Effect of gene dose and parental origin on bone histomorphometry in X-linked Hyp mice

Z.Q. Qiu, a R. Travers, b F. Rauch, b,c F.H. Glorieux, b,c,d,e C.R. Scriver, c,e,f and H.S. Tenenhouse a,c,e,f,*

*Department of Biology, McGill University, Montreal, Quebec H3A 1B1, Canada
bGenetics Unit, Shriners Hospital for Children, Montreal, Quebec H3G 1A6, Canada
cDepartment of Pediatrics, McGill University, Montreal, Quebec H3H 1P3, Canada
dDepartment of Surgery, McGill University, Montreal, Quebec H3A 1A1, Canada
eDepartment of Human Genetics, McGill University, Montreal, Quebec H3A 1B1, Canada
fMontreal Children’s Hospital Research Institute, Montreal, Quebec H3Z 2Z3, Canada

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Abstract

X-linked hypophosphatemia (XLH) is characterized by rickets and osteomalacia and arises from mutations in the Phex and PHEX genes in mice (Hyp) and humans, respectively. The present study was undertaken to examine the effect of gene dose on the skeletal phenotype using a histomorphometric approach. Metrical traits (vertebral length, growth plate thickness, cancellous osteoid volume per bone volume, and cancellous, endocortical, and periosteal osteoid thickness) were compared in caudal vertebrae of mutant female (Hyp+/+, Hyp/Hyp) and male (Hyp/Y) mice and their normal female (+/+) and male (+/Y) littermates. Mutant animals had trait values that differed significantly from those of normal animals. However, with the exception of vertebral length and cancellous osteoid thickness, values were not significantly different between the three mutant genotypes. We also examined the effect of gamete-of-origin on histomorphometric parameters in obligate Hyp/+ females derived from male or female transmitting parents. The metrical trait values in both groups of Hyp/+ mice were similar, with the exception of vertebral length and cancellous osteoid volume per bone volume. In summary, we demonstrate that the amount of osteoid per bone volume is similar in the three mutant genotypes and conclude that the extent and magnitude of the mineralization defect is fully dominant and likely not affected by gene dose. The differences in vertebral length in the mutants suggest that rickets and osteomalacia are not the only causes of decreased vertebral growth in Hyp mice and that Phex protein may influence bone growth and mineralization by distinct pathways.

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Introduction

X-linked hypophosphatemia (XLH), a dominant disorder of phosphate homeostasis, is characterized by renal defects in the reabsorption of filtered phosphate and metabolism of vitamin D [1]. The most obvious clinical manifestation of XLH is caused by a mineralization defect affecting growth plates (rickets) and bone tissue (osteomalacia). The gene responsible for XLH was identified by positional cloning and designated PHEX (formerly PEX) to depict a PHosphate regulating gene with homology to Endopeptidases on the X chromosome [2]. The PHEX gene encodes a protein that exhibits significant homology to the M13 family of zinc metallopeptidases, membrane glycoproteins involved in the activation, or inactivation of biologically active peptides [3]. However, the nature of endogenous PHEX substrate(s) is not known. PHEX is expressed predominantly in osteoblasts, osteocytes, and odontoblasts, but not in kidney [4–9], suggesting that the renal abnormalities in this disorder are secondary to a skeletal defect. The 171 mutations in the PHEX gene that have been identified in XLH patients are catalogued in a locus-specific database [10] (www.phexdb.mcgill.ca) and are consistent with loss of PHEX function.
The Hyp mouse model of XLH exhibits all the features of the human disorder, namely, hypophosphatemia, renal defects in phosphate reabsorption, and vitamin D metabolism, as well as rickets and osteomalacia [11–15]. Hyp mice harbor a large 3' deletion in the PheX gene [5,16], which does not include the downstream Sat gene and therefore is a true homologue of XLH [17].

The PheX and PHEX genes in mouse and human, respectively, map to loci on the X chromosome that undergo dosage compensation by random X chromosome inactivation [18]. Thus, a gene dose effect was expected in patients with XLH, namely that affected females should have a more variable and less severe clinical and biochemical phenotype than hemizygous male patients. Evidence for a gene dose effect in XLH rests on the demonstration that female patients tend to have a wider variation in overt bone disease, from severe to none at all, while males appear to have severe overt bone manifestations [19–21]. Furthermore, untreated male adults have more severe skeletal radiographic and scintigraphic findings than untreated female adults [21]. In addition, female patients exhibit a higher tubular reabsorption rate for phosphate than male patients [22] and have dental pulp profile area values in secondary dentin that are intermediate between values for age-matched affected male patients and normal subjects [23].

On the other hand, there are several lines of evidence for the absence of a gene dose effect in XLH patients and in the Hyp mouse counterpart. Serum phosphate values and skeletal mass changes are similar in affected male and female patients [19,20,24]. The decrement in the rate of Na+-dependent phosphate transport across the renal brush border membrane is similar in heterozygous Hyp/+ and homozygous Hyp/Hyp female mice [25]. The pattern of craniofacial abnormalities is not more severe in Hyp/Hyp mice than in Hyp/+ animals [26]. Sequential measurements of tail length, serum phosphate concentration up to 150 days of age, and increase in renal mitochondrial 24-hydroxylase activity are not different in Hyp/+ Hyp/Hyp and Hyp/Y mice [27]. Finally, serum phosphate and alkaline phosphatase values were similar in Hyp/+, Hyp/Hyp, and Hyp/Y mice in an in vitro fertilization, interspecific backcross study [28].

The aim of the present study was to determine whether the mineralization defect in Hyp mice is subject to a gene dose effect. The hallmark of mineralization defects is the accumulation of unmineralized tissue [29]. Rickets is characterized by the accumulation of growth plate cartilage, whereas osteomalacia leads to the accumulation of osteoid in bone tissue. We therefore quantified the thickness of growth plates and the amount of osteoid in the caudal vertebrae in three Hyp genotypes (Hyp/+, Hyp/Y and Hyp/Hyp) and their normal littersmates (+/+ , +/Y). In addition, we examined the effect of gamete-of-origin of the mutant allele in obligate heterozygous females derived from male (Hyp/Y) or female (Hyp/Hyp) transmitting parents.

Materials and methods

Animals

Mice were bred and raised in the Animal Facility at the Montreal Children’s Hospital. The original breeding pairs (Hyp/+ × +/-Y on C57BL/6J background) were obtained from the Jackson Laboratory (Bar Harbor, ME). All animals were fed Teklad–Wayne Breeder Blox (#8626, Teklad, Madison WI) and received water ad libitum. Mice were weaned at 25 days of age and identified as to genotype by serum phosphate values and body weight [11]. Six mice per genotype were used in this study. At 46–60 days of age, the animals were weighed and sacrificed by decapitation. Blood was collected for the measurement of serum inorganic phosphate using a commercial kit (Stanbio Laboratory Inc., San Antonio, TX). All animal experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of McGill University.

Breeding strategies

Gene dose effects and the effect of parental origin of the mutant allele on offspring were examined using mice generated by the following four breeding strategies: (a) normal mice were derived by breeding +/- females with +/-Y males; (b) Hyp/+ offspring of Hyp/Y males were derived by breeding +/- females with Hyp/Y males; (c) Hyp/+ offspring of Hyp/Hyp females and Hyp/Y mice were derived by breeding Hyp/Hyp females with +/-Y males; and (d) Hyp/Hyp mice were derived by breeding Hyp/Hyp females with Hyp/Y males. To control for intrauterine environment in the gene dose study, Hyp/Hyp females were compared with Hyp/+ females derived from a transmitting female parent (Hyp/Hyp).

Bone histology and histomorphometry

Samples of the three proximal caudal vertebrae were collected from the two normal and three mutant genotypes. After fixation, dehydration, and embedding, 6-μm-thick undecalcified sections were cut on a Polyvar E microtome (Reichert–Jung, Heidelberg, Germany). Sections were analyzed with a Polyvar microscope (Reichert–Jung) at an 80-fold magnification. The samples were analyzed in a blinded fashion. The following parameters were measured: vertebral length, growth plate thickness, osteoid thickness (separately analyzed for cancellous, endocortical, and periosteal surfaces), and cancellous osteoid volume per bone volume. Cancellous bone parameters were measured 200 μm away from the growth plate. Quantitation was performed using a digitizing tablet and the Osteomeasure® software (Osteometrics, Atlanta, GA, USA). Nomenclature follows the recommendations of the American Society for Bone and Mineral Research [30].
Statistics

Differences between two groups were tested for significance using unpaired t tests. Differences between more than two groups were evaluated using analysis of variance (ANOVA). Post-hoc analyses of differences between individual groups were performed using Bonferroni’s adjustment. The gene dose study had a power of 80% to detect the following differences between the three hypophosphatemic groups (Hyp+/+, Hyp/Hyp, and Hyp/Y): serum phosphate, 0.33 mmol/l; vertebral length, 0.15 mm; growth plate thickness, 40 µm; cancellous osteoid volume per bone volume, 11.0%; cancellous osteoid thickness, 2.6 µm; endocortical osteoid thickness, 4.4 µm; periosteal osteoid thickness, 15.4 µm. The study on the effect of paternal origin had a power of 80% to detect the following differences between paternal and maternal transmission: serum phosphate, 0.27 mmol/l; vertebral length, 0.13 mm; growth plate thickness, 32 µm; cancellous osteoid volume per bone volume, 9.0%; cancellous osteoid thickness, 2.2 µm; endocortical osteoid thickness, 3.6 µm; periosteal osteoid thickness, 12.6 µm.

Results

Serum phosphate

All mutant animals had a significantly lower phosphate concentration than normal animals, but the values were not significantly different among the three mutant genotypes (Hyp+/+, Hyp/Hyp, and Hyp/Y) (Table 1). Thus, there was no evidence of a gene dose effect on the serum phosphate concentration in Hyp mice. The gamete of origin of the mutant allele did not influence serum phosphate values, as heterozygous Hyp+/ females, derived from either a transmitting affected female or an affected male, had similar values (Table 2).

Qualitative bone histology

The overall length of the proximal caudal vertebrae appeared shorter in mutant Hyp+/+, Hyp/Hyp, and Hyp/Y mice than in wild-type mice (Fig. 1). In addition, the growth plate appeared thicker in the mutant animals when compared to normal counterparts (Fig. 1). The accumulation of osteoid was evident in all three Hyp genotypes.

Quantitative bone histomorphometry

Vertebral length was significantly reduced in all mutant genotypes when compared to normal counterparts (Table 1). In female mice, there was a significant gene dose effect in vertebral length, with values in heterozygous Hyp+/+ females intermediate between normal and Hyp/Hyp females (Table 1). In addition, vertebral length in Hyp/Y males was similar to that in Hyp/Hyp females (Table 1), consistent with the absence of a normal X chromosome in both mutant genotypes.

Growth plate thickness, a parameter that directly reflects rickets, was significantly increased in the three mutant genotypes when compared to wild-type mice (Table 1). However, in contrast to vertebral length, growth plate thickness was not significantly different in Hyp+/+ and Hyp/Hyp females (Table 1). Growth plates were significantly thinner in Hyp/Y than in Hyp/Hyp mice (Table 1).

The parameters that reflect osteomalacia, namely cancellous osteoid volume per bone volume, and cancellous, endocortical, and periosteal osteoid thickness were all significantly increased in Hyp+/+, Hyp/Hyp, and Hyp/Y mutants.

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>+/+</th>
<th>Hyp+/+</th>
<th>Hyp/Hyp</th>
<th>+/Y</th>
<th>Hyp/Y</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Serum phosphate (mmol/l)</td>
<td>2.72 ± 0.13abcde</td>
<td>1.57 ± 0.09cde</td>
<td>1.44 ± 0.16abcde</td>
<td>2.81 ± 0.32abcde</td>
<td>1.38 ± 0.10abcde</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Histomorphometry

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/+</th>
<th>Hyp+/+</th>
<th>Hyp/Hyp</th>
<th>+/Y</th>
<th>Hyp/Y</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertebral length (mm)</td>
<td>3.35</td>
<td>2.43</td>
<td>2.15</td>
<td>3.44</td>
<td>2.20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Growth plate thickness (µm)</td>
<td>115</td>
<td>162</td>
<td>144</td>
<td>113</td>
<td>175</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cancellous osteoid volume per bone volume (%)</td>
<td>5.6</td>
<td>34.2</td>
<td>35.5</td>
<td>2.8</td>
<td>33.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cancellous osteoid thickness (µm)</td>
<td>4.2</td>
<td>10.4</td>
<td>12.9</td>
<td>3.4</td>
<td>10.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endocortical osteoid thickness (µm)</td>
<td>4.1</td>
<td>14.9</td>
<td>14.7</td>
<td>3.3</td>
<td>13.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Periosteal osteoid thickness (µm)</td>
<td>7.5</td>
<td>45.7</td>
<td>47.3</td>
<td>5.9</td>
<td>53.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The Hyp+/+ group represents mice that resulted from a crossing of Hyp/Hyp and +/+ mice. Values are means ± SD. P values indicate the significance of the difference between groups by ANOVA. Results of post-hoc analyses of differences between individual groups (using Bonferroni’s adjustment) are indicated by letters in superscript.

a Significant difference to Hyp+/+ group.
b Significant difference to Hyp/Hyp group.
c Significant difference to Hyp/Y group.
d Significant difference to +/+ group.
ec Significant difference to +/+ group.
when compared to wild-type male and female mice (Table 1). In female mice, there was a significant gene dose effect on cancellous osteoid thickness. However, values in heterozygous Hyp+/+ females were closer to those in Hyp/Hyp females than normal females (Table 1). No gene dose effect was observed for cancellous osteoid volume per bone volume and endocortical and periosteal osteoid thickness (Table 1).

**Effect of parental origin of mutant allele**

Heterozygous Hyp/+ females derived from a transmitting female (Hyp/Hyp) had significantly shorter vertebrae and a higher amount of osteoid within cancellous bone (i.e., cancellous osteoid volume per bone volume) than Hyp/+ females derived from transmitting males (Hyp/Y) (Table 2). No significant differences were found for the other histomorphometric parameters. However, for each single parameter, there was a trend for a more severe phenotype in Hyp/+ mice that were derived from a transmitting female.

**Discussion**

The present study provides some evidence to support the hypothesis that the mineralization defect in Hyp mice is not influenced by gene dose. Only one of several histomorphometric parameters reflecting bone matrix mineralization, namely cancellous osteoid thickness, was significantly different in Hyp/+ and Hyp/Hyp females. However, both Hyp/+ and Hyp/Hyp mice had accumulated a similar amount of cancellous osteoid volume per bone volume, suggesting that the mineralization defect was of the same severity in both mutant genotypes. Moreover, there appeared to be no evidence for a gene dose effect on the severity of rickets, as Hyp/+ and Hyp/Hyp mice had similar values for growth plate thickness. These findings are in accordance with our earlier studies, where no gene dose effect on either serum phosphate concentration or renal mitochondrial 24-hydroxylase activity was evident in Hyp mice [27]. It must be acknowledged, however, that more subtle gene dose effects may have escaped detection in the present study.

In contrast to the absence of a major gene dose effect on the amount of osteoid and the extent of growth plate thickness, vertebral length was clearly influenced by gene dose. Vertebral length was higher in Hyp/+ mice than in Hyp/Hyp and Hyp/Y mice. Thus, the presence of one normal X chromosome in heterozygous females allowed for higher vertebral growth rate but did not improve the mineralization defect.

The discrepancy between the growth and mineralization defects raises the possibility that rickets and osteomalacia are not the only causes for decreased vertebral growth in Hyp mice. One explanation for these findings is that Phex protein may influence bone growth via pathways that are distinct from those affecting mineralization. Indeed, Phex may be involved in the activation or inactivation of more
than one endogenous substrate, with different affinities for the enzyme and different target tissue actions. There is unequivocal evidence that Phex can function as an endopeptidase [31], although its endogenous peptide substrates have not yet been identified. Accordingly, in the heterozygous state, the threshold necessary for normal Phex function may vary for each Phex substrate and target tissue action.

There are several possible mechanisms to explain the absence of a gene dose effect [25]. These include functional inactivation by a dominant negative effect. The latter, however, is not compatible with the X-linked pattern of inheritance, given that either the mutant or wild-type allele is transcribed in Phex-expressing cells in heterozygous Hyp/+ females. Moreover, there is no evidence for the synthesis of a truncated Phex protein in the mutants [7]. Another possibility, preferential X chromosome inactivation, was shown not to occur in heterozygous female patients with XLH [32]. Selective imprinting determined by parental origin of the mutant allele can also account for the absence of a gene dose effect. However, this appears to be unlikely as discussed below.

Growth plate thickness, and cancellous, endocortical, and periosteal osteoid thickness are not significantly different in heterozygous Hyp/+ females derived from either a transmitting female (Hyp/Hyp) or male (Hyp/Y) mice, consistent with an absence of imprinting. However, Hyp/+ mice derived from Hyp/Hyp females had significantly shorter vertebrae and a higher amount of osteoid within cancellous bone than Hyp/+ females derived from transmitting Hyp/Y males. This might be interpreted as a gamete of origin effect on phenotype. Nevertheless, it is also possible that the more severe skeletal manifestations may be because heterozygous mice derived from Hyp/Hyp mothers were exposed to a low-phosphate environment during in utero development, whereas those derived from Hyp/Y fathers were exposed to a normal in utero environment. However, it is not possible to distinguish between these two possibilities from the present results.

The effect of gene dose was also recently addressed in mice harboring a point mutation in the Phex gene (Ska1 model) that was introduced by random mutagenesis with ethyl-nitrosourea [33]. This mutation, which causes skipping of exon 8, results in clinical and biochemical phenotypes that are similar to those in Hyp mice and XLH patients, including rickets and osteomalacia [33]. In agreement with the present study, the serum phosphate concentration in all three mutant genotypes (Ska1/+; Ska1/Ska1 and Ska1/Y) is similar and significantly lower than that in wild-type counterparts (+/+ and +/Y) [33]. In contrast, serum alkaline phosphatase activity exhibited a significant gene dose effect, with values in Ska1/+ females intermediate between those of wild-type and Ska1/Ska1 counterparts [33]. Since alkaline phosphatase is a marker of osteoblast function, these data are suggestive of a gene dose effect on skeletal mineralization. However, in a study of Hyp mice backcrossed to Mus spretus, serum alkaline phosphatase activity was equivalently elevated in heterozygous and homozygous mutants when compared to wild-type females [28]. Clearly, bone histomorphometric studies are necessary to determine whether the mineralization defect in Ska1 mice is subject to a gene dose effect. In addition, comparative studies in Ska1 and Hyp mice may provide novel information about the relationship between genotype and phenotype.

The apparent absence of a gene dose effect on the skeletal phenotype in the Hyp mouse model reported here are compatible with results of earlier studies in adults and children with XLH. Reid et al [20] demonstrated that most clinical, histological, and biochemical indices of severity are not significantly different in adult male and female XLH patients. Furthermore, in a study of a large number of untreated children with XLH, Whyte et al [34] demonstrated that biochemical parameters and height z scores are similar in affected males and females. Moreover, consistent with our findings in Hyp mice, no significant phenotypic differences were found in XLH females when maternal and paternal transmissions of XLH were compared [34]. It should be noted that studies in the Hyp mouse model provide several advantages over those in XLH patients. Hyp mice are highly inbred and, thus, are not subject to phenotypic variation arising from differences in modifying genes or the severity of the PHEX mutation. In addition, the lack of availability of homozygous female XLH patients precludes comparisons of females carrying two, one, and no copies of the mutant allele and restricts comparisons to affected males and females, the findings of which may be confounded by differences in sex hormones.

Taken together, the present data suggest that the dominant pattern of inheritance in Hyp mice can be ascribed to Phex haploinsufficiency, as previously suggested [35]. Thus, calvarial Phex protein, produced from the wild-type allele in heterozygous Hyp/+ females and detected on western blots [35], is not sufficient for normal function. Future studies aimed at the identification of endogenous Phex substrates and products, and their function in target tissues is necessary to understand the underlying mechanisms for haploinsufficiency.

In summary, quantitative bone histomorphometric analysis suggests that the mineralization defect in Hyp mice is not influenced by gene dose and that parental origin of the mutant allele probably does not account for these findings. Thus, other mechanisms likely contribute to the variation in severity of the skeletal phenotype in male and female patients with XLH.

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References


