Genetic examination of diseases affecting bone development and structure in newborns

Examination of molecular genetic markers in osteopenic preterm infants

PhD Thesis

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1. Introduction

Technical advances and novel treatment modalities in the care of very low birth weight (VLBW) infants have increased their chance of survival and created new medical diseases such as the bone disease of preterm infants. The definition and name of the disorder affecting bones in premature infants is not clear. Since several pathophysiological conditions may be the cause for the same clinical picture, the nonspecific term „bone disease of premature infants" was suggested. The forms of bone disease include *osteomalacia* (incorporation of minerals into the organic bone matrix is disturbed), *osteopenia* (decreased amount of bone tissue without radiological signs and the sign of rickets), and *osteoporosis* (in paediatric: history of fractures after minor trauma). The incidence of the metabolic bone disease in VLBW infants is about 30%. Hormonal factors (parathormon, calcitonin, vitamin D, growth hormon, cortisol) plays an important role in the mineralisation of the bony system. Factors contributing to diminished synthesis or increased resorption of organic bone matrix include inadequate calcium and phosphorus supply, severe systemic disease (e.g. bronchpulmonary dysplasia), side effects of drugs (corticosteroid, diuretics, methylxanthines), and lack of mechanical stimulation. Recently, maternal parity and gender has been described as risk factors for bone disease in preterm infants**.**

Osteoporosis is a multifactorial skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue occurring due to different environmental, hormonal, nutritional and genetic factors. Adult twin studies suggested that up to 75% of the variance in bone mass density (BMD) is genetically determined. In adults, association has been found between certain genetic polymorphisms [vitamin-D-receptor (VDR), estrogen-receptor (ER), collagen Iα1 (COLIA1)] and the occurrence of osteoporosis. Few studies have addressed possible interactions among the different osteoporosis candidate genes. To date, only one study has considered the genetic predisposition to bone mass content (BMC) in premature infants. It was suggested that multiple genes may be involved in the regulation of bone mass during childhood.

It is important to emphasize that not only VLBW infants but term infants may be affected. Long-term treatment with drugs influencing bone-turnover may lead to bone disease in term, or near term infants too. It has been reported, that somatostatin

analogs (octreotide, Sandostatin), recently used in the treatment of chylothorax, have long-term effects on calcium homeostasis and markers of bone metabolism.

2. Objectives

The main objectives of this thesis were:

1. Is the incidence of metabolic bone disease in Hungarian VLBW infants in agreement with the previously reported data? Is it possible to diagnose metabolic bone disease based on biochemical parameters and radiological findings?
2. Among fetal and maternal risk factors such as gestational age, birth weight, height, gender, Clinical Risk Index for Babies (CRIB) score, Apgar score, maternal parity, and length of hospitalization, which factors play an important role in developing bone disease?
3. Does an association exist between bone disease in VLBW infants and the three main candidate genes for osteoporosis?
4. Is it possible to identify the exposed population? How to prevent severe metabolic bone disease? What are the cornerstones of successful treatment?
5. Has the short-term use of somatostatin analog (octreotide, Sandostatin) an effect on bone metabolism?

3. Patients

This study included preterm infants with body weight below 1500 g and born at gestational age 31 weeks or less at the Department of Obstetrics and Gynaecology, Medical School, University of Pécs, and admitted to our NICU between January 1, 2002 and June 30, 2005. Written consent was obtained from the parents. The 104 infants enrolled to our study had mean (±SEM) birth weight 1080 ± 38 g and gestational age 28,5 ± 0,4 weeks; 50 were girls and 54 were boys. Gestational age was determined from the mother's last menstrual period or ultrasound examination during pregnancy and confirmed by the new Ballard examination.

Based on laboratory and radiological findings, we divided the infants into two groups - infants with and without bone disease.

With regard to the duration of assisted ventilation and supplemental oxygen therapy, we did not find any significant difference between the two groups. The mean duration of assisted ventilation was 4.5 days in the group with bone disease and 4.0 days in the

group without bone disease. The mean duration of oxygen supplementation was 13 days in the bone disease group and 11 days in the comparison group. Five infants in the bone disease group and four in the comparison group received steroid therapy (0.5 mg/kg/day for 10 days) for bronchopulmonary dysplasia. Enteral feeding was generally introduced on the second postnatal day with preterm formula (containing 75 mg calcium, 48 mg phosphorus, 8 mg magnesium per 100 ml) or fortified human milk (calcium, phosphorus, magnesium contents were 81 mg, 49 mg, 5.5 mg per 100 ml, respectively). Supplementary parenteral nutrition was given for about 15 days in both groups (bone disease group 16 days, no bone disease group 14 days). Full enteral nutrition (150 ml/kg/day) was generally achieved by the 16^{th} postnatal day. All infants received a supplement of 400 IU/day vitamin D (cholecalciferol) starting from the 7^{th} day of life.

4. Method

4.1. Determination of biochemical parameters

In all cases at the ages of 1, 2, 3, 6, and 12 months, in 92 infants at the age of 2 years and in 68 infants at the age of 3 years the following analyses were performed: Serum calcium, inorganic phosphorus, alkaline phosphatise, magnesium, osteocalcin, parathyroid hormone was determined under standard conditions. At the same time, bone resorption was assessed by the measurement of urinary calcium and pyridinium crosslinks corrected for creatinine concentration, expressed as nmol/mmol creatinine.

For infants with bone disease follow-up x-rays of the chest and wrist (together with the distal portions of associated long bones) were obtained at the ages of 2 and 6 months. Radiographic diagnoses were based on the Koo score**.**

In infants treated with somatostatin because of chylothorax serum calcium, inorganic phosphorus, alkaline phosphatase, osteocalcin, parathyroid hormone was determined before and after treatment.

4.2. Identification of genetic polymorphisms

Genetic analysis for the polymorphisms of the VDR, ER and COLIA1 genes was performed using genomic DNA isolated from EDTA-treated peripheral blood.

To determine vitamin-D-receptor polymorphisms, polymerase chain reaction (PCR) was used to amplify the 740-base-pair region of exon 9 known to contain a constant and a polymorphic TaqI endonuclease site. PCR products were digested with TaqI

endonuclease and resolved by agarose gel electrophoresis. Restriction fragment length polymorphisms (RFLP) were coded TT, homozygotes, absence of the Taq I restriction fragment sites, Tt, heterozygotes, tt, homozygotes and presence of the polymorphic size.

To identify estrogen receptor dinucleotide repeat polymorphisms, PCR was performed using oligonucleotide primers designed to amplify the polymorphic [thymine-adenine $(TA)_n$] repeat of the human ER gene at 1174-base-pair upstream (15). The number of $(TA)_n$ repeats in each amplified products was determined by the comparison of the length of PCR products to the sequence ladder of control DNAs.

Collagen Iα1 gene genotype was determined after restriction of endonuclease digestion with BALI. The genotype was classified as CC, homozygotes, absence of the restriction site resulting in one fragment 255 bp, Cc, heterozygotes exhibiting fragments of 255 bp, 236 bp and 19 bp, and cc, homozygotes, presence of the restriction site results in two fragments of 236 bp and 19 bp.

5. Results

5.1. Changes in biochemical parameters depending on metabolic bone disease

Bone disease was diagnosed in 30 out of 104 VLBW infants (28.8 %) based on serum alkaline phosphatase, osteocalcin, parathyroid hormone, urinary pyridinium cross link, and calcium excretion. Radiological signs were present in all cases except for two twin pairs, who all had unambiguously abnormal biochemical parameters. Radiological abnormalities were generally noted in the second postnatal month; improvement in radiological signs was generally observed at the age of 6 months. Radiological abnormalities were grade 1 or 2 on the Koo score. No infants had bone fractures.

At the age of 1 month, infants with bone disease had significantly lower serum phosphorus values (1.96 ± 0.04 vs. 2.27 ± 0.04 mmol/l, $p < 0.05$) and significantly higher alkaline phosphatase levels (920 ± 51 vs. 709 ± 23 IU/l, $p < 0.001$) than infants without bone disease. Alkaline phosphatase values remained high during the entire study period (Fig. 1). Osteocalcin levels, also markers of bone formation, were significantly higher at all ages (Fig. 2). The differences in the urinary pyridinium crosslinks levels, markers of bone resorption, were the most striking at ages of 2, 3, 6 and 12 months (Fig. 3). We detected significantly higher urinary calcium excretion

(expressed as mmol/mmol creatinine) at ages of 2, 3, 6 and 12 months in infants with bone disease (Fig. 4). Parathyroid hormone levels were significantly higher in the bone disease group at ages of 2, 3, 6 and 12 months. There was no significant difference between groups in serum calcium and magnesium levels.

At ages of 6, 24 and 36 months significant differences in weight and length were revealed between the two groups. Infants not suffering from bone disease were heavier and taller.

At the age of half, two and three years of age we determined the serum estradiol levels. In infants diagnosed with bone disease lower estradiol values were found.

5.2. Identification of clinical and genetic risk factors

Clinical risk factors: Investigating the influence of gestational age, birth weight, birth height, gender, CRIB score (a marker of illness severity), duration of hospitalization, 1 min and 5 min Apgar scores, maternal parity as possible clinical risk factors, male gender ($p < 0.001$), high CRIB score ($p < 0.05$), duration of hospitalization ($p=0.05$) and high maternal parity ($p < 0.05$) were found to correlate with bone disease (Table 1).

Genetic factors:

The genotypic distribution of $(TA)_n$ dinucleotide repeat polymorphism in the first exon upstream of the estrogen-receptor α (ESR1) gene, the Taq 1 polymorphism in the exon of the VDR gene as well as G-to-T polymorphism in the first intron of the COLIA1 gene in 65 VLBW infants are shown in Table 2. Examining the distribution of the VDR gene and COLIA1 gene polymorphisms separately, we could not observe any significant difference (Table 2). However, a statistically significant correlation between $(TA)_n$ repeat allelic variant and bone disease was observed. According to the distribution pattern of $(TA)_n$ alleles, we divided the infants into three groups: (i) group HoH including homozygous alleles with a high number of $(TA)_n$ repeats [$(TA)_n > 18$]; (ii) group HeHL including heterozygous alleles with a high and a low number of $(TA)_n$ repeats; (iii) group HoL including homozygous alleles with low numbers of $(TA)_n$ repeats [$(TA)_n < 19$]. Infants in the HoL group suffered significantly more often from bone disease ($p < 0.01$). In contrast, infants with a high number of $(TA)_n$ repeats in both alleles were protected against bone disorder ($p < 0.01$). Using a logistic regression forward stepwise analysis this correlation between bone disease and HoH group members was shown to remain significant.

To analyze the combined influence of polymorphisms in these three candidate genes in determining bone disease, we performed a logistic regression analysis. The genotype combinations found most commonly are listed in Table 5. We observed significant interaction ($p < 0.05$) between VDR and COLIA1 genotype effects. The common occurrence of the heterozygote Tt RFLP of the VDR gene and CC polymorphism of the COLIA1 gene was a protective factor with regard to developing bone disease (OR: 0.05, 95% CI: 0.005 - 0.55) (Table 4). Logistic regression forward stepwise analysis revealed this interaction as an independent factor in developing bone disease.

The association of the above mentioned Tt or CC polymorphism with homozygous carriers of a high number of $(TA)_n$ repeats (HoH allele) was found to be overrepresented in infants without bone disease ($p < 0.05$). In spite of this, the association of Cc polymorphism with homozygous carriers of a low numbers of $(TA)_n$ repeats (HoL allele) was correlated with bone disease ($p = 0.01$).

Investigating clinical and genetic risk factors using multivariate analysis, male gender, duration of hospitalization, the $(TA)_n$ polymorphism of the ER gene, interaction between VDR and COLIA1 and VDR and ER gene remained as risk factors (Table 4).

5.3. Changes in biochemical parameters after treatment with somatostatin

In term infant no changes were revealed in laboratory findings before and after treatment with octreotide. All values were within the physiological range.

Slightly elevated ALP, OC values and higher urinary calcium excretion were determined in the premature infant.

6. Discussion

6.1. Metabolic bone disease of VLBW infants

With a better survival rate of VLBW infants, the importance of investigating the organic and functional abnormalities of the children surviving has been increasing. The analysis of risk factors for one of these abnormalities, bone disease, is of special significance for two reasons: 1) osteoporosis in later life is a public health challenge; and 2) a better understanding of the natural course of bone abnormalities would result in preventive measurements and provide a base for accurate prognosis.

In our study, 30 infants (28.8 %) were diagnosed with bone disease. This rate is in agreement with the reported incidence of bone disease in VLBW infants.

This study was conducted primarily to investigate the influence of genetic factors on bone disease in prematurely born infants. The results of our study indicate that bone disease in preterm infants is associated with certain genetic factors, namely, the $(TA)_n$ polymorphism of the ERα gene and the locus interaction between VDR and COLIA1 genes which may influence the development of bone disease. The molecular mechanisms responsible for this observation are not yet known. $(TA)_n$ polymorphism may directly affect gene expression through transcription regulation or may be linked with other exonic polymorphisms regulating ESR1 protein function directly. At least three different promoters have been identified in the ESR1 gene. The different $(TA)_n$ dinucleotide region lies between promoter A and B. It is possible that the different length of this polymorphism might have physiological relevance by affecting promoter usage.

The locus interaction between VDR and COLIA1 genes observed by logistic regression analysis is worthy of discussion. Few studies have addressed the interaction between the different osteoporosis candidate genes. An interaction between VDR and COLIA1 gene as well as an association between VDR and ESR1 gene may play a role at least in print in the pathogenesis of fractures. Examining the VDR and COLIA1 genotypes separately, we found no significant difference in genotype distribution between infants with and without bone disease.

However, we observed a significant association between VDR and COLIA1 genotype. The common occurrence of CC genotype (absence of the thymine allele) of the COLIA1 gene and Tt genotype (heterozygotes) of the VDR gene protected the infants from developing bone disease. VDR is a steroid transcription factor and regulates the expression of the COLIA1 gene. Genetic variations in the VDR gene can be expected to influence the effects of COLIA1 gene polymorphisms in regard to bone disease.

Among clinical risk factors logistic regression analysis revealed that infants with a longer hospitalisation suffer more often from metabolic bone disease. Longer hospitalisation means long-time medical treatment (corticosteroids, diuretics, parenteral nutrition), severe diseases (ventilation, BPD. NEC) resulting in elevated csont-turnover.

Furthermore, our results indicate that boys are more likely to be affected with bone disease than girls. This may be related to the observation that the estrogen level in

VLBW preterm boys is lower than in girls. In adults, common allelic variants of ESR1 gene are related to variation in responsiveness to estrogen. It has been proposed that compensatory hyperestrogenism can regulate this relative resistance and may be disturbed in menopause leading to osteoporosis. Thus, lower estrogen levels may predispose boys to bone disorders.

In conclusion, this study shows that the development of bone disease in VLBW infants is related to clinical factors such as gender and duration of hospitalisation.

We have demonstrated an association in VLBW infants between bone disease and certain genetic factors. The $(TA)_n$ polymorphism of the ERα gene determines the development of bone disease. The locus interaction between the VDR and COLIA1 genes may play an important part in the protection of preterm infants from bone disease.

Primer prevention and successful treatment of metabolic bone disease lead to decreased numbers of pathological fractures and later complications, such as short stature and severe osteoporosis in adulthood.

6.2. Effects of long-lasting somatostatin analogue on bone-turnover

In critically ill infants treated with drugs affecting bone-turnover metabolic bone disease may develop. We studied the effect of octreotide on bone-turnover in two cases suffering from chylothorax. The goal of our examination was to determine whether one week treatment with octreotide may influence bone metabolism. In term infant no differences were revealed in biochemical parameters before and after treatment. Slightly elevated bone formation and bone resorption parameters were determined in the premature infant, which is characteristic at this stage of development and probably is not related to the treatment applied. Our results indicate that short–time treatment with octreotide did not influence bone-turnover in newborn infants.

7. Thesis

1. In our study 30 infants (28.8%) were diagnosed with metabolic bone disease. This rate is in agreement with the reported incidence of bone disease in VLBW infants. Our results indicate that the diagnosis based on bone formation and bone resorption markers is reliable. It is not necessary to perform X-ray examination. At the age of one month significant differences were revealed among infants with

and without bone disease, which is in agreement with previously reported data. I propose to start screening for bone disease VLBW infants at the age of one month. It is suggested to evaluate the efficacy of the treatment weekly with determination of bone resorption markers in the urine. Initially bone formation markers in the blood should be detected monthly. After effective treatment bone disease of prematurity will be resolved within 2-3 years. At 3 years of age DEXA should be performed.

2. Among risk factors male gender and duration of hospitalization are significant. High maternal parity and high CRIB score also correlate with bone disease. Early preventive treatment of VLBW infants having more than one risk factors is strongly suggested. Physical activity intervention should be started as early as possible, 2-3 times daily. Determination of biochemical parameters of bone metabolism at two weeks of age can be helpful to assess presence and severity of bone disease.

3. Our results indicate that bone disease in premature infants is also associated with certain genetic factors. The genotype analysis is especially important in infants without any clinical risk factors. The occurrence of certain genotype may predispose the VLBW infant to develop bone disease.

4. Premature infants identified with higher risk to develop metabolic bone disease should receive appropriate treatment to prevent severe bone disease as early as possible.

5. It is important to realize that long-term treatment with drugs affecting bone-turnover may affect not only premature infants but term infants also. In such cases biochemical parameters should be monitored throughout the treatment. Our results indicate that short-term treatment with octreotide does not affect bone metabolism in neonates.