Examination of glucocorticoid sensitivity in patients with adrenal tumors using biochemical and molecular biologic markers

Ph.D thesis

Dr. Judit Majnik

Semmelweis University
School of Ph.D Studies

Supervisor: Dr Károly Rácz, professor, D.Sc

Opponents: Dr Csaba Barta, assistant professor, Ph.D
Dr Péter Gergely, assistant professor, Ph.D

Final exam Committee:
Head: Dr Béla Szende, professor, D.Sc
Members: Dr András Váradi, scientific advisor, D.Sc
Dr Sándor Alföldi, associate professor, Ph.D

Budapest, 2006.

1. INTRODUCTION

The physiologic answer to glucocorticoids, and glucocorticoid sensitivity can vary widely among different species, individuals, tissues and cell types, and even in the same cell during cell cycle. Human studies have shown large variability of plasma cortisol concentration among healthy individuals, which suggests the possibility of genetic influence on the feedback sensitivity of the hypothalamo-pituitary-adrenal (HPA) axis.

Genetic variants of the glucocorticoid receptor (GR) can have an impact on receptor function; some GR gene polymorphisms frequently present in the healthy population are associated with a susceptibility to metabolic disorders. N363S polymorphism has been shown to increase receptor sensitivity and to be associated with obesity, tendency to lower bone mineral density, coronary artery diseases, higher cholesterol level, and increased cortisol response to stress. ER22/23EK polymorphism has been shown to decrease receptor sensitivity, and to be associated with lower insulin concentration and smaller insulin resistance, lower serum cholesterol level, increased body height and muscle strength in men, longer survival, lower CRP concentration in the elderly and less white matter lesion and dementia.

The majority of adrenal tumors are non-hyperfunctioning adrenal adenomas, which are mainly diagnosed incidentally during abdominal imaging. Their incidence is between 0,34-4,36%. These

incidentalomas are usually characterized by the absence of hormonal alterations. Nevertheless, metabolic abnormalities which are typically related to increased glucocorticoid effect, are very common in these patients. Among incidentally discovered adrenal masses, the proportion of bilateral tumors is unexpectedly high, ten fold higher than what would be statistically expected. This suggests the possibility of systemic effects, perhaps genetic alterations, in the background of bilateral adenoma formation.

Bone metabolism is influenced in several ways by glucocorticoid hormones. A large number of serum bone marker tests has been developed, which can detect changes in bone metabolism much faster than bone mineral density measurement. These serum markers are nowadays widely used in routine laboratory diagnostics. Several papers have studied the short term effect of glucocorticoids on serum bone markers, but the change of marker concentrations during dynamic testing of the HPA axis has never been previously examined.

2. OBJECTIVES

During my PhD studies at the 2nd Department of Medicine of Semmelweis University, I performed clinical, biochemical and molecular biological studies in patients with adrenal incidentalomas and in control subjects, to study certain aspects of glucocorticoid sensitivity. My objectives were:

1. To develop a fast, simple and reliable method for the detection of the most frequently studied polymorphism of the glucocorticoid receptor gene, N363S, which makes the determination of N363S genotype available in large number of samples.

2. To determine the allele-frequency of GR N363S and ER22/23EK polymorphisms in patients with hormonally inactive adrenal tumors and in healthy controls. My aim was to examine whether the N363S polymorphism that increases glucocorticoid sensitivity, or the ER22/23EK polymorphism that decreases glucocorticoid sensitivity could play a role in the pathomechanism of these adrenal tumors. Within this research, an emphasis was set on the examination of patients with bilateral adrenal tumors, whose pathogenesis suggests the role of possible systemic factors influencing both adrenal glands.

3. To study the possible association of GR N363S and ER22/23EK genotypes with metabolic abnormalities in patients with non-hyperfunctioning adrenal adenomas. My objective was to determine whether the polymorphisms that increase or decrease glucocorticoid sensitivity could be involved in the pathogenesis of metabolic alterations frequently associated with these tumors.

4. To determine the allele-frequency of GR N363S and ER22/23EK genotypes in type 2 diabetes mellitus (which is also frequently associated with other metabolic abnormalities), and to study whether

these polymorphisms could be associated with the presence of type 2 diabetes mellitus.

5. To study the effect of dynamic tests (dexamethasone, ACTH and metyrapon tests) currently used in the assessment of the HPA axis on the concentration of serum osteocalcin and β-Crosslaps concentrations, with the aim of determining and comparing a rapid glucocorticoid effect on bone in patients with non-hyperfunctioning adrenal tumors and in patients with Cushing's syndrome.

3. PATIENTS AND METHODS

Patients involved in the validation of the new PCR method:

302 patients (84 men and 217 women, age range: 19-84 years) treated at the 2nd Department of Medicine of Semmelweis University.

Patients involved in the study of glucocorticoid receptor gene polymorphisms:

-143 patients with non-hyperfunctioning adrenal adenomas (99 patients with unilateral tumors, among them 75 women and 24 men, age range 22-78 years; and 44 patients with bilateral tumors, among them 30 women and 14 men, age range: 34-74 years)

-102 control subjects (65 women and 37 men, age range: 28-79 years)

-100 patients with type 2 diabetes, in whom no sign of HPA axis abnormality could be observed (42 women and 58 men, age range: 28-82 years).

Patients involved in the study of bone markers:

-40 healthy subjects (35 women and 5 men, age range: 18-69 years),

-49 patients with non-hyperfunctioning adrenal adenomas (34 women, and 15 men, age range: 19-77 years)

-8 patients with Cushing's syndrome (3 women and 5 men, age range 19-70 years), among them 5 with adrenal, and 3 with pituitary origin.

Molecular biologic methods:

-DNA isolation from peripheral blood leukocytes,

-for the detection of the N363S polymorphism, in addition to the RFLP described in the literature, we developed a new allele-specific PCR method,

-for the detection of the ER22/23EK polymorphism, we used the PCR-RFLP method described in the literature,

-both polymorphisms were verified by direct sequencing.

Routine laboratory and hormone laboratory measurements

were carried out according to the protocols used at the 2nd Department of Medicine.

Bone marker measurements:

Measurement of serum OC and β-CTx was carried out with electrochemoluminescens immunoassay. The osteocalcin assay measured both the intact molecule and the N-mid fragment, the β-Crosslaps assay recognized an 8 amino acid fragment of the C-terminal isomerized telopeptid of type I collagen.

Measurement of bone mineral density

was carried out with DEXA method on the lumbar spine and femoral neck.

Statistical analysis included:

-χ2–test and Fischer exact test

-Student t-test and Mann-Whitney U-test

-one-way ANOVA and Dunnet's post-hoc test

Softver used: SPSS 12.0

Level of significance: $p<0,05$.

4. RESULTS

1. Development of a new, allele-specific PCR method

For the detection of N363S polymorphism in large number of samples, we developed a new method that is fast, cost-saving and easy to apply. In brief, using the new method the presence or absence of the polymorph allele can be detected with one simple PCR reaction, containing two control primers and a primer specific for the polymorphism. Afterwards, in the minority of positive samples, a second PCR reaction must be carried out to decide whether the polymorphism is heterozygous or homozygous. In the case of an average allele-frequency of 4%, the N363S genotype of 100 samples can be determined in 108 PCR reactions.

Apart from the cost-effectiveness, fast and easy applicability, further advantage of this method is that it does not require expensive instrumentation. Our results obtained with this allele-specific PCR showed 100% accuracy compared to previous methods. Therefore this new method can be recommended for widespread use.

2. Association of the GR N363S polymorphism with bilateral adrenal tumors

In the group of patients with bilateral adrenal adenomas, the carrier frequency for the N363S polymorph allele (20,5%) was significantly higher when compared both to control subjects (7,8%) and to patients with unilateral tumors (7,1%). The relative risk for the occurrence of bilateral adrenal adenomas in N363S carriers is 3,02 (95% CI, 1,04-9,45) when compared to subjects carrying no polymorph allele.

Carrier frequency of the ER22/23EK polymorphism did not show any difference between controls (4,9%) and patients with uni- or bilateral adrenal adenomas (6,2% and 4,5%).

Our result supports the hypothesis, that bilateral adrenal tumor formation can be related to mechanisms other than unilateral tumor formation, and it suggests the possible pathogenetic role of N363S polymorphism.

3. Association of the N363S polymorphism with altered glucose metabolism in patients with bilateral adenomas

Metabolic abnormalities (hypertension, increased blood lipid level, obesity, coronary artery diseases) are frequent in patients with both unilateral and bilateral adenomas, and altered glucose metabolism occurs more often in patients with bilateral than unilateral tumors. When examining the association of GR polymorphisms with metabolic abnormalities, we found that in patients with adrenal adenomas who are carriers of the N363S polymorphism, the frequency of altered glucose metabolism (DM or IGT) was significantly higher (93,8%) than in non-carriers (53,5%). When examined separately, we found a significant association between N363S polymorphism and altered glucose metabolism in patients with bilateral tumors: the frequency of DM or IGT was 57,1% patients with wild genotype, and 100% in carriers. On the contrary, in patients with unilateral adrenal adenomas, we found no significant association between the N363S polymorphism and altered glucose metabolism.

We found no association between N363S polymorphism and other metabolic abnormalities.

The ER22/23EK polymorphism did not show any association with altered glucose metabolism or other metabolic abnormalities.

4. Lack of association of GR gene polymorphisms with type 2 diabetes

In patients with type 2 diabetes mellitus without any sign of disruption of HPA axis function, the carrier frequency of the N363S and ER22/23EK polymorphisms (13% and 6%) did not differ significantly from the carrier frequency observed in controls and patients with adrenal tumors. We found no association between N363S and ER22/23EK polymorphisms and body mass index, hypertension or need for insulin therapy in patients with type 2 diabetes.

The lack of association between N363S polymorphism and type 2 diabetes underlies the fact, that the overrepresentation of N363S polymorphism in patients with bilateral adrenal adenomas cannot be explained by direct association between altered glucose metabolism and the N363S variant. Nevertheless, it cannot be entirely ruled out that the increased transactivating capacity of the N363S variant influences pathogenetic factors leading to both bilateral adrenal tumor formation and the development of altered glucose metabolism in these patients. Increased transactivating capacity of the glucocorticoid receptor may enhance transcription of target genes involved in both the formation of bilateral adrenal incidentalomas and the frequent occurrence of type 2 diabetes in these patients.

5. Change of bone maker concentrations during dynamic testing of HPA axis

Serum "baseline" osteocalcin concentrations in the morning did not show any difference between patients with hormonally inactive adrenal adenomas and controls (29,8±15,9 ng/ml és 28,3±12,2 ng/ml), whereas in patients with Cushing's syndrome serum morning OC (17,7±9,6 ng/ml) was significantly decreased when compared to both other groups. Serum baseline β-Crosslaps concentrations did not show any difference between the three groups.

After dexamethasone suppression test, serum OC concentration was significantly decreased both in healthy subjects (from 28.3±12.2 to 21.8±9.5 ng/ml) and in patients with hormonally inactive adrenal adenomas (from 29.8±15.9 to 24.1±14.1 ng/ml), whereas in patients with Cushing's syndrome, the low morning OC levels remained unchanged after dexamethasone administration. Serum β-Crosslaps concentration showed a subtle decrease after dexamethasone administration, but this change was not significant.

After ACTH administration, serum OC concentration were even more markedly decreased than after dexamethasone test both in healthy subjects (from 28.3±12.2 to 12.5±4.6 ng/ml) and in patients with adrenal incidentalomas (from 29.8±15.9 to 12.2±6.5 ng/ml). Serum β-Crosslaps concentration showed a subtle decrease after ACTH administration, but this change was not significant, either.

Inhibition of cortisol biosynthesis by metyrapon did not have an impact on serum OC and β-CTx concentrations.

Our results suggest, that by physiologic adrenal function, administration of a single small dose of exogenous steroid, or the acute stimulation of endogenous steroid formation exert a marked inhibitory effect on osteocalcin synthesis. These results confirm that the catabolic effect of glucocorticoids on bones is mainly related to the inhibition of osteoblast activity.

5. CONCLUSIONS

1. The new allele-specific PCR reaction developed to detect the N363S polymorphism of the glucocorticoid receptor gene is a reliable and accurate method for the detection of this variant. The new method is easy to apply, cost-saving with no need for expensive instrumentation, and it is suitable for testing large number of samples.

2. In patients with non-hyperfunctioning bilateral adrenal adenomas, carrier frequency of the N363S polymorphism of the glucocorticoid receptor gene is significantly higher than in control subjects from the same population, or in patients with unilateral adrenal tumors. These observations support the hypothesis, that this polymorphism could play a role in the pathogenesis of hormonally inactive adrenal adenomas, although the mechanism is unknown. In the case of ER22/23EK

polymorphism of the glucocorticoid receptor gene, such association could not be found.

3. In patients with hormonally inactive bilateral adrenal adenomas, the frequency of altered glucose metabolism is significantly higher than in patients with unilateral tumors. In patients with bilateral adrenal tumors, altered glucose metabolism is associated with the presence of the N363S polymorphism. Obesity, hyperlipidemia and hypertension do not show any association with the presence of N363S or ER22/23EK polymorphisms in these patients.

4. In patients with type 2 diabetes, the frequency of N363S or ER22/23EK polymorphisms does not differ from the frequency of these polymorphisms in control subjects. There is no association between N363S and ER22/23EK polymorphisms and the age at onset of diabetes, body mass index, hypertension or need for insulin therapy in these patients.

5. Among the dynamic tests currently used in the assessment of the hypothalamic-pituitary-adrenal axis, the low-dose dexamethasone and the ACTH test induce a significant decrease of serum osteocalcin concentration in healthy subjects and in patients with hormonally inactive adrenal adenomas. However, in patients with Cushing's syndrome, the low-dose dexamethasone does not influence serum osteocalcin concentration. The low-dose dexamethasone and ACTH test do not alter serum β-Crosslaps concentration in any groups examined. Administration of a single dose of metyrapon, an inhibitor of cortisol biosynthesis, does not have any impact on serum osteocalcin and β-Crosslaps concentrations. These results confirm that the catabolic effect of glucocorticoids on bones is mainly related to the inhibition of osteoblast activity.

6. LIST OF PUBLICATIONS

Publications directly related to the PhD thesis

1. Majnik J, Patocs A, Balogh K, Toth M, Gergics P, Szappanos Á, Mondok Á, Borgulya G, Panczel P, Prohaszka Z, Racz K. Overrepresentation of the N363S variant of the glucocorticoid receptor gene in patients with bilateral adrenal incidentalomas**.** J Clin Endocrinol Metab 91:2796-2799, 2006. **IF: 6,020.**

2. Majnik J, Szücs N, Patócs A, Tóth M, Balogh K, Varga I, Gláz E, Rácz K. Effect of single doses of dexamethasone and adrenocorticotrop hormone on serum bone markers in healthy subjects and in patients with adrenal incidentalomas and Cusing's syndrome. Journal of Endocrinological Investigation, 27:747-753, 2004. **IF: 1,525.**

3. Majnik J., Patócs A., Balogh K., Tóth M., Rácz K. A rapid and simple method for detection of Asn363Ser polymorphism of the human glucocorticoid receptor gene. Journal of Steroid Biochemistry and Molecular Biology, 92(5):465-468, 2004. **IF: 2,715**.

4. Majnik J, Patócs A, Balogh K, Luczay A, Török D, Szabó V, Borgulya G, Gergics P, Szappanos Á, Bertalan R, Belema B, Tőke J, Sereg M, Nagy Z.Zs, Sólyom J, Tóth M, Gláz E, Rácz K, Németh J, Fekete Gy, Tulassay Zs. Nucleotide sequence variants of the glucocorticoid receptor gene and their significance in determining glucocorticoid sensitivity. Orvosi Hetilap, 147 (44):2107-2115, 2006.

Publications not directly related to the PhD thesis

1. Majnik J. Clinical significance of biologic markers of bone metabolism. Characteristics of bone metabolism in psoratic arthritis. Magyar Reumatológia, 44:93-104, 2003.

2. Gergics P, Patocs A, **Majnik J**, Balogh K, Szappanos A, Toth M, Racz K. Detection of the BclI polymorphism of the glucocorticoid receptor gene by single tube allele-specific polymerase chain reaction. J Steroid Biochem Mol Biol., 100:161-6, 2006. **IF: 2,866**

3. Luczay A, Török D, Ferenczi A, **Majnik J**, Sólyom J, Fekete Gy. Potential advantage of N363S glucocorticoid receptor polymorphism in 21-hydroxylase deficiency. Eur. J. Endocrinol. 154: 859-864, 2006, **IF: 2,962.**

4. Balogh K, Hunyady L, Patócs A, Valkusz Zs, Bertalan R, Gergics P, **Majnik J**, Tőke J, Tóth M, Szűcs N, Gláz E, Fűtő L, Horányi J, Rácz K, Tulassay Zs. Clinical symptoms, diagnosis and treatment of multiple endocrine neoplasia type 1. Results of genetic screening in Hungarian patients. Orvosi Hetilap, 146(43):2191-2197, 2005.

5. Balogh K., Patócs A., **Majnik J.**, Varga F., Illyés Gy., Hunyady L., Rácz K. Unusual presentation of multiple endocrine neoplasia type 1 in a young woman with a novel mutation of the MEN1 gene. Journal of Human Genetics, 49(7):380-386, 2004. **IF: 2,316.**

6. Patócs A, Karádi É, Tóth M, Varga I, Szücs N, Balogh K, **Majnik J**, Gláz E, Rácz K. Clinical and biochemical features of sporadic and hereditary phaeochromocytomas: an analysis of 41 cases investigated in a single endocrine center. European Journal of Cancer Prevention, 13:403-409, 2004. **IF: 1,785.**

7. Balogh K, Patócs A, **Majnik J**, Rácz K, Hunyady L. Genetic screening methods for the detection of mutations responsible for multiple endicrine neoplasia type 1. Molecular Genetics and Metabolism, 83:74-81, 2004. **IF: 2,502**.