MPS I: Early diagnosis, and treatment of bone disease
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Chapter
Secondary storage and alterations in composition and distribution of glycosaminoglycans in MPS I and III fibroblasts

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ABSTRACT
The mucopolysaccharidoses (MPSs) are caused by the deficiency of lysosomal glycosaminoglycan (GAG) degrading enzymes and subsequent GAG accumulation. Clinical manifestations may include shortened lifespan, neurological deterioration, multi-organ failure and bone dysplasia. Differences in clinical phenotype between different MPSs with the same accumulating GAGs, and phenotypic differences within the same type of MPS and the same disease causing mutations, suggest the contribution of secondary processes involved in the pathophysiology. We studied secondary GAG storage, extracellular GAG distribution and gene-expression of enzymes involved in GAG synthesis in fibroblasts (FBs) from MPS I (heparan sulfate (HS) and dermatan sulfate (DS) accumulation) and MPS III (HS accumulation) patients. We demonstrate secondary accumulation of chondroitin sulfate (CS) in both MPS I and III FBs and secondary accumulation of DS in MPS III FBs. Also, we observed differences in the fractions of HS-derived disaccharides and CS-derived disaccharides between MPS I and MPS III FBs. In addition, we observed alterations in the extracellular distribution of sulfated HS domains in MPS I FBs and decreased abundance in MPS III FBs, as compared to healthy control FBs. Finally, gene-expression levels of HS, DS and CS synthesis enzymes were increased in MPS I and MPS III FBs, as compared to healthy control FBs. These secondary effects may contribute to clinical heterogeneity and poor genotype-phenotype correlations in MPS patients. In the future, treatments that aim to decrease synthesis or increase degradation of GAGs not typically associated with a certain MPS subtype might assist in altering the clinical course of MPS patients.
INTRODUCTION

The mucopolysaccharidoses (MPSs) comprise a group of heterogenic lysosomal storage disorders, characterized by glycosaminoglycan (GAG) accumulation. Each MPS subtype is caused by the deficiency of a single lysosomal enzyme required for the degradation of the GAGs heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), keratan sulfate (KS) or hyaluronan. GAG accumulation results in cellular damage which affects multiple organ systems. Clinical manifestations may include reduced lifespan, multi-organ failure, bone dysplasia and cognitive impairment. Disease severity is thought to depend on the degree of enzyme deficiency and the storage product. Differences in phenotype between the MPSs in which the same GAGs accumulate or even between patients with the same disease causing mutations, however, remain largely unexplained. This indicates that there may be secondary biochemical and cellular pathways involved in the pathogenesis of these diseases, initially triggered by the accumulation of non-degraded or partially degraded GAGs. Such processes include inflammation, autophagy, growth factor signaling defects and the storage of secondary metabolites. Secondary metabolites may include gangliosides, which have been reported to accumulate in several MPSs or GAGs not typically associated with a certain MPS subtype. The cause of secondary accumulation remains unknown, but probably involves the disruptive effects of storage products on lysosomal enzymes and cellular trafficking. Also, changes in gene-expression of GAG synthesis enzymes have been suggested to contribute to secondary storage and disease manifestations.

In order to better understand the pathogenesis of the MPSs, which is needed for the identification of new therapeutic targets, it is essential to understand the complex cascade of events leading to secondary substrate accumulation. As far as we know, no studies have been reported in MPS I or MPS III on increased GAG synthesis as a cause of secondary GAG storage, nor on alterations in GAG content and distribution in the extracellular matrix (ECM).

In order to explore the potential role of secondary GAG synthesis and storage in the MPSs, we studied secondary GAG storage and GAG composition in fibroblasts (FBs) from MPS I (OMIM 607014, HS and DS accumulation) and MPS IIIA, B and C (OMIM 252900, 252920, 252930, HS accumulation) patients. In addition, we performed immunohistochemistry to study GAG distribution in the ECM. Finally, we studied gene-expression of HS, DS and CS synthesis enzymes.

MATERIALS AND METHODS

Chemicals and materials

Primers were from Biolegio or Sigma Aldrich. Goat serum was from Dako. The antibodies for specific HS (HS4C3, EV3C3, HS4E4, NS4F5, LKIV69), DS (LKN1, GD3A12), CS domains (IO3H10,
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GD3G7) and anti-VSV antibody were a kind gift from dr. van Kuppevelt (Radboud University Medical Center, Nijmegen, The Netherlands). Brightvision DPV8-AP kit was from Immunologic and SIGMA Fast 3,3-diaminobenzidine (DAB) tablets and DPX mounting medium were from Sigma. Phosphate buffered saline (PBS), $H_2O_2$, paraformaldehyde, eosin and haematoxylin were of analytical grade.

Cell culture

Informed consent for the use of FBs was obtained from all patients or parents. Cell culture was performed as described earlier. Experiments were performed 12 days after plating.

GAG analysis

GAG levels were determined by measuring HS, DS and CS-derived disaccharides using HPLC-MS/MS, as described previously, with a minor modification: 40μg of FB lysate was used. As D0a4 was the only detectable DS-derived disaccharide, only this value is given. Significant differences in GAG levels or fraction of ≥25% were considered biologically relevant.

Immunohistochemistry

FBs were grown on coverslips. All steps of the immunohistochemical procedures were carried out at room temperature unless otherwise stated and coverslips were washed three times with PBS for 5 minutes between each step. FBs were fixed in 4% w/v paraformaldehyde in PBS for 15 minutes. Subsequently, coverslips were incubated for 3 minutes with 3% v/v $H_2O_2$ 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. 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 GaM/R (Brightvision Kit) for 30 minutes. Staining was visualized with DAB substrate. Finally, coverslips were stored in PBS at 4°C for at least 1 day, dehydrated, and mounted in DPX. Cell morphology was analyzed with routine hematoxylin/eosin (HE) staining.

Quantitative real-time PCR
RNA isolation and qPCR analysis was performed as described earlier. Primer sequences are described in Table 1. Significant differences in gene expression of ≥2 fold gene expression were considered biologically relevant.

Statistical analysis
Statistical analysis was performed using nonparametric tests: Kruskal Wallis tests for comparison between more than 2 groups and two-tailed Mann-Whitney-U tests for comparison between 2 groups and post-hoc analysis. SPSS Statistics software version 21 (IBM Corp.) was used. Significance was assumed where p values were <0.05.

RESULTS
GAG analysis

GAG levels
As expected, HS levels in MPS I, MPS IIIA, MPS IIIB and MPS IIIC FBs were increased (10, 19, 14 and 16 fold respectively, p<0.05), as compared to healthy control FBs (fig. 1A). DS levels were 11 fold (p<0.05) increased in MPS I FBs, but also 6 fold (p<0.05) and 4 fold (p<0.05) increased in MPS IIIA and MPS IIIC FBs, respectively, as compared to healthy control FBs (fig. 1B). Surprisingly, CS levels were 6 fold (p<0.05), 6 fold (p<0.05) and 4 fold (p<0.05) increased in MPS I, MPS IIIA and MPS IIIC FBs, respectively, as compared to healthy control (fig. 1C).

FBs from all MPS IIIB patients followed the same trends, but due to large variation between cell lines, changes were not significant. KS was not detectable in any of the studied FBs (results not shown).

Kruskal Wallis tests were performed to study differences between 3 groups: healthy control, MPS I and MPS III FBs. Because MPS IIIA, MPS IIIB and MPS IIIC are characterized by HS accumulation and the same clinical manifestations, and because the values were not significantly different between the MPS III subtypes, we combined these groups together for the Kruskal Wallis tests. Total DS levels were significantly different between healthy control, MPS I and MPS III FBs (p<0.01).
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GAG fractions

No biologically relevant differences (>25%) in fractions of the HS-derived disaccharides D0a0, D0S0, D0A6-D2A0 and D0S6-D2S0 were observed between healthy control and MPS FBs (results not shown). As compared to healthy controls, fractions of the highly sulfated HS-derived disaccharide D2S6 were 64% ($p<0.05$) and 109% ($p<0.05$) increased in MPS IIIA, and MPS IIIC FBs, respectively (fig. 1D). No difference in D2S6-fraction between

Figure 1. HS levels (A), DS levels (B), CS levels (C), expressed as milligrams GAGs per gram of protein. HS-derived disaccharide D2S6 (D), expressed as percentage of total HS (tHS) levels. CS-derived disaccharides D0a0 (E) and D0a6 (F), expressed as percentage of total CS (tCS). All values are mean ± standard deviation of 3 cell lines. Each sample was analyzed in duplicate. * $p<0.05$, asterisks above bars: as compared to healthy control FBs.
healthy controls and MPS I FBs were observed. The fractions of CS-derived disaccharides D0a0 (fig. 1E) and D0a6 (fig. 1F) were not significantly different in MPS FBs, as compared to healthy control FBs.

Kruskal Wallis tests and subsequent post-hoc analysis revealed significant differences in fractions of CS-derived disaccharides D0a0 \( (p<0.05) \) and D0a6 \( (p<0.05) \) between MPS I and MPS III FBs \( (p<0.05) \). Because the fraction of HS-derived disaccharide D2S6 was significantly different between MPS III subtypes (increased in MPS IIIC FBs as compared to MPS IIIA and B, \( p<0.05 \)), no Kruskal Wallis test was performed to study differences between healthy control, MPS I and all types of MPS III FBs. However, D2S6 fraction was significantly higher in MPS IIIA and MPS IIIC FBs \( (p<0.05) \), as compared to MPS I FBs.

**GAG staining**

Staining with antibodies against specific HS, DS and CS domains was performed to study GAG content and distribution in the ECM. Using the anti-HS antibody LKIV69, which binds to 2-\( \Omega \)-, and N-sulfated HS domains \(^{16}\), differences between healthy controls, MPS I and MPS III FBs were observed (fig. 2). In healthy control FBs (fig. 2A), a typical pattern of HS staining could be observed in the ECM, which was present in ridges in a fishnet pattern. In MPS I FBs (fig. 2B), HS staining was distributed equally and evenly in the ECM. HS staining was less abundant in the ECM of MPS IIIA (fig. 2C), MPS IIIB (fig. 2D) and MPS IIIC (fig. 2E) FBs, as compared to healthy control and MPS I FBs. We observed no differences in morphology or growth patterns between healthy control, MPS I and MPS III FBs (fig. 2F-J).

**Figure 2.** Immunostaining of healthy control (A), MPS I (B), MPS IIIA (C), MPS IIIB (D) and MPS IIIC (E) FBs with the anti-H5 antibody LKIV69. HE staining of healthy control (F), MPS I (G), MPS IIIA (H), MPS IIIB (I) and MPS IIIC (J) FBs. For each group, the experiment was performed in 3 different cell lines, 1 representative figure per group is shown.
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Gene expression of enzymes involved in GAG synthesis

Gene expression of enzymes involved in GAG synthesis was measured using RT-qPCR (table 2). Expression levels of the following genes involved in HS synthesis were significantly different (p<0.05) in the MPSs, as compared to healthy control: EXT2 (MPS IIIA 3 fold), HS6ST1 (MPS I 4 fold, MPS IIIC 4 fold), NDST1 (MPS I 13 fold, MPS IIIA 8 fold, MPS IIIC 6 fold) and NDST2 (MPS I 7 fold, MPS IIIC 3 fold). Expression levels of the DS synthesis gene CHST14 was 5 fold increased in MPS I, 5 fold in MPS IIIA and 4 fold in MPS IIIC FBs (p<0.05). There was a significant increase (p<0.05) in the following genes involved in both DS and CS synthesis: CHPF2 (MPS I 10 fold, MPS IIIC 4 fold), CHST12 (MPS I 4 fold, MPSIIIC 3 fold) and CHSY1 (MPS I 5 fold, MPS IIIC 3 fold). Expression of the KS synthesis gene CHST2 was 13 fold increased in MPS IIIC FBs (p<0.05). Expression levels of other KS synthesis genes were below detection level in FBs. FBs from all MPS IIIB patients followed the same trends, but due to large variation between cell lines, changes were not significant.

DISCUSSION

The MPSs are characterized by clinical heterogeneity and, for most disease causing mutations, poor genotype-phenotype correlations. In order to better understand the pathogenesis of the MPSs and to identify new therapeutic targets, further studies on the processes that may contribute to these striking differences between patients with the same accumulating substrates are warranted. We used cultured FBs from MPS I and MPS III patients to study

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<th>MPS IIIA</th>
<th>MPS IIIB</th>
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Table 2. Percentage change ± standard deviation in mRNA expression of GAG synthesis enzymes. Expression levels in healthy control FBs are set at 100%. All values were normalized against the housekeeping gene PPIB. All measurements were performed in duplicate in 3 different cell lines, * p<0.05.
several potential factors in the disease pathogenesis of the MPSs: secondary storage of 
GAGs, alterations in the composition and distribution of GAGs and gene-expression of 
enzymes involved in GAG synthesis.

We demonstrate secondary storage of CS in MPS I, MPS IIIA and MPS IIIC FBs. We also 
confirm the results of Lamanna et al. on secondary DS storage in MPS IIIA and MPS IIIC 
FBs which was, thus far, the only report on secondary GAG storage in MPS FBs. Secondary 
elevations of different GAGs in urine and plasma from MPS patients have been reported 
more frequently.

Interestingly, we observed differences in the fractions of GAG derived disaccharides 
between FBs from MPS I and MPS III patients. The fractions of the 2-, 6-O-, N-sulfated HS-
derived disaccharide D2S6 and CS-derived disaccharide D0a6 were increased in MPS III FBs, 
as compared to MPS I FBs. In contrast, the unsulfated CS-derived disaccharide D0a0 was 
increased in MPS I FBs, as compared to MPS III FBs. Earlier studies have shown increased 
sulfation of HS in brain from MPS I and particularly MPS III mice, and it was suggested that 
this may contribute to the severity of neuropathology.

GAGs regulate many biological processes, such as inflammation and growth factor signaling, 
by interaction with a number of proteins present in the ECM. The function of GAGs is 
dependent on its extracellular distribution, structure and sulfation pattern. However, very 
little is known about extracellular GAG composition and distribution in the MPSs. To study 
whether MPS FBs exhibit abnormalities in GAG content or distribution in the ECM, FBs were 
stained with antibodies against specific HS, DS and CS domains. We show altered distribution 
of 2-O-, and N-sulfated HS-domains in the ECM of MPS I FBs, and decreased abundance in 
the ECM of MPS III FBs. These results seem in contrast to our GAG analysis results, in which 
we observed no difference in the fraction of the 2-O-, N-sulfated sulfated D2S0 and a higher 
fraction of 2-, 6-O-, N-sulfated D2S6 in MPS III FBs. GAG fractions, however, may be different 
between the intracellular and the extracellular compartment. Also, the results of McGlynn 
et al. , which suggest that GAG accumulation leads to defective cellular trafficking of 
secondary metabolites, may explain the differences that we observed in GAG composition 
and storage between the intracellular and extracellular compartment.

To investigate whether GAG synthesis is a contributing factor to secondary storage and 
differences in GAG composition in FBs, we studied gene-expression of several GAG synthesis 
enzymes. We observed increased gene expression of HS6ST1 (6-O-sulfation), NDST1 
(N-sulfation) and NDST2 (N-sulfation) in MPS I and MPS IIIC FBs, which are all necessary 
for the synthesis of the HS-derived disaccharide D2S6. Also, we observed an increase in 
expression of genes involved in DS and CS synthesis. We did not observe any decreases
in gene-expression of GAG synthesis enzymes. These results suggest that increased GAG synthesis may indeed contribute to the secondary GAG storage and abnormalities in GAG composition that we observed in MPS FBs. Accumulated GAGs are known to induce pro-inflammatory responses \(^2^6\), which may in turn stimulate GAG synthesis \(^2^7\). In addition, because GAGs interact with growth factors, alterations in GAG distribution may lead to alterations in growth factor distribution and signaling, including growth factors that regulate GAG synthesis. 2-O- and N-sulfated HS domains, for instance, are known to bind growth factors \(^1^6\).

An important limitation of this study was the considerable difference in GAG levels and gene-expression between MPS IIIB cell lines, due to variation in 1 cell line. Therefore, future studies should be initiated, studying more cell lines. Also, in addition to GAG synthesis, GAG degradation and trafficking may be studied for a more complete overview of the contribution of several pathways on secondary GAG storage and alterations of GAG distribution. Finally, in vitro studies in other cell types and in vivo studies are needed to confirm that secondary GAG storage and alterations in GAG composition and distribution may contribute to disease manifestations in the MPSs.

CONCLUSION

In conclusion, our studies show, for the first time, secondary storage of CS, abnormalities in HS composition and distribution and increased gene expression of GAG synthesis enzymes in FBs from MPS I and III patients. These secondary pathological effects may influence disease progression and contribute to clinical heterogeneity and poor genotype-phenotype correlations in MPS patients. In the future, therapies that increase degradation or decrease synthesis of GAGs not typically associated with a MPS subtype, may improve the efficacy of current disease modifying treatments.

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