumulating GAGs 33. Also, as chondrocytes are derived from mesenchymal cells, mesenchymal stem cell transplantation may increase the therapeutic efficacy of current HSCT protocols 7. Our results suggest that targeting growth factor regulation or the interaction between growth factors and GAGs may also be a promising therapeutic strategy for the prevention of MPS bone disease. Growth factor therapy, such as monoclonal antibodies against growth factor receptors is currently used as a therapy for some forms of cancer 34. These therapies, however, are focused on achieving cell death, and may have severe adverse effects. For the prevention of MPS bone disease, only drugs with an excellent safety profile that can be used for a long term period at and a very young age, are acceptable. Furthermore, the role of different growth factors and the contribution of different types of GAGs in the pathogenesis of MPS bone disease first need to be elucidated. Also, because growth factors regulate growth and development of all tissues, the interaction between growth factors and GAGs in other tissues than bone should be studied in the different MPSs.

CONCLUSION

Our results suggest that altered interaction and distribution of accumulated GAGs and growth factors contributes to the pathogenesis of MPS bone disease. In the future, targeting growth factor regulation or the interaction between certain growth factors and GAGs might be a therapeutic strategy for MPS bone disease.

ACKNOWLEDGEMENTS

We thank Ronald Wanders and Rene Leen for technical assistance and helpful discussions. We thank Marie-Louise Kingma and Jan Ruijter for help with statistical analysis. We thank Toin van Kuppevelt for kindly providing the GAG antibodies. This work was funded by the foundation ‘Steun Emma Kinderziekenhuis AMC’ and the ‘WE Foundation’.
REFERENCES


