PATHOGENETIC STUDIES OF VESICOURETERIC REFLUX, JUVENILE ESSENTIAL HYPERTENSION AND UREMIC HEMOLYSIS

Summary of Ph.D. Thesis

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2004

1. INTRODUCTION

1.1. Mutations of the renin-angiotensin system genes and renal scarring in primary vesicoureteral reflux

Primary vesicoureteric reflux (VUR) is a common familial disorder, but its genetic background has not been clarified. Primary VUR appears to be a dominant disorder with incomplete penetrance and variable expression. Previous studies have suggested a urinary tract malformation locus on chromosome 6p on the basis of a balanced translocation which was found in a patient with multicystic renal dysplasia and linkage of VUR families to HLA markers on 6p. VUR can be a part of a complex syndrome such as the renal-coloboma syndrome, which is caused by mutations of PAX2 on chromosome 10q. In the first genomwide search of VUR no significant linkage was found to the previously reported VUR candidate loci, including 6p and 10q. Whereas a 20 cM locus on the short arm of the chromosome 1 was identified as a possible locus of primary VUR with a number of candidate genes.

Reflux nephropathy (RN) is defined by the presence of renal scars in children with VUR. Renal scarring secondary to VUR, which is called RN, has been affected by genetic and environmental factors. It has been reported that adequate medical management of urinary tract infections (UTI) and follow-up of children with VUR could significantly reduce the incidence of nephropathy. RN is an important cause of hypertension, and chronic renal failure (CRF) in children. RN accounts for up to 25 % of children and young adults with CRF. Shimada et al reported that most children who developed renal insufficiency due to RN have either two small kidneys or one small kidney and a contralateral large kidney with severe scars. Mackie and Stephens proposed that renal dysplasia and/or hypoplasia related to abnormal development of the embryonic ureteral bud, was the basis for some pathology associated vesicoureteral reflux (VUR). Current knowledge indicates that the evolution of VUR is not equal in all patients, suggesting the influence of

different factors including genetics. The RAS has been postulated to play an important role in the regulation of glomerular sclerotic processes as well as in renal development. Thus, genes encoding components of the RAS have attracted much interest as risk factor of renal scarring. The insertion/deletion (I/D) polymorphism in the angiotensin-converting enzyme (ACE) gene is located on chromosome 17. Three possible genotypes are determined by the absence (deletion, D) or presence (insertion, I) of a 287-base pair DNA fragment at intron 16: DD, ID, II. The deletion allele is associated with higher ACE plasma levels. An association between I/D polymorphism of the angiotensin converting enzyme (ACE) and scar formation in different nephropathies (IgA nephropathy, diabetic nephropathy, autosomal dominant polycystic kidney disease, focal glomerulosclerosis and finally, reflux nephropathy) were reported. Generally the DD genotype of ACE was correlated with the development of the diseases or the decline of renal function in these pathological states. Other gene polymorphisms of the RAS such as the M235T polymorphism of the angiotensinogen (ATG) gene or the A1166C polymorphism of the angiotensin II type 1 receptor (AT1) gene are supposed to be involved in renal scar formation.

1.2. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children

In human essential hypertension, endothelial dysfunction has been documented in peripheral and coronary macro- and microcirculation and in renal circulation. The mechanism appears to be the activation of an alternative pathway involving cyclooxigenase, which reduces NO availability through production of oxidative stress. Reduced NO availability can increase the biological activity of endothelin-1 (ET-1). Besides the endothelial damage platelet hyperactivity may also play a role in the vascular complications of essential hypertension. Nitric oxide not only causes vessel relaxation but also inhibits platelet aggregation, smooth

Musclecell proliferation, monocyte adhesion, adhesion molecules expression, and ET-1 production which is a potent vasoconstrictor and has inotropic a mitogenic properties.

During hypertension very important alterations have been shown in rheological, mechanical and biochemical characteristics of erythrocytes and of blood flow. In hypertensive adults Cicco et al found a decrease in erythrocyte deformability, in erythrocyte aggregation time, an increase of shear rate to disaggregate erythrocytes and in fibrinogenaemia, a decrease in cellular oxygen delivery and tissue oxygenation and an impairment of microcirculation. These patterns were not related with the age of the patients but with the patient hypertension age. Carroll et al demonstrated that obesity and poor cardio respiratory fitness might be important in the development of haemorheological abnormalities associated with metabolic syndrome. Increased plasma viscosity is associated with the increased clustering of metabolic markers in middle-aged people.

Most of non-genetic predisposing factors for adult essential hypertension - like smoking, atherosclerosis, hyperlipidemia, diabetes, and alcohol consumption - are absent in paediatric population. Therefore the pathogenetic role of platelets, nitric oxide, and endothelin can be more precisely investigated in adolescent hypertensive patients. Nevertheless the occurrence of obesity in the general and also in paediatric population has been extensively growing, and its role in juvenile essential hypertension (JEHT) has not been cleared yet.

In our previous study platelet aggregation and the thromboxane B2 (TxB2) level were significantly higher while the plasma total cholesterol, triglyceride and LDL cholesterol concentrations were normal in non-obese children with JEHT. Obesity and hypertension are double risks for cardiovascular morbidity in adults. Beside high blood pressure, platelet aggregation, blood and plasma viscosity, plasma lipid concentrations and endothelial nitric oxide (NO) production are frequently investigated factors in adult hypertension.

1.3. The effects of vitamin E on uraemic erythrocytes in the early period of erythropoietin treatment

Anaemia is a common feature of chronic renal failure (CRF), particularly with chronic haemodialysis (HD), and causes a significant morbidity. An erythropoietin (EPO) deficiency and hypoproliferative bone marrow are the principal factors of the anaemia. A further important contributory phenomenon is the shortened red blood cell (RBC) survival, which may be due to circulating plasma factors accumulating during the uraemic state. Additionally, a growing body of evidence has accumulated indicating that a disturbance of the balance between oxidative stress and antioxidant defence mechanisms plays a major role in the pathomechanism of oxidative haemolysis of chronic uraemic patients undergoing chronic HD therapy. The plasma lipid peroxidation, malonyl dialdehyde (MDA) production and haemoglobin (Hb) oxidation are increased, and the activities of antioxidant enzymes [superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (Gpx)] are decreased. The vitamin E concentration in the erythrocytes is low and vitamin C is depleted during HD. The increased MDA production results in changes in cation permeability and a reduced deformability of the RBCs. During HD therapy the availability of free oxygen radicals (FOR) originating from activated neutrophils is increased. The production of reduced glutathione (GSH), one of the main scavenging materials of FOR, is decreased. Furthermore, the activity of the RBC hexose monophosphate shunt, which is responsible for GSH regeneration from oxidized glutathione (GSSG), is depressed. All these factors make the free radical scavenging system ineffective.

In a previous paper we reported on the early effect of rh EPO therapy on the changes in the glutathione redox system and Hb oxidation causing haemolysis in 10 children with CRF undergoing chronic HD. A rapid increase in the reticulocyte count was accompanied by a slower rise in total Hb concentration. The mean level of GSSG was increased from 13.2±5.3

nmol/g Hb to 56.7±15.8 nmol/g Hb 4 weeks after the start of rh EPO ($p<0.001$), followed by a fall to the basal value. The GSH levels showed a smaller though constant elevation during rh EPO therapy ($p<0.001$). Before rh EPO treatment, incubation of RBCs for 1 h with acetylphenylhydrazine induced a decrease in GSH concentration as compared with the controls ($p<0.001$), which became more pronounced in the first few weeks of rh EPO therapy ($p<0.001$). In addition, the levels of Hb derivatives (metHb and haemichrome) increased in the first 4 weeks of rh EPO treatment ($p<0.001$). Although there was no significant difference between the values before and during EPO treatment, the MDA levels were continuously higher and the SOD, Cat and Gpx activities were lower than in the control ($p<0.001$). These results were compatible with oxidative damage to the RBCs in the early period of rh EPO therapy in children with end-stage renal failure. The GSH-GSSG system as an important cellular defence mechanism of the RBCs appears to be severely affected.

2. AIMS OF THE STUDY

The objectives of this study were to investigate

2.1. Mutations of the renin-angiotensin system genes and renal scarring in primary vesicoureteral reflux

2.1.1. Is there any role of ACE gene *I/D* polymorphism in the occurrence of VUR?

2.1.2. Is there any difference in the M235T polymorphism of the angiotensinogen (ATG) gene between VUR patients and controls?

2.1.3. Is there any contribution of A1166C polymorphism of the angiotensin II type I receptor *(AT1)* gene to the development of primary VUR?

2.1.4. Do these ACE, ATG and AT1 gene mutations contribute to the progression of VUR associated renal scarring?

2.2. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children

2.2.1. How obesity and hypertension can separately and in combination influence platelet aggregation, blood and plasma viscosity in children?

2.2.2. Are there any changes in plasma lipid levels in obese (O) and obese hypertensive (OHT) patients at this age?

2.2.3. How endothelial NO change in essential hypertension (EHT), O and OHT as compared with the controls.

2.3. The effects of vitamin E on uraemic erythrocytes in the early period of erythropoietin treatment

2.3.1. The aim of the present study was to investigate the effects of vitamin E on the glutathione redox system.

2.3.2. We studied the effect of vitamin E and erythropoietin combined therapy on the Hb oxidation and the changes in haematocrit (Htc) and Hb in the same patients.

3. PATIENTS

3.1. Mutations of the renin-angiotensin system genes and renal scarring in primary vesicoureteral reflux

77 children with VUR and 80 healthy controls were studied. 38 patients had low grade (I_III) VUR and 39 patients had high grade (IV-V) or severe VUR. Renal scarring was found with ^{99m}Tc-DMSA scan in 43 VUR patients.

3.2. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children

32 patients (mean age: 14.4±3.1 years, male/female 17/18) with juvenile essential hypertension (JEHT) 17 of them with >25 body mass index (BMI kg/m^2 obese hypertension, OHT), 15 with <25 BMI (essential hypertension, EHT) and 35 age and sex matched controls (15 with >25 BMI (obese, O) and 20 with <25 BMI, C) were investigated.

3.3. The effects of vitamin E on uraemic erythrocytes in the early period of erythropoietin treatment

This study included 10 children with chronic renal failure (5 boys and 5 girls, age range 7-17 years). The patients had been on 4-h bicarbonate hemodialysis (HD) (3 times a week) for 2-6 years. Following 1 year of rh EPO therapy (supported by CILAG) 4 weeks break was introduced because of a lack of further supply by the National Insurance Company. From the restart of rhEPO treatment the same dosage was used but from the 3rd week the treatment was completed with vitamin E.

4. METHODS

4.1. Mutations of the renin-angiotensin system genes and renal scarring in primary vesicoureteral reflux

Genomic DNA was isolated from peripheral blood leucocytes by a standard phenol/chloroform method. The I/D polymorphism in intron 16 of the ACE gene was determined according to the previously method of Chiu et al. using polymerase chain reaction.

The M235T polymorphism in exon 2 of the ATG gene was detected by single-step LightCycler technology published by Malin et al.

Determination of the A1166C polymorphism of the AT1 gene was also carried out with LightCycler technology according to the detection of the M235T polymorphism, which was optimized for the analysis of A1166C polymorphism by us.

4.2. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children

Platelet aggregation was carried out with a laser rheoaggregometer using collagen stimulation at a final concentration of 2 µg/ml platelet rich plasma by the method of Jung et al.

Measurement of whole blood and plasma viscosity was carried out in a Hevimet-40 viscosimeter.

Nitric oxide end-products in the plasma were determined as nitrite and nitrate imultaneously by anion-exchange HPLC.

Plasma lipid peroxides were quantified as malondialdehyde (MDA)-thiobarbituric acid adducts by HPLC.

Plasma free thiol (SH) groups were assayed with 5.5-dithiobis (2-nitrobenzoic acid) at 412 nm.

Plasma cholesterol and triglicerides were assayed using standard methods.

4.3. The effects of vitamin E on uraemic erythrocytes in the early period of erythropoietin treatment

GSH and GSSG determinations were carried out after hemolysis of two samples of whole blood collected with EDTA by the method of Tietze.

Carbonmonoxide-Hb (COHb), as an indirect measure of hemolysis, was estimated in heparinized blood with a hemoximeter (Radiometer Copenhagen. Denmark) and expressed as a percentage of the total Hb.

5. RESULTS

5.1. Mutations of the renin-angiotensin system genes and renal scarring in primary vesicoureteral reflux

We found the significant over-representation of the DD genotype in patients with renal scarring (44%) compared to normal controls (22.5%; $P<0.05$) and patients with no scar formation (21%; $P<0.05$). A significantly higher D and significantly lower I allele frequencies were presented in VUR patients with scarred kidneys (D allele: 0.64 and I allele: 0.36) compared to controls (D allele: 0.53 and I allele: 0.47; $P<0.05$) and patients with unscarred kidneys (D allele: 0.4 and I allele: 0.6; $P<0.05$). No differences in the ATG and AT1 genotype distributions and allele frequencies were observed in VUR patients compared to normal population. The DD genotype and D allele of ACE may be a genetic susceptibility factor contributing to scar formation in VUR. There is no linkage of genetic polymorphisms of ATG and AT1 to VUR and VUR associated renal scarring.

5.2. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children

The plasma cholesterol was significantly higher in OHT and O groups as compared to C and trigliceride was higher in OHT also ($p<0.05$). In vitro platelet aggregation was significantly higher in OHT, EHT and O as compared to controls ($p<0.001$). A significant positive correlation was observed between the BMI values and the platelet aggregation ($r=0.47$, $p<:0.05$). NO level was significantly lower in EHT and OHT as compared to controls ($p<0.01$). A significant negative correlation was observed between the results of platelet aggregation and NO levels ($r=-0.53$ and -0.504 for EHT and OHT groups respectively). Plasma MDA was significantly high in OHT, and free thiols were low in OHT ($p<0.001$) and EHT groups ($p<0.01$). Blood viscosity was higher in OHT ($p<0.01$), and plasma viscosity was higher in OHT ($p<0.01$) and O ($p<0.05$) as compared to controls.

5.3. The effects of vitamin E on uraemic erythrocytes in the early period of erythropoietin treatment

The Hb concentration was significantly higher than the initial level 2 weeks after the introduction of vitamin E. A significant rise in Hb level was observed only in the 8^{th} week, when they were treated with rh EPO alone. Rh EPO + vitamin E resulted in a more rapid increase in the Htc too, which was significant in the 4^{th} week (2 weeks after the start of vitamin E), as compared with only the 5^{th} week with rh EPO alone. The mean level of oxidized glutathione increased from 10.9±3.1 nmol/g Hb (x±SD) to 26.7±5.7 nmol/g Hb 2 weeks after the start of rh EPO, but decreased significantly 1 week after vitamin E introduction, 10.1±4.9 nmol/gHb ($p<0.001$), and remained at this level subsequently. The reduced glutathione (GSH) level displayed a smaller elevation ($p<0.05$), but was also decreased significantly 1 week after the introduction of vitamin E ($p<0.05$). Similar changes were observed in the percentages of carboxy Hb and met Hb ($p<0.01$).

6. CONCLUSIONS

6.1.1. There was a significant over-representation of the DD genotype in patients with renal scarring compared with normal controls and patients with no scar formation.

6.1.2. Significantly higher D and significantly lower I allele frequencies were present in VUR patients with scarred kidneys compared with controls and patients with unscarred kidneys. The DD genotype and D allele of ACE may be a genetic susceptibility factor contributing to scar formation in VUR.

6.1.3. No differences in the ATG and AT1 genotype distributions and allele frequencies were observed in VUR patients compared with the normal population. We detected no linkage of genetic polymorphism of ATG and AT1 to VUR and VUR-associqated renal scarring.

6.2.1. Obese children (hypertensive and controls) had significantly higher concentrations of total cholesterol and triglicerides.

6.2.2. The levels of high density lipoprotein (HDL)-cholesterol were lower in obese hypertensive children than their non-obese counterparts.

6.2.3. There was a significant increase in platelet aggregation and a decrease in NO levels in hypertensive patients (obese and non-obese) reflecting a significant negative correlation. An increased tendency to aggregation was also evident in obese normotensive patients. A significant positive correlation was observed between the platelet aggregation and BMI.

6.2.4. Plasma free thiols were decreased in hypertensive children independent of their BMI.

6.2.5. An increased lipid peroxidation and higher blood and plasma viscosity were found only in obese patients with hypertension.

6.2.6. Multivariate analysis revealed significant interactions in the effects of obesity and hypertension on platelet aggregation and thiol oxidation. In obese children an increased platelet aggregation and oxidative insult contribute to the development of hypertension and to the promotion of vascular damage.

6.3.1. In uremic children with chronic haemodialyisis the rhEPO and vitamin E combined therapy resulted in a significant decrease in the high ratio of GSSG/GSH concentration indicating an oxidative stress at the beginning of rhEPO therapy.

6.3.2. The level of COHB also decreased following the introduction of vitamin E during rhEPO treatment. This indicates a hemolysis that could be prevented by vitamin E.

6.3.3. A significant increase in Hb and hematocrit was achieved within 2 weeks of starting the combined therapy, while similar results occurred only at 8^{th} and 5^{th} weeks without vitamin E. Antioxidant vitamin E supplementation improved the therapeutic effect of rhEPO in patients with chronic renal failure on hemodialysis.

LIST OF PUBLICATIONS

List of publications included in the thesis

1. Németh I., Túri S., **Haszon I.**, Bereczki Cs.: Vitamin E alleviates the oxidative stress of erythropoietin in uremic children on hemodialysis
   Pediatric Nephrology 2000 14: 13-17.

2. **Haszon I,** Friedman A, Papp F, Bereczki Cs, Baji S, Bodrogi T, Károly É, Endreffy E, Túri S.: ACE gene polymorphism and renal scarring in primary vesicoureteric reflux
   Pediatric Nephrology 2002. 17: 1027-1031.

3. **Haszon I,** Papp F, Bors M, Bereczki Cs, Túri S: Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children.
   European Journal of Pedaitrics 2003. 162: 385-390

List of publication not closely related to the subject of the thesis

1. Túri S., **Haszon I.**, Bodrogi T.: Plasma exchange and immunosuppressive therapy in a paediatric patient with systemic lupus erythematosus.
   Acta Paed. Acad.Sci. Hung. 1989, 29, 293-298.

2. Túri S., Nagy J., **Haszon I.**, Havass Z., Németh M., Bereczki Cs.: Plasma factors influencing PGI2-like activity in patients with IgA nephropathy and Henoch - Schönlein purpura.
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7. F. Papp, A. Friedman, Cs. Bereczki, **I. Haszon**, É. Kiss, E. Endreffy, S. Túri: Renin angiotensin gene polymorphism in children with uremia and essential hypertension
   Pediatric Nephrology 2003 18:150-154.