低フォスファターゼ症の診断指針

主症状

1. 骨石灰化障害
   骨単純 X 線所見として骨の低石灰化、長管骨の変形、くる病様の骨幹端不整像
2. 乳歯の早期脱落（4歳未満の脱落）

主検査所見

1. 血清アルカリフォスファターゼ(ALP)値が低い（年齢別の正常値に注意）

参考症状

1. ビタミン B6依存性けいれん
2. 四肢短縮、変形

参考検査所見

1. 尿中フォスフォエタノールアミンの上昇（尿中アミノ酸分析の項目にあり）
2. 血清ピロリン酸値の上昇
3. 乳児における高カルシウム血症

遺伝子検査

1. 確定診断、病型診断のために組織非特異的 ALP (*TNSALP*)遺伝子検査を行う事が望ましい

参考所見

1. 家族歴
2. 両親の血清 ALP 値の低下

診断基準

主症状一つ以上と血清 ALP 値低値があれば遺伝子検査を行う。参考症状、参考検査所見、参考所見があれば、より確実である。

症例の御相談、遺伝子検査の御依頼、血清ピロリン酸測定の御依頼は、厚生労働省難治疾患克服事業「低フォスファターゼ症」研究班事務局(hypophos@ped.med.osaka-u.ac.jp)までお問い合わせください。

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特許

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Successful gene therapy *in utero* for lethal murine hypophosphatasia

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Abstract

Hypophosphatasia (HPP), caused by mutations in the gene *ALPL* encoding tissue-nonspecific alkaline phosphatase (TNALP), is an inherited systemic skeletal disease characterized by mineralization defects of bones and teeth. The clinical severity of HPP varies widely from a lethal perinatal form to mild odontohypophosphatasia showing only dental manifestations. HPP model mice (*Akp2*$^{-/-}$) phenotypically mimic the severe infantile form of human HPP; they appear normal at birth but die by two weeks of age due to growth failure, hypomineralization, and epileptic seizures. In the present study, we investigated the feasibility of fetal gene therapy using the lethal HPP model mice. On day 15 of gestation, the fetuses of HPP model mice underwent transuterine intraperitoneal injection of AAV serotype 9 expressing bone-targeted TNALP. Treated and delivered mice showed normal weight gain and seizure-free survival for at least 8 weeks. Vector sequence was detected in systemic organs including bone at 14 days of age. ALP activities in plasma and bone were consistently high. Enhanced mineralization was demonstrated on X-ray images of the chest and forepaw. Our data clearly demonstrates that systemic injection of AAV9 *in utero* is an effective strategy for the treatment of lethal HPP mice. Fetal gene therapy may be an important choice after prenatal diagnosis of life-threatening HPP.

Introduction

Hypophosphatasia (HPP), caused by a deficiency in tissue-nonspecific alkaline phosphatase (TNALP), is an inherited disease characterized by mineralization defects. HPP is a clinically heterogeneous disease and is classified according to severity and age at diagnosis (Mornet, 2007; Whyte, 2010). Perinatal and infantile forms of HPP are usually severe, and life expectancy is less than one year in most cases. The major cause of death is respiratory failure associated with a narrow chest and pyridoxine-responsive seizures are also observed in some severely affected cases (Whyte, 2010; Nakamura *et al.*, 2010). The childhood and adult forms of HPP show milder phenotypes and odontohypophosphatasia causes premature loss of deciduous teeth without evidence of skeletal disease. Perinatal HPP is more common in Japan than in other countries and is the fifth most common form of fetal-diagnosed skeletal dysplasia (Satoh *et al.*, 2009).

There is no established treatment for HPP, but several experimental approaches have been attempted to treat TNALP knockout mice (*Akp2*$^{-/-}$). The phenotype of these mice mimics that of severe infantile HPP; the animals appear normal at birth, but rapidly develop growth failure, epileptic seizures, and hypomineralization, and die by two weeks of age. Recently, Millán et al. reported that *Akp2*$^{-/-}$ mice can be treated by repeated injection of bone-targeted TNALP with deca-aspartates at the C terminus (TNALP-D10) (Millán *et al.*, 2008). Based on these data, new clinical trials of enzyme replacement therapy (ERT) for patients with infantile and childhood HPP have been initiated (http://clinicaltrials.gov/). Another important possibility for treatment of HPP is gene therapy. We have recently demonstrated that a single

injection of either lentiviral or adeno-associated viral (AAV) vector expressing TNALP-D10 into postnatal HPP mice resulted in prolonged seizure-free survival and phenotypic correction (Yamamoto *et al.*, 2010; Matsumoto *et al.*, in press). This gene therapy approach is referred to as viral vector-mediated ERT, and it is more practical than classical ERT that requires repeated injections. Neonatal gene therapy may be an important option for treatment of severe HPP.

Due to the remarkable progress of prenatal diagnosis with clinical imaging, including echography and computed tomography, as well as molecular testing, the chance of diagnosis of perinatal lethal HPP during the fetal period is increasing. Fetal gene therapy may be the only choice options of treatment for perinatal HPP and severe infantile HPP in the future. In the present study, we evaluated the feasibility of gene therapy during the fetal period. Here, we report that systemic injection of AAV vector *in utero* is an effective strategy for treatment of perinatal orearly-onset lethal genetic diseases.

Materials and Methods

Construction and preparation of recombinant adeno-associated viral vector

The AAV vector plasmid containing cDNA for TNALP-D10 was described previously (Matsumoto *et al.*, in press). Recombinant AAV serotype 9 (AAV9) vector was generated by the triple transfection method (Salvetti *et al.*, 1998) and purified as described previously (Hermens *et al.*, 1999; Miyake *et al.*, 2011). The titer of each AAV vector was determined by real-time PCR (7500 Fast; Applied Biosystems, Tokyo, Japan). AAV9-EGFP (Miyake *et al.*, 2011) was used to analyze the biodistribution of the vector.

Animal procedures and vector injection

All animal procedures were performed in accordance with the guidelines approved by the Nippon Medical School Animal Ethics Committee. *Akp2*$^{-/-}$ mice were obtained by mating *Akp2*$^{+/-}$ mice with a mixed genetic background of 129J and C57Bl/6J, which were generated by the Millán laboratory (Fedde *et al.*, 1999; Narisawa *et al.*, 1997). AAV9 vector was intraperitoneally injected into fetal or postnatal mice. For fetal vector injection, pregnant dams on day 15 of gestation were anesthetized by intramuscular injection of 0.6 mg of pentobarbital in 100 μl of PBS and inhalation of isoflurane. A midline laparotomy was performed and the uterus was exposed. Vector (8.3×10^{10} viral genomes [vg] of AAV9-TNALP-D10 or AAV9-EGFP/body weight [g]) was injected transuterine, intraperitoneally into each fetus using a 33-gauge Ito syringe (Ito Corporation, Shizuoka, Japan). The amount of vector for fetal injection was 1.0×10^{11} vg/10 μl/body. After the

injections, the peritoneum was filled with 300 μl of PBS and the abdominal muscle layer and the skin layer were closed with 5-0 nylon sutures. After normal delivery, the newborn mice stayed for 1 month with the dam. The genotype of treated fetuses was determined after delivery. For neonatal injection, 2.0×10^{11} vg/20 μl/body was intraperitoneally injected into the mice on postnatal day 1.

ALP activity in plasma and organs

Blood samples were collected from the orbital sinus using heparinized capillaries on days 14, 28, and 56 after birth. After plasma separation, ALP activity in the plasma was quantified using a colorimetric assay for ALP as described previously (Goseki *et al.*,1988). ALP activity was described in units (U) defined as the amount of enzyme needed to catalyze production of 1 μmol p-nitrophenol formed per minute and calculated as U/ml.

Brain, heart, liver, quadriceps, abdominal rectus muscle, and tibial bone were dissected out under deep anesthesia following perfusion with 20 ml of PBS containing 10 U/ml of heparin followed by 20 ml of PBS. Bone marrow and soft tissues were carefully removed from the isolated bone. Each organ was homogenized with 500 μl of dH_2O using a Percellys 24 bead-beating homogenizer (Bertin Technologies, Paris, France) and centrifuged at 14000 × *g* for 5 min. ALP activity of the supernatant was analyzed as described above and standardized by 1 mg of protein. Protein concentration was determined using a DC protein assay kit (Bio-Rad, Tokyo, Japan).

Histochemical examination of bone

Knee joints were removed under deep anesthesia following perfusion with 20 ml of PBS containing 10 U/ml of heparin and 20 ml of PBS, followed by embedding in SCEM compound (Leica Microsystems, Tokyo, Japan) and freezing, without fixation or decalcification. Sections (14 μm thick) were cut with the Kawamoto film method (Leica Microsystems) and washed with 99.5% ethanol and dH_2O. ALP activity was assayed by incubating the tissue with 0.1 mg/ml naphthol AS-MX phosphate as a substrate and 0.6 mg/ml fast blue BB salt as dye in 20 ml of 0.1M Tris-HCl buffer (pH 8.5) for 15 min at 37°C, as described previously (Sugiyama *et al.*, 2003). After mounting on silane-coated slides (Muto Pure Chemicals Ltd., Tokyo, Japan), they were examined under a light microscope (BX 60; Olympus Ltd., Tokyo, Japan).

For immunostaining, sections (10 μm thick) of the knee joint were cut with the Kawamoto film method (Leica Microsystems) and fixed with 10% formarine. The sections were incubated with 1% skim milk in PBS for 60 minutes (blocking). After the blocking, the sections were incubated with primary antibodies for 60 minutes at room temperature followed by 48 hours at 4°C. The primary antibodies were rabbit anti-collagen II (1:100; Abcam, Cambridge, UK) and mouse anti-GFP (1:400; Invitrogen, OR). After washing with PBS, they were incubated with secondary antibodies, which were goat anti-rabbit IgG conjugated with alexa 568 (1:500; Invitrogen) and donkey anti-mouse IgG conjugated with alexa 488 (1:500; Invitrogen), at room temperature for 2 hours. After mounting on silane-coated slides (Muto Pure Chemicals Ltd.), they were examined with fluorescence microscopy (BX60; Olympus Ltd.).

X-ray analysis

Radiographic images were obtained using μFX-1000 (Fujifilm, Tokyo, Japan) with energy of 25 kV and exposure time of 10 s, and imaged with FLA-7000 (Fujifilm).

Biodistribution of AAV vector

Biodistribution of AAV9 vector was determined by real-time PCR of genomic DNA of *Akp2*$^{+/+}$ mice after intraperitoneal (IP) injection of AAV9-EGFP. Heart, liver, quadriceps, abdominal rectus muscle, tibial bone, brain, and gonads were removed under deep anesthesia following perfusion with 20 ml of PBS containing 10 U/ml of heparin followed by 20 ml of PBS at 14 days of age. Each tissue of the mice was homogenized using a Percellys 24 bead-beating homogenizer (Bertin Technologies) followed by DNA extraction using a Gentra Puregene kit (Qiagen Sciences, Germantown, MD). Real-time PCR was performed using primers designed to amplify part of the CMV promoter of AAV9 (sense, 5'-GACGTCAATAATGACGTATG-3'; antisense, 5'-GGTAATAGCGATGACTAATACG-3'). Reaction was carried out with 100 ng of template, 0.2 μmol/l of each primer, SYBR Premix Ex Taq (Perfect Real Time; Takara, Tokyo, Japan) and Rox Reference Dye II (Takara). The amplification conditions were 95°C for 10 s, followed by 40 cycles of 95°C for 15 s and 60°C for 34 s. Dissociation was performed at 95°C for 15 s, 60°C for 1 min, and 95 °C for 15 s. GFP expression in the tissues of the mice treated with AAV9-EGFP was visualized by immunohistochemical staining using anti-EGFP antibody (MBL, Aichi, Japan) and the avidin/biotinylated enzyme complex method using diaminobenzidine dihydrochloride (DAB) as described previously (Iwamoto *et al.*, 2009).

Statistical analyses

Differences between groups were tested for statistical significance using Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. The survival rate was analyzed by the Kaplan–Meier method, and differences in survival rate were assessed by the Wilcoxon's test.

Results

Fetal injection of AAV9-TNALP-D10 prolonged survival and improved development of Akp2$^{-/-}$ mice

Fetal mice were obtained by mating *Akp2*$^{+/-}$ heterozygous mice, which are healthy and have a normal lifespan. AAV9-TNALP-D10 (1×10^{11} viral genomes/10 μl) was injected intraperitoneally into a total of 88 fetuses on embryonic day 15 from 11 pregnant dams. Among the treated mice, 47 (53%) were live-born, while 41 (47%) did not live longer than half a day because they were eaten by the dam. Genotyping of live neonates showed that 9 of 47 were *Akp2*$^{-/-}$. Non-treated *Akp2*$^{-/-}$ mice were born with a normal appearance but showed growth failure with epileptic seizures and did not live longer than 11 days. In contrast, the weight and growth rate of treated *Akp2*$^{-/-}$ mice (7/9) were indistinguishable from those of wild-type littermates (17.1 ± 1.4 g [n = 3] vs. 16.9 ± 1.9 g [n = 3] on day 28 [Fig. 1A], 19.3 ± 2.8 g [n = 3] vs. 21.7 ± 1.7 g [n = 3] on day 56) and the lifespan was significantly extended up to at least 56 days (the time of sacrifice for analysis) (Fig. 1B). Seizures were not observed in these surviving mice throughout the experimental period. Two of the *Akp2*$^{-/-}$ fetuses failed to respond to treatment. They showed severe weight loss and convulsions, and died on postnatal day 21.

ALP activities were elevated in the fetal treated mice

To measure the levels of TNALP-D10 expression in the bloodstream and in the tissues after fetal gene therapy, ALP activity was analyzed by colorimetric assay. The plasma

ALP activities of the *in utero*-treated mice were markedly increased and significantly remained more than 10 times higher than those in wild-type mice ($Akp2^{+/+}$) during the observation period (9.3 ± 2.5 U/ml vs. 0.15 ± 0.085 U/ml [$n = 3$] on day 14, $P < 0.01$, 3.7 ± 2.0 U/ml vs. 0.13 ± 0.037 U/ml [$n = 3$] on day 28, $P < 0.05$, and 3.5 ± 1.5 U/ml vs. 0.065 ± 0.020 U/ml [$n = 3$] on day 56, $P < 0.05$) (Fig. 2A). Despite the super-physiologically high levels of ALP activity, no gross deformities of the bones or abnormal calcification were observed on X-ray images (data not shown).

ALP activities in the tissues of treated $Akp2^{-/-}$ mice were measured 14 days after birth (20 days after vector injection). ALP activity was elevated in heart, quadriceps, abdominal rectus muscle, and surprisingly in the bone (Fig. 2B), which was not observed in our previous study with neonatal injection of AAV vector.

Elevated ALP activities were detected in the bone after fetal gene therapy

The cartilage zone of the proximal tibia was analyzed histochemically for ALP activity using the azo-dye technique. In the epiphysis of $Akp2^{+/+}$ mice, strong ALP activity was observed in the hypertrophic chondrocytes and on the surface of the endosteal bones on days 14 (Fig. 3A) and 56 (Fig. 3B). The ALP staining in the bone of $Akp2^{+/+}$ mice appeared to be strengthened with age. In the corresponding area of the tibial bone of untreated $Akp^{-/-}$ mice on day 10, growth of the trabecula was significantly inhibited and no ALP signal was detected (Fig. 3C). After fetal gene therapy, strong ALP signals were detected mainly on the surface of the endosteal bones on day 14, although the density of the trabecula is still less than that of

Akp2$^{+/+}$ *mice*(Fig. 3D). Faint scattered ALP signals were also observed in the cartilage zone of fetal treated *Akp2*$^{-/-}$ mice on day 56 (Fig. 3E).

X-ray images revealed the skeletal phenotypic correction after fetal gene therapy

Mineralization of fetal treated *Akp2*$^{-/-}$ mice was evaluated by X-ray examination. The radiographic changes in *Akp2*$^{-/-}$ mice became apparent during the first 7 to 10 days of life, although the severity of the mineralization defects was highly variable. X-ray images of the most severely affected cases on day 10 showed that a lack of secondary ossification centers and reduced numbers of carpal bones compared to *Akp2*$^{+/+}$ mice, indicating ossification incompetence in HPP mice. In contrast, apparent ossification centers were detected in all treated mice on day 10 (n = 3, Fig. 4A). No differences in skeletal structure or mineralization, including the size of the thoracic cage, were observed between *Akp2*$^{+/+}$ and treated *Akp2*$^{-/-}$ mice on day 56 (n = 3, Fig. 4B).

Chondrocytes were transduced by fetal injection of AAV9

To confirm whether the elevated ALP activity in the tissues (especially bones) was derived from other tissues via the bloodstream or was expressed in the original tissues, the biodistribution of AAV9 vector was determined using real-time PCR on genomic DNA. AAV9-EGFP, which does not have special affinity to the bone, was used to confirm the vector biodistribution. DNA was isolated from each tissue of the *Akp2*$^{+/+}$ mice treated by fetal or neonatal IP injection of AAV9-EGFP. After neonatal IP injection, high copy numbers of AAV9 were observed in the heart and liver, whereas transduction into the bone was very low. This

distribution pattern was similar to that obtained after neonatal intravenous (IV) injection of AAV8 vector (Matsumoto *et al.*, in press). On the other hand, after fetal IP injection, AAV9 vector was widely distributed to all organs examined except the gonads. Importantly, AAV sequence was significantly elevated in brain and bone compared with neonatal IP injection (Fig. 5A, $P < 0.01$). No vector sequence was detected in the gonads of the pups (data not shown). AAV9 mediated transduction of the bone tissue *in utero* was confirmed by histochemical staining (Fig. 5B, 5C). EGFP expression detected by DAB staining was observed especially in chondrocytes in the growth cartilage area. The double-positive cells with EGFP and collagen II of the treated mice confirm that These data suggest that chondrocytes are susceptible to AAV9 transduction at least during the fetal period.