Impaired bone remodeling and its correction by combination therapy in a mouse model of mucopolysaccharidosis-I

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Abstract

Mucopolysaccharidosis-I (MPS-I) is a lysosomal storage disease (LSD) caused by inactivating mutations of IDUA, encoding the glycosaminoglycan-degrading enzyme α-1-iduronidase. Although MPS-I is associated with skeletal abnormalities, the impact of IDUA deficiency on bone remodeling is poorly defined. Here we report that Idua-deficient mice progressively develop a high bone mass phenotype with pathological lysosomal storage in cells of the osteoblast lineage. Histomorphometric quantification identified shortening of bone-forming units and reduced osteoclast numbers per bone surface. This phenotype was not transferable into wild-type mice by bone marrow transplantation (BMT). In contrast, the high bone mass phenotype of Idua-deficient mice was prevented by BMT from wild-type donors. At the cellular level, BMT did not only normalize defects of Idua-deficient osteoblasts and osteocytes but additionally caused increased osteoclastogenesis. Based on clinical observations in an individual with MPS-I, previously subjected to BMT and enzyme replacement therapy (ERT), we treated Idua-deficient mice accordingly and found that combining both treatments normalized all histomorphometric parameters of bone remodeling. Our results demonstrate that BMT and ERT profoundly affect skeletal remodeling of Idua-deficient mice, thereby suggesting that individuals with MPS-I should be monitored for their bone remodeling status, before and after treatment, to avoid long-term skeletal complications.
Introduction

Lysosomal storage diseases (LSDs) represent a heterogeneous group of metabolic disorders caused by defects of specific lysosomal enzymes or general disturbances of lysosomal functions (1). Since lysosomes play a key role in macromolecule degradation by different cell types in various organs, LSDs are typically characterized by intracellular accumulation of non-digested macromolecules that interfere with the respective cellular functions (2). Of note, many LSDs are associated with various defects of skeletal development, growth or modeling, often summarized as dysostosis multiplex (3). These skeletal pathologies are well documented by radiology, but their cellular and molecular causes are poorly defined (4). In addition, there is still limited knowledge about the effects of lysosomal dysfunction on the two cell types responsible for bone remodeling, a physiologically relevant process ensuring long-term skeletal stability. Bone remodeling is mediated through the continuous and physiologically balanced activities of bone-forming osteoblasts and bone-resorbing osteoclasts (5). While osteoblasts are derived from mesenchymal progenitor cells and act in large groups of cells simultaneously producing the bone matrix, osteoclasts develop by fusion of hematopoietic progenitor cells and resorb bone by extracellular acidification and secretion of matrix-degrading enzymes (6,7). Based on these fundamental differences between the two bone remodeling cell types, it is likely that they are differentially affected in specific LSDs. Moreover, since available pharmacologic treatment options can either target osteoblasts to increase bone formation or inhibit bone resorption by osteoclasts, it is highly relevant to define the specific cellular defects in the respective individuals (8).

Mucopolysaccharidosis-I (MPS-I) is a prototypical LSD caused by deficiency of a single lysosomal enzyme and occurs at a frequency in the range of 1:100,000 (9–11). The disease is caused by homozygous inactivating mutations of the IDUA gene, encoding α-L-iduronidase, an enzyme required for degradation of heparan and dermatan sulfate, representing two highly abundant glycosaminoglycans (GAGs). Depending on the type of mutation, there is a wide range of disease severity. The most severe forms of MPS-I (commonly termed Hurler syndrome) are characterized by mental retardation, hepatosplenomegaly, dysostosis multiplex, corneal clouding, cardiac dysfunction and death within the first decade of life. The mildest forms (commonly termed Scheie syndrome) are characterized by normal intelligence and life expectancy, yet corneal clouding, dysostosis multiplex and mild visceral storage are still causing serious problems in the affected patients. The existence of a third intermediate form of MPS-I, the high bone mass phenotype of Idua-deficient mice is pre-77706176 | Human Molecular Genetics, 2015, Vol. 24, No. 24-

In terms of understanding MPS-I pathologies, it was an important step that an Idua-deficient mouse model was generated in 1997 (15). These mice, lacking detectable α-L-iduronidase activity, display many features of MPS-I, albeit they are apparently not as severely affected as patients with Hurler syndrome. More specifically, Idua-deficient mice neither displayed increased lethality until the age of 20 weeks, nor obvious growth deficiencies. Histologically, however, pathologic lysosomal storage was detectable in various organs, and urinary GAG levels were found significantly increased. Subsequent analyses additionally identified neurological pathologies and significantly shortened life span in Idua-deficient mice, thereby demonstrating their value as a model of MPS-I (16). With respect to the skeleton, features of dysostosis multiplex, such as enlargement of craniofacial bone structures and widening of the ribs, were observed in Idua-deficient mice, and their severity increased with age (17,18). Histologically, alterations of growth plate maturation as well as abnormalities of bone matrix organization have been described, and a recently published study demonstrated that the high bone mass phenotype of Idua-deficient mice is prevented by neonatal BMT (19,20). Importantly however, none of these studies provided a quantitative cellular analysis of bone remodeling cell types on the basis of undecalcified histology and histomorphometry.

We have previously analyzed a mouse model with mucolipidosis-II (ML-II), an LSD with severe lysosome dysfunction. Here we found, unexpectedly, that the osteoporotic phenotype of these mice was primarily caused by increased osteoclastogenesis and thus correctable by anti-resorptive bisphosphonate treatment (21,22). In the present manuscript, we show that Idua-deficient mice progressively develop a high bone mass phenotype, in sharp contrast to ML-II mice. The Idua-deficient phenotype was accompanied by impaired formation of osteoblasts and osteoclasts, and it was remarkably influenced by BMT. At the cellular level, BMT did not only normalize osteoclastogenesis, but instead dramatically increased the number of osteoclasts per bone surface specifically in Idua-deficient mice. Since this latter aspect of the phenotype was corrected by ERT, our results are potentially relevant for the clinical management of MPS-I.

Results

Impaired skeletal remodeling in Idua-deficient mice

We first stained skeletons from 4-week-old wild-type and Idua-deficient littermates with alcian blue and alizarin red. Here we failed to detect obvious differences between the two groups, demonstrating that Idua deficiency does not interfere with skeletal development or growth (Fig.1A). Since enlargement of craniofacial bone structures is a major pathology of human MPS-I, we applied X-ray and µCT analysis, where we observed a remarkable porosity of the jawbones in 24-week-old Idua-deficient mice (Fig.1B). To quantify the development of this phenotype over time, we performed µCT (micro-computed tomography) scanning of 6-, 24- and 52-week-old wild-type and Idua-deficient littermates (Fig.1C). While we did not observe significant differences of jawbone volume and porosity at the age of 6 weeks, 24- and 52-week-old Idua-deficient mice displayed a progressive increase of the jawbone volume with persistent porosity (Fig.1D). These analyses suggested that the overgrowth of craniofacial bones is not a consequence of impaired skeletal development or growth, but of altered skeletal remodeling.

We next analyzed other skeletal elements to address the question, whether Idua-deficient mice would develop phenotypic
abnormalities over time. To include bones of the axial and appendicular skeleton, we focused on spine and tibia, where we found no length differences between wild-type and *Idua*-deficient littermates (Fig. 2A). To analyze differences in bone mass, we applied undecalcified histology followed by von Kossa/van Gieson staining of spine and tibia sections. Here we observed that the amount of trabecular bone (stained in black) was progressively increasing in *Idua*-deficient mice (Fig. 2B).

Figure 1. Postdevelopmental onset of the *Idua*-deficient skeletal phenotype. (A) Alcian blue/alizarin red staining of skeletons from 6-week-old wild-type (+/+) and *Idua*-deficient (−/−) mice reveals the absence of gross abnormalities in the latter ones. (B) X-ray analysis (left panels) and μCT scanning (right panels) reveal jawbone porosity in 24-week-old *Idua*-deficient (−/−) mice. (C) Cross-sectional μCT scanning of the jawbones from wild-type and *Idua*-deficient mice at 6, 24 and 52 weeks as indicated. (D) Quantification of the jawbone tissue volume (left) and jawbone porosity (right) in wild-type and *Idua*-deficient mice at 6, 24 and 52 weeks of age as indicated. Bars represent mean ± SD (n ≥ 4 mice per group; *P < 0.05 versus wild type).
Subsequent histomorphometric quantification revealed that 24 and 52 weeks old, but not 6-week-old Idua-deficient mice displayed an increased trabecular bone volume in spine and in tibia sections (Fig. 2C). With respect to non-mineralized bone matrix (i.e. osteoid), we observed a decrease of the osteoid surface per bone surface in 24-week-old Idua-deficient mice, indicative of decreased osteoblast-mediated bone formation, yet there was no sign of osteomalacia, as the osteoid thickness was not different towards wild-type littermates (Supplementary Material, Fig. S1). The high trabecular bone mass phenotype of 24-week-old Idua-deficient mice was further confirmed by µCT scanning of the femora (Fig. 2D). Here we additionally found that cortical porosity was not significantly increased relative to wild-type littermates (Fig. 2E), unlike the case in MLII mice (22).

To understand the cellular cause of the high bone mass in Idua-deficient mice, we analyzed undecalciﬁed sections stained with toluidine blue. Here it was most evident that the length of bone-forming units (BFUs) at trabecular bone surfaces was shortened in 24-week-old Idua-deficient mice compared with wild-type littermates (Fig. 3A). In addition, there was pathologic enlargement of osteocytes (Supplementary Material, Fig. S2), representing terminally differentiated bone matrix-embedded osteoblasts (23,24). Likewise, electron microscopy revealed that osteocytes from 24-week-old Idua-deficient mice displayed lysosomal accumulation of storage material, which was also found in osteoblasts and less pronounced in osteoclasts (Fig. 3B). We additionally observed that the ruffled border, a specialized membrane structure required for exocytosis and bone resorption (25,26), was

Figure 2. Progressive increase of trabecular bone mass in Idua-deficient mice. (A) Length of the lumbar spine (left) and the tibia (right) from 6-, 24- and 52-week-old wild-type and Idua-deficient mice. Bars represent mean ± SD (n ≥ 4 mice per group). Statistical analysis was performed using Student’s t-test. (B) Von Kossa/van Gieson staining of undecalciﬁed spine (left panels) and tibia (right panels) sections from 6-, 24- and 52-week-old wild-type and Idua-deficient mice reveals a progressive increase of trabecular bone mass in the latter ones. (C) Quantification of the trabecular bone volume (BV/TV, bone volume per tissue volume) in spine (left) and tibia (right) sections from wild-type and Idua-deficient mice at 6, 24 and 52 weeks as indicated. Bars represent mean ± SD (n = 4 mice per group; *P < 0.05 versus wild type). (D) µCT scanning of the femora from 24-week-old wild-type and Idua-deficient mice. Quantification of the trabecular bone volume (BV/TV) is given on the right. Bars represent mean ± SD (n = 4 mice per group; *P < 0.05 versus wild type). (E) Quantification of the cortical porosity in femora from 24-week-old wild-type and Idua-deficient mice. Bars represent mean ± SD (n = 4 mice per group).
less developed in Idua-deficient osteoclasts. Taken together, these findings demonstrated that Idua deficiency causes distinct cellular defects in the osteoblast and osteoclast lineage and suggested that Idua-deficient mice display an osteopetrotic phenotype, i.e. increased bone mass due to impaired bone resorption (27).

**Absence of a cell-autonomous defect of Idua-deficient osteoclasts**

Since osteoclasts derive from hematopoietic progenitor cells, we addressed the question whether the phenotype of Idua-deficient mice is transferable into wild-type recipient mice by BMT, or vice versa. Transplantation was performed at 8 weeks of age, at which time lysosomal storage was already observed in Idua-deficient osteoblasts and osteocytes (Supplementary Material, Fig. S3), and the skeletal phenotype was analyzed by undecalciﬁed histology at 24 weeks of age (Fig. 4A). Histomorphometric quantiﬁcation revealed that BMT did not affect trabecular bone mass in wild-type mice, and that the skeletal phenotype of Idua-deﬁcient mice was not transferable into wild-type recipients by BMT (Fig. 4B). On the other hand, when BMT was performed with wild-type donor cells and Idua-deﬁcient recipient mice, the increase of trabecular bone mass was fully prevented. We additionally studied ex vivo osteoclastogenesis of wild-type and Idua-deﬁcient bone marrow cells and found that TRAP-positive multinucleated cells were formed in cultures from both genotypes (Fig. 4C). There were neither signiﬁcant differences regarding expression of osteoclastogenesis markers (Acp5, encoding tartrate-resistant acid phosphatase, TRAP; Calcr, encoding the calcitonin receptor) (Fig. 4D), nor in the resorptive capacity of cells that were cultured on dentin chips (Fig. 4E).

To analyze for defects in the osteoblast lineage, we cultured primary calvarial cells and analyzed matrix mineralization at Day 10 of differentiation (Fig. 4F). Here we observed a moderate reduction in Idua-deficient cultures (Fig. 4G). To determine GAG turnover, we labeled the primary osteoblasts by [35SO4] incorporation followed by a chase period of 48 h. We found that Idua-deficient osteoblasts displayed signiﬁcant enrichment of cell-associated GAGs, which were fully degraded by chondroitinase, a bacterial lyase degrading chondroitin and dermatan sulfate. Since heparinase treatment only had a moderate effect, these data indicated that dermatan sulfate, not heparan sulfate, is the primary GAG type accumulating in Idua-deficient osteoblasts (Fig. 4H). Collectively, these findings demonstrated that the high bone mass phenotype of Idua-deficient mice is not caused by a cell-autonomous osteoclast defect and suggested that an altered composition of the Idua-deficient bone matrix interferes with physiological bone remodeling.

**BMT causes pathologically increased osteoclastogenesis in Idua-deficient mice**

To conﬁrm this hypothesis, we applied histomorphometry to address the question whether the defects of the osteoblast lineage are corrected by BMT treatment of Idua-deficient mice. By quantifying the number of BFUs, we found that there were only few BFUs in untreated Idua-deficient mice exceeding a length of 50 µm, whereas the number of smaller BFUs was signiﬁcantly increased (Fig. 5A). Both parameters were normalized by BMT, and the same was the case for the osteoblast surface per bone surface (Fig. 5B). Electron microscopy additionally revealed that the lysosomal storage defect in Idua-deficient osteocytes was corrected by BMT (Fig. 5C), which was supported by quantiﬁcation of the osteocyte lacunar area (Fig. 5D).

With respect to bone resorption, we found that histomorphometric parameters of osteoclastogenesis, i.e. osteoclast number per bone perimeter and osteoclast surface per bone surface, were signiﬁcantly reduced in untreated Idua-deficient mice compared with wild-type littermates (Fig. 5E). Importantly however, both parameters were >8-fold increased by BMT treatment speciﬁcally in Idua-deficient mice, thereby causing a non-physiological state with high abundance of osteoclasts displaying normal morphology (Supplementary Material, Fig. S4). These ﬁndings demonstrated that BMT profoundly affects the bone remodeling status of Idua-deficient mice, thereby raising the question, if the same applies for human MPS-I, where BMT is one of the currently applied therapies.
In an attempt to address this question, we analyzed an iliac crest biopsy from an 8-year-old boy with MPS-I, which was taken during orthopedic surgery (Fig. 6A). Here we found that the trabecular bone volume (Fig. 6B) was lower than the reported children’s reference range (28), and that osteoblast and osteoclast surfaces were not pathologically affected (Fig. 6C). Although these findings were not confirming our results obtained in Idua-deficient mice, the medical history of the patient provided a potential explanation, as he received BMT at 13 months of age and additional treatment by ERT for the last 3 years before biopsy (Fig. 6D). Of note, whereas urinary concentrations of desoxypyridinoline crosslinks, a biomarker of bone resorption, were pathologically increased at the age of 4 years (i.e. before ERT), they were within the children’s reference range (29) at the time of biopsy.

Combination therapy normalizes bone remodeling in Idua-deficient mice

Based on these clinical observations, we utilized the Idua-deficient mouse model to study the impact of ERT, alone and in combination with BMT, on their skeletal remodeling phenotype. ERT was achieved by weekly injection of recombinant IDUA enzyme (0.58 mg/kg) starting at 12 weeks of age, and the skeletal phenotype of the treated mice was analyzed by undecalciﬁed histology at 24 weeks of age (Fig. 7A). Histomorphometric quantiﬁcation revealed that ERT alone did not reduce trabecular bone mass in Idua-deficient mice, whereas combination therapy normalized trabecular bone mass to wild-type levels (Fig. 7B). Similar findings were made with respect to the jawbone volume, which was signiﬁcantly reduced by BMT or combination therapy, but not by ERT alone (Fig. 7C). When we quantiﬁed the percentage of BFUs exceeding the length of 50 µm, we found that ERT alone only caused partial normalization in Idua-deficient mice, while combination therapy fully normalized this pathology (Fig. 7D). Finally, whereas ERT treatment of Idua-deficient mice resulted in increased osteoclastogenesis, both osteoclast number per bone perimeter and osteoclast surface per bone surface were reduced to wild-type levels by combination therapy (Fig. 7E, Supplementary Material, Table S1). Taken together, these findings suggest that sufﬁcient enzyme replacement, achieved by combination

![Image](https://via.placeholder.com/150)
Figure 5. BMT treatment of Idua-deficient mice causes increased osteoclastogenesis. (A) Quantification of the BFU number with more or less than 50 µm length in 24-week-old untreated wild-type and Idua-deficient mice and BMT-treated Idua-deficient mice. Bars represent mean ± SD (n ≥ 4 mice per group; *P < 0.05 versus wild type). (B) Quantification of the osteoblast surface (ObS/BS, osteoclast surface per bone surface) in the same groups of mice. Bars represent mean ± SD (n ≥ 4 mice per group; *P < 0.05 versus wild type). (C) Electron microscopy on sections from 24-week-old untreated (control) or BMT-treated Idua-deficient mice reveals that the lysosomal storage defect in osteocytes is corrected by BMT. (D) Quantification of the osteocyte lacunar area (OtLcAr) in 24-week-old untreated wild-type and Idua-deficient mice and BMT-treated Idua-deficient mice. Bars represent mean ± SD (n ≥ 4 mice per group; *P < 0.05 versus wild type). (E) Quantification of the osteoclast number (OcN/Bpm, osteoclast number per bone perimeter) and osteoclast surface (OcS/BS, osteoclast surface per bone surface) in untreated and BMT-treated wild-type and Idua-deficient mice. Bars represent mean ± SD (n ≥ 4 mice per group; *P < 0.05 versus untreated wild type).

Figure 6. Analysis of an iliac crest biopsy of an individual with MPS-I after treatment by BMT and ERT. (A) Von Kossa/van Gieson (left) and toluidine blue (right) staining of an undecalcified section from an iliac crest biopsy derived from an 8-year-old boy with MPS-I. (B) Quantification of the trabecular bone volume. The orange box indicates the reference range reported for age-matched control biopsies (29). (C) Quantification of osteoblast surface and osteoclast surface. The orange boxes indicate the reference ranges reported for age-matched control biopsies (29). (D) Schematic presentation showing the medical history of the respective individual. Urinary levels of desoxypyridinoline per creatinine (Dpd/Crea) were determined twice as indicated.
therapy, is a prerequisite to fully normalize bone remodeling in MPS-I.

Discussion

Since the bone remodeling status in MPS-I has not been assessed yet, we performed a thorough skeletal phenotyping of Idua-deficient mice. We thereby identified specific cellular defects in all bone remodeling cell types, together with a reduction of osteoblast and osteoclast numbers in untreated Idua-deficient mice. Cross-transplantation experiments demonstrated that the high bone mass phenotype of Idua-deficient mice was not caused by an intrinsic osteoclast defect, yet it was fully prevented by BMT with wild-type donor cells. We additionally found that BMT, and also ERT, caused increased osteoclastogenesis in Idua-deficient mice, whereas bone remodeling was fully normalized by combination therapy. Based on these findings, it will be important to address the question, if insufficient enzyme replacement causes excessive bone resorption also in human MPS-I, which could principally result in long-term skeletal fragility.

We have previously analyzed the skeletal phenotype of a mouse model for ML-II, another LSD associated with dysostosis multiplex and hypersecretion of lysosomal enzymes, and obtained several unexpected findings of key significance (22). More specifically, while the function of growth plate chondrocytes and bone-forming osteoblasts was impaired in ML-II mice, bone-resorbing osteoclasts were not functionally affected. Instead, osteoclastogenesis was markedly increased, thereby causing an osteoporotic phenotype that was prevented by bisphosphonate administration (22). In the present study, we focused our analysis on Idua-deficient mice and observed that their skeletal phenotype was remarkably different compared with ML-II mice. Idua-deficient mice were not growth retarded, and they did not display increased osteoclastogenesis and/or osteoporosis. Instead, they progressively developed a high bone mass phenotype, and their osteoclastogenesis parameters were significantly reduced.

Although some skeletal abnormalities of untreated Idua-deficient mice have been described previously (15–20), our study was the first to apply quantitative histomorphometry to follow the phenotype development over time. The fact that 6-week-old Idua-deficient mice did not display the abnormalities found in 24- and 52-week-old animals clearly demonstrated that both craniofacial overgrowth and high trabecular bone mass are the consequence of altered bone remodeling, and not of impaired skeletal development. Since osteoblasts in Idua-deficient mice were not only affected by lysosomal storage, but also numerically decreased, the progressive bone mass increase in untreated Idua-deficient mice is likely explained by impaired osteoclast differentiation and/or function. To address the question, whether this mild osteopetrotic phenotype is caused by an intrinsic defect of Idua-deficient osteoclasts, we performed bone marrow cross-transplantation experiments, since BMT is known to selectively replace the pool of hematopoietic cells, including the osteoclast progenitors (30). Here we found that BMT from wild-type donors into Idua-deficient recipients prevented development of a high bone mass phenotype, thereby suggesting an intrinsic osteoclast defect caused by Idua deficiency. Surprisingly however, BMT from Idua-deficient donors into wild-type recipients did not cause a high bone mass phenotype, as one would have predicted based on classical experiments demonstrating that osteopetrotic phenotypes are transferable into wild-type mice by hematopoietic cell transfer, when caused by an intrinsic osteoclast defect (30–33).

These inconsistent findings led us to perform ex vivo experiments, where we did not observe a cell-autonomous defect of osteoclastogenesis in cultures of Idua-deficient bone marrow cells. In contrast, Idua-deficient osteoblasts displayed a significant
enrichment of cell-associated GAGs, which were identified as dermatan sulfate. These data are in principal agreement with the fact that MPS-IH, an LSD caused by inactivation of the heparan sulfate-degrading enzyme N-sulfoglucosamine sulfohydrolase, is not associated with severe skeletal manifestation (34). Although our functional analysis of cultured Idua-deficient osteoblasts revealed that they were only moderately impaired with respect to matrix mineralization, it is reasonable to speculate that their long-term inability to degrade dermatan sulfate is a major pathology causing impaired bone remodeling in vivo. More specifically, since a lack of dermatan sulfate degradation should alter the composition of the Idua-deficient bone matrix, it is likely that the shortening of BFUs, the reduced osteoclast number and the impairment of ruffled border formation observed in untreated Idua-deficient mice are caused by defective GAG degradation by cells of the osteoblast lineage. This hypothesis is also in agreement with our findings obtained by cross-transplantation experiments, since Idua-deficient osteoblasts should be able to resorb a bone matrix that was produced and/or modified by wild-type osteoblasts and/or osteocytes.

Our most important observation, however, was that treatment of Idua-deficient mice by BMT and/or ERT significantly influenced their bone remodeling status. With respect to BMT, we principally confirmed the results of a recent study, where the authors demonstrated prevention of a high bone mass phenotype in 37-week-old Idua-deficient mice by neonatal BMT (20). Since the authors did not perform a histomorphometric analysis however, our results identified an unexpected pathology in BMT-treated Idua-deficient mice, i.e. increased osteoclastogenesis. We additionally found that defects in the osteoblast lineage were essentially corrected and/or prevented by BMT. This was a truly unexpected finding, since the mesenchymal osteoprogenitor cells are generally considered to be non-transplantable, even under our experimental conditions, where we did not specifically enrich the hematopoietic cells before transplantation (since this was not done for the described patient either). One hypothesis to explain this observation was therefore an Idua secretion by descendants of the transplanted cells, as was reported previously (35). For that purpose, we determined serum Idua activities in wild-type and Idua-deficient mice that received BMT from wild-type donors. Here we found, using 4-methylumbelliferyl-α-L-iduronide (4-MU) as a substrate, that serum from transplanted Idua-deficient mice contained ~30% of the activity found in wild-type controls (19.3 ± 2.5 versus 63.7 ± 28.5 nmol MU/µl serum), whereas such activity was undetectable in untreated Idua-deficient mice. This observation confirms previously published data (35) and provides a possible explanation for the correction of a mesenchymal cell defect by hematopoietic stem cell transfer. Apparently however, BMT alone did not fully restore the GAG degradation defects in Idua-deficient bones, as it caused excessive osteoclastogenesis.

At present, it is difficult to find a molecular explanation for this latter observation, especially since the cell culture defects were rather moderate. Interestingly however, there is recent experimental evidence for an influence of sulfated GAGs on osteoclastogenesis, which was molecularly explained by interferon with complex formation between Opg and Rankl, the two most critical regulators of osteoclast differentiation known to date (36). More specifically, whereas Rankl is an osteoblast-derived cytokine promoting osteoclastogenesis and bone resorption, Opg antagonizes the Rankl influence by acting as a decoy receptor (37). It is therefore conceivable to speculate that the release of non-physiological amounts of sulfated GAGs in treated Idua-deficient mice activates Rankl by sequestering Opg, which in turn causes, at least transiently, excessive bone resorption. Not only based on this argument one of the key questions to be addressed in future studies is whether the increased osteoclastogenesis obtained by treatment of Idua-deficient mice is transient or permanent. Since we only analyzed one time point (i.e. 24 weeks of age) in the present study, we cannot answer this question, and it might be useful to study BMT-treated mice at different ages (i.e. 16 and/or 52 weeks of age). On the other hand, it remains questionable, if and how the results of such experiments would translate into the human situation, especially since the phenotype of Idua-deficient mice, for reasons that remain to be clarified (15,16,38), mostly resembles the milder forms of MPS-I. Therefore, it is probably more appropriate to assess bone remodeling in individuals with Hurler syndrome at different time points after BMT.

Although there are certainly more experiments needed to truly understand the molecular mechanisms underlying the observed phenotypes, there is an immediate clinical relevance of our findings. In fact, since a large number of individuals with MPS-I are currently treated by BMT and/or ERT, it will be important to monitor their bone remodeling status, not only before but also after treatment. This should include the determination of specific biomarkers, such as the urinary Dpd crosslinks, as they represent an excellent non-invasive readout for the rate of bone resorption in humans. In our opinion, such a monitoring process is truly required to answer the question, if BMT and/or ERT causes long-term skeletal complications, such as osteoporosis, in human MPS-I. Given the various non-skeletal pathologies of MPS-I patients, it is certainly no option to avoid BMT for the treatment of Hurler syndrome or ERT for the treatment of Hurler/Scheie and Scheie syndrome. However, since there are possibilities to prevent increased osteoclastogenesis after treatment, i.e. anti-resorptive drugs or BMT/ERT combination therapy, a monitoring process should be advantageous. In any case, addressing this question in humans is a prerequisite to draw any conclusions about the superiority of combination therapy and/or the necessity of anti-resorptive intervention.

Materials and Methods

Mice

Idua-deficient mice were obtained from the Jackson Laboratory (#004068). They were first mated with C57Bl/6 mice to obtain heterozygous animals. These were again mated to obtain wild-type and Idua-deficient littermates for analysis. Offspring from heterozygous matings was genotyped by PCR with the primers 5′-GGA ACT TTG AGA CTT GAG ATG AAC CAG-3′, 5′-CAT TGT AAA TAG GGC TAT CCT TGA ACT C-3′ and 5′-GGA TTG GGA AGA CAA TAG CAG GCA TGC T-3′ to amplify a 550 bp and/or a 350 bp fragment, indicative of the wild-type and mutant allele, respectively. After initially confirming that the observed abnormalities are found in male and female Idua-deficient offspring, we focused on female mice for the remaining analysis.

Skeletal analysis

Staining of skeletons from 6-week-old mice with alcin blue and alizarin red was performed using standard protocols (39). To determine the length of the lumbar spine and the tibia, all mice were analyzed by contact X-ray. Before histology, dissected skeletons were fixed in 3.7% PBS-buffered formaldehyde for 18 h, before they were stored in 80% ethanol. The lumbar vertebral bodies L1-L4 and one tibia were dehydrated in ascending alcohol concentrations and then embedded in methylmethacrylate as
described previously (40). Sections of 4 μm thickness were cut in the sagittal plane and stained by toluidine blue or von Kossa/van Gieson staining procedures as described (40). Histomorphometry was performed according to the ASBMR guidelines using the OsteoMeasure histomorphometry system (Osteometrics Inc., USA) (41). For μCT scanning, we used a μCT 40 desktop cone-beam microCT (Scanco Medical) with a voxel size of 10 μm. Reconstructed slices were inspected using the Scanco MicroCT software suite. Ultrastructural analysis by electron microscopy was performed according to standard protocols (42). Acid-etching was performed with specimens embedded into methylmetacrylate by immersion in 9% phosphoric acid as described (43).

Treatment of IdUA-deficient mice
For BMT, we used 8-week-old female recipient mice and 12-week-old male donor mice (44). The recipient mice were irradiated with a dose of 10.5 Gy 12 h prior to transplantation. Bone marrow cells from the donor mice were obtained from femur and tibia. These cells (500,000 in 150 μl) were injected into the tail vein of the recipient mice. The success of BMT was monitored at sacrifice of the recipient mice (24 weeks of age) by qRT-PCR for the Sry gene in DNA being extracted from whole blood as described (45). DNA extracted from blood of non-transplanted male and female mice served as one control and female mice used as a reference. ERT was started at 12 weeks of age until sacrifice (24 weeks of age). These mice received weekly injections of recombinant human IDUA (Aldurazyme®), kindly provided by Dr M. Vellard (Biomarin/Genzyme LLC, Cambridge, MA, USA), at a dose of 0.58 mg/kg and in a volume of 20 μl. All animal experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the ‘Amt für Gesundheit und Verbraucherschutz’ (32/12, 102/12, 121/13, Org529).

Primary bone cells
For osteoclastogenesis, the bone marrow was flushed out of the femora from 24-week-old mice. Cells were plated at a density of 5 × 10⁴ cells/ml in the presence of 10 nm 1,25 dihydroxyvitamin D₃. On the following day, the adherent cells were cultured for 3 days, before M-CSF (Peprotech) and RANKL (Peprotech) were added to a final concentration of 20 and 40 ng/ml, respectively. For TRAP (tartrate-resistant acid phosphatase) activity staining, the cells were washed with PBS and then fixed for 5 min in cold methanol. After two washing steps with distilled water, the fixed cells were first dried for 2 min, before they were subjected to the TRAP-specific substrate Naphtol ASMX-Phosphate (Sigma). To determine the resorptive activity, cells were plated on dentin chips, that were stained by toluidine blue to quantify the resorbed area as described previously (46). Primary osteoblasts were isolated by sequential collagenase digestion from the calvariae of 5-day-old mice and differentiated in the presence of 500 000 in 150 µl) were injected into the tail vein of the recipient mice. The success of BMT was monitored at sacrifice of the recipient mice (24 weeks of age) by qRT-PCR for the Sry gene in DNA being extracted from whole blood as described (45). DNA extracted from blood of non-transplanted male and female mice served as one control and female mice used as a reference. ERT was started at 12 weeks of age until sacrifice (24 weeks of age). These mice received weekly injections of recombinant human IDUA (Aldurazyme®), kindly provided by Dr M. Vellard (Biomarin/Genzyme LLC, Cambridge, MA, USA), at a dose of 0.58 mg/kg and in a volume of 20 μl. All animal experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the ‘Amt für Gesundheit und Verbraucherschutz’ (32/12, 102/12, 121/13, Org529).

Expression analysis
RNA was isolated using the RNeasyMini kit (Qiagen), and DNase digestion was performed according to manufacturer’s instructions. Concentration and quality of RNA were measured using a NanoDrop ND-1000 system (NanoDrop Technology). Expression analysis by qRT-PCR was performed using a StepOnePlus system and predesigned TaqMan gene expression assays (Applied Biosystems). Gapdh expression was used as an internal control.

Glycosaminoglycan analysis
For GAG incorporation, the cultures were incubated with [35SO₄] sulfenic acid for 48 h and then switched to non-radioactive medium for additional 48 h. GAGs were then purified from cell homogenates by DEAE-Sepharose anion exchange chromatography and subjected to digestion with heparinase or chondroitinase as described (47).

Human bone biopsy specimen
A transiliac crest biopsy was taken during orthopedic surgery from an 8-year-old boy with MPS-I (Hurler type) after receipt of informed consent by the parents. The biopsy specimen was embedded undecalciﬁed into methylmetacrylate. Sections were stained toluidine blue or von Kossa/van Gieson staining procedures as described (40). Histomorphometric analysis was performed using the OsteoMeasure system (Osteometrics Inc., USA) and set into relation to the age- and gender-matched reference range (29). The bone resorption parameter Dpd/Creatinine had been determined in the Department of Clinical Chemistry according to standard procedures and were compared with the age- and gender-matched reference range (30).

Statistical analysis
All data are presented as means ± standard deviations. Statistical analysis was performed using unpaired, two-tailed Student’s t-test, and P-values below 0.05 were considered statistically significant. For the multiple comparisons shown in Supplementary Material, Table S1, we applied one-way ANOVA with Turkey’s post hoc multiple comparison test.

Supplementary Material
Supplementary material is available at HMG online.

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