Role of abnormal neutral endopeptidase-like activities in Hyp mouse bone cells in renal phosphate transport

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Dubois, Stéphanie G., Andréa Frota Ruchon, Aline Delalandre, Guy Boileau, and Daniel Lajeunesse. Role of abnormal neutral endopeptidase-like activities in Hyp mouse bone cells in renal phosphate transport. Am J Physiol Cell Physiol 283: C1414–C1421, 2002—We investigated whether the absence of Phex (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) in the Hyp mouse affects the expression and activity of neprilysin (NEP) and of endothelin-converting enzyme-like endopeptidase (ECEL1/DINE) in bone marrow stromal cells (BMSC) and osteoblasts (Ob). Total NEP-like activity was higher in Ob than in BMSC regardless of genotype, and Hyp cells showed higher activities than normal. Conditioned media (CM) from Hyp BMSC and Ob inhibited inorganic phosphate (Pi) uptake by mouse proximal tubule cells, and incubating Hyp Ob with phosphoramidon prevented the production of the inhibitor of renal Pi uptake. A linear relationship was observed between the NEP-like activity of Hyp and normal cells and the inhibition of Pi uptake. NEP and ECEL1/DINE mRNA levels were higher in Hyp cells than in normal cells, and in situ hybridization of ECEL1/DINE confirmed higher levels of expression in the Hyp mouse than in normal cells. In conclusion, we observed a correlation between the inhibition of Pi uptake by CM from Hyp cells and elevated NEP-like activities.

X-linked hypophosphatemia; Phex; putative inhibitor of renal phosphate uptake

RENAL PHOSPHATE WASTING in human X-linked hypophosphatemia (XLH) is attributable to a specific reduction of phosphate (Pi) reabsorption in the proximal tubule (reviewed in Ref. 37). Phenotypic characteristics of XLH involve paradoxically low 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] levels in response to hypophosphatemia, osteomalacia, rickets, and lower limb deformities that sometimes require surgery. The murine Hyp model reproduces these characteristics (15, 18, 48, 49) and has been extensively used to study the cause of hypophosphatemia. In recent years it has become evident that the observed hypophosphatemia is not due to an intrinsic renal cell defect but results from humoral mediation (25). This hypothesis was first proposed by Meyer et al. (30) following the observation that parabiosis between a normal and a Hyp mouse results in progressive hypophosphatemia in the normal animal in association with a diminished renal reabsorption of phosphate. Kidney transplantation from normal to Hyp mice and from Hyp to normal mice and measurements of Pi uptake by immortalized cell cultures from the renal proximal tubule of normal and Hyp mice also argued in favor of a humoral mediation (32, 33). More recently, we demonstrated the direct intervention of a humoral factor, present in Hyp mice, on Pi uptake inhibition by primary mouse proximal tubule cells in vitro, and we also showed that Hyp osteoblasts could produce and/or modify this factor (25).

The PHEX/Phex (human/mouse) gene (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) has been shown to be mutated in XLH patients (16) and in Hyp mice (9). Because PHEX/Phex codes for a putative neutral endopeptidase, it was suggested that the enzyme could either inactivate an inhibitor of phosphate reabsorption or hydrolyze a propeptide that stimulates phosphate reabsorption in the kidney. The putative in vivo substrate(s) of PHEX/Phex remains unknown, but it was recently demonstrated that parathyroid hormone (PTH)-related peptide107–139 (PTHrP) was an in vitro substrate of soluble PHEX (3), suggesting that, in addition to this role in modulating the activity of a phosphaturic peptide, Phex also appears to be involved in bone cell differentiation and/or mineralization. Furthermore, increasing evidence indicates that a primary bone cell defect is present in XLH and in the Hyp mouse. Indeed, although high Pi diet and pharmacological doses of calcitriol improve phosphorus balance (4, 5, 8, 41, 48), rickets never completely heal even in the face of active mineralization (4, 5, 19). Reduction, but not normalization, of the osteoid thickness and volume, indexes of abnormal mineralization, are observed following bone cell transplants from Hyp mice into normal recipients, whereas abnormal bone formation is observed when

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normal bone cells are transplanted into mutant mice (13, 14). This role of Phex is further supported by the observations that the inability of Hyp osteoblasts to produce normal bone is not due to prior exposure to an abnormal low-phosphate environment (12) and that primary cell cultures of Hyp osteoblasts show abnormal phenotypic expression compared with normal mouse osteoblasts (7, 25).

PHEX/Phex is a member of a family of structurally related zinc-containing endopeptidases that includes, among others, nephrisin (NEP) and the endothelin-converting enzymes (ECEs) (50). Besides PHEX/Phex, two more enzymes of the family, NEP and endothelin-like converting enzyme 1/damage-induced neuronal endopeptidase (ECEL1/DINE), have been shown to be present in bone (10, 40, 43, 52, 53), suggesting an important role for these enzymes in bone metabolism. The relation between PHEX/Phex expression and expression of other members of the family has never been investigated. In the present paper, we used specific inhibitors to probe the role of NEP on the elaboration of the inhibitor of phosphate uptake by Hyp mouse bone marrow stromal cells (BMSC) and osteoblasts (Ob).

Finally, because Phex is absent from Hyp BMSC and Ob, we evaluated whether this absence had an effect on the total NEP-like activity of these cells and on the expression of NEP and ECEL1/DINE. Our results show that total NEP-like activity was higher in Ob than in BMSC, regardless of genotype, and that Hyp BMSC and Ob showed higher NEP-like activities than normal cells. We also showed that the expression of both NEP and ECEL1/DINE is increased in BMSC and Ob from Hyp mice compared with normal mice and that these enzymes may be involved in the elaboration of the putative inhibitor of phosphate uptake by Hyp BMSC and Ob.

METHODS

Animals

Both normal (C57BL/6J+/+) and Hyp (C57BL/6JHyp+/+) 6- to 8-wk-old male mice were obtained from our own breeding colony. Hyp mice were identified by their shorter body length, shorter tail length, lower weight, and reduced serum phosphorus levels (Table 1).

### Preparation of Primary BMSC Cultures, Ob, and Mouse Proximal Tubule Cells

Total bone marrow was recuperated by flushing posterior limbs twice with 5 ml of Ham’s F-12/DMEM (Sigma, St. Louis, MO) containing 5% penicillin-streptomycin (Pen-Strep; GIBCO BRL, Grand-Island, NY), cutting bones longitudinally, and vortexing twice for 30 s in the same medium by the method of Levite et al. (27). The cell suspensions were pooled and centrifuged at 1,200 g for 2 min at room temperature three times with Ham’s F-12/DMEM containing 5% Pen-Strep. The last pellet was resuspended in Biggers, Gwatkin, and Judah medium (BGJb; Sigma) containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD). Total bone marrow cells were plated at an initial density of 2 × 10^6 cells/cm^2 in T25 flasks (Starsted). After 3 days of culture, adhering BMSC were washed twice with Ham’s F-12/DMEM containing 5% Pen-Strep. The cells were then fed with BGJb medium containing 10% FBS and changed every 2 days thereafter. At day 10, BMSC were split once with 0.025% trypsin and 0.05% EDTA (Sigma), and cells distributed were in 24-well plates. Primary Ob cultures and mouse proximal tubule cell (MPTC) cultures were prepared following our previously published protocols (24, 25).

### Preparation of Conditioned Media From BMSC and Ob

After BMSC and Ob were plated at 50,000 cells/cm^2 in 24-well plates, they were fed with BGJb medium containing 10% FBS for 4 days with one change of medium at day 2. At day 4, cells were washed once with Ham’s F-12/DMEM (1:1) without FBS and then fed with Ham’s F-12/DMEM containing 10% FBS for conditioning. The conditioned media (CM) were recuperated and used to treat MPTC cultures for 48 h before Pi uptake, as previously described (25). BMSC and Ob were used to determine alkaline phosphatase and NEP-like activities, protein content and/or cell count, or to extract RNA. No differences were noted in protein content or cell number between normal and Hyp cells under these conditions. CM in the absence of cells or conditioned in the presence of Chinese hamster ovary (CHO) cells were prepared in parallel to BMSC-CM and Ob-CM to determine the specificity of the conditioning (negative control).

### Alkaline Phosphatase and Neutral Endopeptidase Activities of BMSC and Ob

Alkaline phosphatase was determined by the hydrolysis of p-nitrophenylphosphate (12.5 mM), as previously described (23, 24). Total NEP-like activity of BMSC and Ob was evaluated following a two-step enzymatic analysis, as described.

### Table 1. Physiological parameters for normal and Hyp mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Weight, g</th>
<th>Serum Phosphorus, mM</th>
<th>Total BMC, 10^6 cells/g body wt</th>
<th>BMSC, 10^6 cells/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>22.43 ± 0.43</td>
<td>2.99 ± 0.22</td>
<td>5.38 ± 0.41</td>
<td>2.78 ± 0.29</td>
</tr>
<tr>
<td>Hyp</td>
<td>16.69 ± 0.38</td>
<td>1.56 ± 0.1</td>
<td>3.47 ± 0.22</td>
<td>1.92 ± 0.16</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.005</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Mice were sacrificed, and their posterior long bones were removed and cleaned of all surrounding tissues. After both ends of the femur and tibia were out, they were flushed with Ham’s F-12/DMEM and total bone marrow cells (BMC) were evaluated. Total BMC plated at 2 × 10^6 cells/cm^2 in T25 flasks were cultured for 3 days in BGJb medium containing 10% FBS, the medium was removed, and adhering cells were washed three times with Ham’s F-12/DMEM alone containing 5% penicillin/streptomycin. They were then fed every 2 days with BGJb medium containing 10% FBS until day 10. These bone marrow stromal cells (BMSC) were split with trypsin and counted. Statistical analysis was performed by using the 2-tailed Mann Whitney U-test; a difference of 0.05 was considered significant.

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by Shipp et al. (45), in the presence or absence of 10 μM phosphoramidon or thiorphan, using the synthetic peptide Glu-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Sigma).

**Phosphate Uptake by MPTC**

Transport experiments with MPTC were performed at 37°C in the incubation buffer composed of 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 10 mM HEPES-Tris buffer at pH 7.4, containing 0.1 mM ³²P (NEN-DuPont, Mississauga, ON, Canada) at 2 μCi/ml, as previously described (25). After three washings with stop solution, cells were covered with 500 μl of 0.5 N NaOH and agitated for 60 min at room temperature on a rotating platform at 100 rpm. Aliquots were counted, after neutralization with 0.5 N HCl, in a beta counter, and protein content was determined by the BCA method (46).

**Detection of Phex, NEP, and ECEL1/DINE in Cell Preparations**

**Detection by RT-PCR.** Total RNA from normal and Hyp cells was extracted by the method of Chomczynski and Sacchi (6), and 4 μg of RNA was used for RT-PCR amplification. Reverse transcription reaction was performed by using pd(N)₆ random hexamers as recommended by the supplier (Pharmacia Biotech, Baie d’Urfé, QC, Canada). Half of the single-stranded cDNA obtained was used to generate, via PCR, specific cDNAs of 670, 732, and 450 bp for ECEL1/DINE, NEP, and Phex, respectively. The second half was used to amplify a cDNA of 983 bp for GAPDH via PCR. PCR reactions used 20 pmol of each primer (sense) 5’-TCATAAAAGATTTGACAGGAGGTGG-3’ and (antisense) 5’-AGGACACTTATGAGCGGGTTC-3’ for ECEL1/DINE, (sense) 5’-AGATGAGTGTAATGGGAGGCAGC-3’ and (antisense) 5’-GTATTCTGAGGAAAGATGTTCCCTG-3’ for NEP, (sense) 5’-AAAATGGAACCTTGGATCCGTG-3’ and (antisense) 5’-ATCTGCTCCTCTGTTCATAGTGG-3’ for Phex, and (sense) 5’-TGAAGTCGGTGAGTTGAGCAG-3’ and (antisense) 5’-CATGTAGGCCATGAGGTCGGTGTGAACG-3’ for GAPDH. Thirty-five cycles (portion of the amplification curve) were needed for Phex and NEP, and forty cycles were requested for ECEL1/DINE, whereas twenty-eight cycles were enough for the amplification of GAPDH. Amplified DNA fragments were then visualized on 1.2% agarose gel.

**Detection by Western blot analysis.** Extracts of normal and Hyp cells were prepared and analyzed by Western blotting essentially as previously described with specific monoclonal antibodies for NEP (40). Protein was detected by ECL-PLUS chemiluminescence kit with Lumigen-PS3 (Amersham Pharmacia Biotech) and visualized with Kodak X-Omat films, and scanning densitometry was performed directly on these films.

**Detection by in situ hybridization.** In situ hybridization (ISH) was performed essentially as previously described (39), except for the RNase A concentration, which was 200 mg/ml instead of 200 mg/ml. Sense and antisense riboprobes labeled with α-35S-uridine triphosphate (UTP; 1,250 Ci/mmol; Amersham, Arlington Heights, IL) were prepared by in vitro transcription of appropriate linearized plasmids. Plasmids used were, for ECEL1/DINE, pSPORT1 plasmid (catalog no. 1058129; American Type Culture Collection, Rockville, MD) containing a 458-bp mouse ECEL1/DINE cDNA insert corresponding to nucleotides 2075–2533 of the nucleotide sequence (GenBank accession no. XM002262), and for PTH/PTHrP receptor, pBlueScript containing a 528-bp cDNA insert corresponding to nucleotides 193–721 of PTH/PTHrP receptor nucleotide sequence (GenBank accession no. X78936).

**Statistical Analysis**

Values represent means ± SE for n individual culture preparations. Transport experiments are performed in quadruplicate dishes per experiment per conditions tested, whereas all other determinations were performed on individual samples in duplicate. Two-tailed Mann-Whitney U-tests were performed, and a level of 0.05 was considered to be significant.

**RESULTS**

**Phenotypic Characterization of BMSC and Ob**

BMSC are the reservoir of the osteoblastic cell lineage. The suggestion that normal mouse bone marrow transplanted into an Hyp mouse can correct the Hyp phenotype (31) could indicate that Hyp BMSC already bear a cell defect leading to abnormal bone metabolism. We therefore investigated whether Hyp BMSC are different from normal cells. Hyp mice showed a significant reduction in total bone marrow cells and in available BMSC compared with normal (Table 1). Moreover, when normal and Hyp BMSC were plated at high initial density at first passage, to eliminate the possibility that growth characteristics may influence enzymatic profiles, alkaline phosphatase activities in confluent cell cultures were always significantly higher in Hyp mouse BMSC compared with normal. Although an increase in alkaline phosphatase activity was also noted in Hyp Ob compared with normal, it never reached statistical significance (Fig. 1).

**Neutral Endopeptidase Activity in BMSC and Ob**

It has been reported that NEP-like activity may vary with bone cell maturation (1). Therefore, we questioned whether an NEP-like activity could be affected in bone lineage cells of the Hyp genotype. Regardless of genotype, NEP-like activity of Ob was much higher than for BMSC (2- to 3-fold, Fig. 2). This activity was also higher in Hyp BMSC compared with normal BMSC.
(89.9 ± 13.5% increase, \( P < 0.001 \)) and in Hyp Ob compared with normal Ob (157.4 ± 49.9% increase, \( P < 0.02 \)) (Fig. 2). Thiophorphan (10 \( \mu \)M) and phosphoramidon (10 \( \mu \)M), both inhibitors of NEP-like activity, significantly inhibited the NEP-like activity in normal and Hyp cells (Fig. 2). Treatment of cells with 10 \( \mu \)M phosphoramidon failed to significantly alter cell growth characteristics in normal and Hyp BMSC and Ob (data not shown). However, the same treatment reduced alkaline phosphatase activity in Hyp BMSC (24.4 ± 8.8% decrease, \( P < 0.025 \)) and Ob (30.7 ± 7.9% decrease, \( P < 0.025 \)) but failed to influence this parameter in normal BMSC (3.7 ± 2% decrease, not significant) and Ob (1.3 ± 0.3% increase, not significant).

**Effect of NEP-Like Activity Inhibition on Production of the Putative Phosphate Uptake Inhibitor**

To determine whether this NEP-like activity is involved in the production of the phosphaturic factor, we measured phosphate uptake by MPTC in the presence of CM from normal or Hyp BMSC and Ob. This biological assay provided a sensitive method to monitor the production of the phosphaturic factor, because we successfully used MPTC to evaluate the time and dose dependence of Hyp mouse serum and CM of Hyp Ob on \( P \), uptake (25) and to monitor the purification of the elusive Hyp humoral phosphaturic factor(s) (Loignon M, Dubois SG, and Lajeunesse D, unpublished observations). Phosphate uptake by MPTC in response to CHO-CM and normal BMSC-CM is not significantly modified compared with cells treated with medium alone (Fig. 3). In contrast, Hyp BMSC-CM produced a significant inhibition of \( P \) uptake by MPTC (−36.1 ± 4.3%, \( P < 0.001 \)). Likewise, normal Ob-CM partly inhibited \( P \) uptake (\( P < 0.005 \)), whereas Hyp Ob-CM inhibited this uptake much more than normal Ob (\( P < 0.001 \)). Because the capacity to inhibit \( P \) uptake in MPTC varies between Hyp BMSC and Ob and normal BMSC and Ob, we evaluated whether a relationship exists between NEP-like activity and \( P \) uptake. A linear relationship was observed between the NEP-like activity of normal and Hyp BMSC and Ob and the inhibition of \( P \) uptake by MPTC (Fig. 4). The involvement of the NEP-like activity in the production of the phosphaturic factor by Ob from Hyp animals is further supported by the addition of phosphoramidon in culture medium. Phosphoramidon inhibited NEP-like activity in both normal and Hyp Ob, and the same treatment during the conditioning of Hyp mouse Ob

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**Fig. 2.** Neprylisin (NEP)-like activity of normal and Hyp mice BMSC and Ob treated with or without 10 mM phosphoramidon (Ph) or 10 mM thiorphan (Th). NEP-like activity of BMSC from normal (13 preparations) and Hyp (8 preparations) mice and primary Ob from normal (10 preparations) and Hyp (8 preparations) was measured. Values are means ± SE and are expressed as nmol·mg protein \(^{-1}·h^{-1}. \) **\( P < 0.001 \) between normal and Hyp BMSC. \(* P < 0.005 \) between Hyp BMSC and Ob. \(* * P < 0.02 \) between normal and Hyp Ob. \(* * * P < 0.05 \) between normal BMSC and Ob.

**Fig. 3.** Effect of conditioned medium (CM) from normal and Hyp mouse BMSC or Ob (see Fig. 2 legend) on renal phosphate uptake by mouse proximal tubule cells (MPTC). CM were prepared for the last 48 h of culture of cells before being added to MPTC for 48 h. Phosphate uptake was then measured with 0.1 mM \(^32\)Pi for 5 min. Values are means ± SE of 4–10 experiments run in quadruplicate dishes per condition. \( P < 0.001; \) **\( P < 0.005 \) vs. medium alone.

**Fig. 4.** Relationship between total neutral endopeptidase activity and phosphate uptake inhibition capacity of both normal and Hyp Ob and BMSC (see Fig. 2 legend). Total neutral endopeptidase activity was measured as described in Fig. 2 and \( P \) uptake as described in Fig. 3. Values are means ± SE of 4–10 experiments per condition run in quadruplicate dishes per condition.
PREPARED ITS INHIBITORY EFFECT ON P3 UPTAKE BY MPTC (Fig. 5), WHEREAS NO CLEAR EFFECTS COULD BE OBSERVED WITH NORMAL MOUSE CM UNDER THESE CONDITIONS.

Levels of Expression of NEP and ECEL1/DINE

In addition to Phex, two members of the NEP family have been reported in bone: NEP and ECEL1/DINE. Both these enzymes have been shown to be sensitive to thorphan and phosphoramidon (22, 38, 50, 51). To test the possibility that expression of these enzymes is increased and thus contributes to the higher level in NEP-like activity observed in Hyp BMSC and Ob, we determined the level of NEP and ECEL1/DINE mRNA and protein. Specific oligonucleotides were used in a RT-PCR strategy to amplify DNA fragments for ECEL1/DINE, NEP, and Phex from total RNA isolated from normal or Hyp BMSC and Ob (Fig. 6). In the conditions used, ECEL1/DINE could not be detected in normal mouse BMSC but was readily detectable in normal Ob and in both Hyp BMSC and Ob, with higher levels in Ob than BMSC. The ratio of ECEL1/DINE to GAPDH mRNA levels measured by RT-PCR was higher in Hyp Ob compared with normal Ob (1.317 ± 0.100 vs. 0.849 ± 0.040, respectively, P < 0.025, n = 3). NEP mRNA levels were also found to be slightly higher in Hyp Ob vs. normal Ob, although this difference did not reach significance (1.056 ± 0.061 vs. 1.019 ± 0.048 NEP-to-GAPDH ratio, respectively, n = 3), and levels were significantly more elevated in Hyp than in normal BMSC (0.994 ± 0.021 vs. 0.841 ± 0.034 NEP-to-GAPDH ratio, respectively, P < 0.025, n = 3). As expected, Phex expression measured by RT-PCR was absent from Hyp cells. Interestingly, we observed higher levels of Phex in normal BMSC than in Ob (4.3-fold increase; 1.531 ± 0.125 vs. 0.393 ± 0.072 Phex-to-GAPDH ratio, respectively, P < 0.01, n = 4).

To determine whether changes in ECEL1/DINE expression observed in primary cultures of Ob and BMSC cells of Hyp mouse are also present in whole bones, we performed ISH on sections of 4-day-old (P4) normal and Hyp animals using an ECEL1/DINE-specific probe. Figure 7 shows an increased expression of ECEL1/DINE in the calvarium of the Hyp mouse compared with normal mouse (Fig. 7, A and B for normal and Hyp, respectively). As a control, the expression of PTH/PTHrP receptor was measured by ISH in parallel in these samples (Fig. 7, D and E for normal and Hyp, respectively). No significant differences were noted for PTH/PTHrP receptor expression between genotypes as opposed to ECEL1/DINE. Similar results were observed in vertebrae (results not shown).

We next used specific antibodies to determine the level of NEP in normal and Hyp cells (Fig. 8). In contrast to mRNA levels, which were only slightly higher in Hyp cells, larger amounts of NEP protein were observed in Hyp than normal cells (Fig. 8).

DISCUSSION

BMSC are the precursor cells of osteoblast lineage cells (17, 35) and differentiate into Ob (2, 26). Several studies have shown that BMSC can form a mineralized collagen matrix and calcified nodules as found in bone (29, 42, 44, 47). The first conclusion of the present study is that BMSC from the Hyp mouse are less numerous, grow at a slower rate, have higher alkaline phosphatase activity, and produce more phosphate uptake inhibitor than cells from a normal mouse. Similar features were previously reported for Hyp Ob (25). Elevated alkaline phosphatase in Hyp BMSC may suggest that these cells are kept at an earlier stage of development, because this activity should decrease as
the cells progress into the osteoblast lineage (36), as illustrated here by the decrease in alkaline phosphatase activity in both normal and Hyp Ob compared with their respective BMSC. However, Hyp cells always showed higher alkaline phosphatase activities than normal cells. These observations suggest that the lack of Phex could result in an intrinsic defect in these progenitor cells that did not disappear as the cells progressed toward mature Ob.

The precise role of Phex is still obscure. Some have proposed that mineralization by osteoblasts may be linked to Phex expression (20, 54), whereas others have suggested that Phex may be involved in osteoblast differentiation, hence possibly leading to abnormal mineralization (11). Recently, Liu et al. (28) demonstrated that in vivo and in vitro expression of Phex in Hyp osteoblasts is not sufficient to fully rescue the Hyp phenotype, hence indicating that additional factors are likely to be important in the pathogenesis of XLH. Indeed, because Phex may be linked to osteoblast maturation, its absence could modify the expression of other genes in BMSC and/or Ob from Hyp animals. The observation that NEP-like activity and expression levels of two NEP-like enzymes are increased in BMSC and Ob of Hyp animals is the second important finding of our study. mRNA levels of Phex in normal BMSC compared with Ob varied oppositely to those of NEP and ECEL1/DINE. Consistent with this observation, the absence of Phex in Hyp cells resulted in increased expression of NEP and ECEL1/DINE. Our results suggest a regulation, direct or indirect, of both NEP and ECEL1/DINE expression in bone cells by the Phex gene product. However, because this increase in NEP expression was not observed in bone extracts of 4-day-old mice (40), it is suggested that the role of Phex in the regulation of NEP expression is more important at an earlier stage of growth.

It has been shown previously that NEP-like activity increases during the differentiation of BMSC (1). Because NEP has been detected in growing bone plates (10, 40) and NEP-like activity is inversely correlated with bone cell growth (1), the present increase in NEP-like activity may explain the reduction in the reservoir of BMSC of the Hyp mouse. However, because NEP and ECEL1/DINE cannot be truly distinguished on the basis of inhibitors used in our study, it is not possible at the present time to determine which of these activities is more important for bone cell growth, differentiation, and/or mineralization. However, the partial normalization of alkaline phosphatase activity in Hyp BMSC and Ob following the inhibition of endopeptidase activities with 10 μM phosphoramidon (not shown), which was not affected in normal cells, suggests that abnormally high NEP-like activity in Hyp cells could contribute to the abnormal cell behavior of these cells in vitro as well as in vivo. It is interesting to note that increases in NEP protein in both Hyp BMSC and Ob are much higher than the increase in mRNA level. This finding could suggest the removal of a post-transcriptional inhibition on NEP synthesis in the Hyp mouse. Other investigators (21) recently indicated that another peptidase is elevated in Hyp mouse bone tissues, namely, cathepsin D, offering another link to abnormal bone mineralization/turnover in these animals.

Our study is the first demonstration of Phex expression in BMSC suggesting that Phex can be involved in prolif-
thiorphan and phosphoramidon are potent inhibitors of neutral enopeptidase activity in primary renal cells, and the elaboration of this product is linked to a cellular defect in Ob, which is already present in BMSC. The presence of higher levels of PHEX endopeptidase catalytic activity in BMSC than in Ob. The production of this inhibitor of renal Pi uptake. Both thiorphan and phosphoramidon are potent inhibitors of neutral enopeptidases that have slightly different specificities. Whereas phosphoramidon is a more general inhibitor of neutral enopeptidases such as NEP and ECE, thiorphan is believed to be a more selective inhibitor of renal Pi uptake with phosphoramidon-sensitive neutral endopeptidase activity in BMSC and Ob.

In conclusion, the results indicate that a cellular defect is present in hypophosphatemia: mouse model for human familial hypophosphatemia (Vitamin D-resistant) rickets. Proc Natl Acad Sci USA 73: 4671–4671, 1976.

REFERENCES


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