GENETIC AND ENVIRONMENTAL APPROACH TO LIPID METABOLISM AND BLOOD PRESSURE IN TWINS

Ph.D. thesis

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Introduction

Cardiovascular diseases cause over 15 million deaths in the world each year. Nearly half (49%) of all deaths from cardiovascular diseases are from coronary heart disease (CHD). CHD by itself is the most common cause of death in Europe: accounting yearly for nearly 2 million deaths in Europe. Death rates from CHD are generally higher in Eastern Europe than in Southern and Western Europe.

Coronary heart disease, particularly at young age, is largely influenced by genetic variability. The influence of genetic variance on the serum lipids is of great interest; however, the heritability data are in part conflicting. Serum total cholesterol (TC), LDL cholesterol (LDL-C), and triglyceride (TG) levels are directly correlate, whereas HDL-cholesterol (HDL-C) is inversely correlate with cardiovascular risk.

LDL-receptor (LDLR) gene mutations have dramatic effect on circulating LDL-C levels in patients with familial hypercholesterolemia, while the influence of the LDLR gene on LDL-C concentrations in the general population is less clear. Data on the influence of the lipoprotein lipase (LPL) gene locus on lipoprotein and TG levels do not uniformly agree.

Hypertension is a major public health problem, and the treatment of which consumes a large portion of the health care budget. Blood pressure itself is a complex variable, influenced by multiple interacting physiologic regulatory systems. Clear evidence for familial transmission has been discovered for almost 80 years. Some unusual forms of hypertension are inherited as a simple, monogenic trait; however, in essential hy[illegible] and multiple genes are likely to be involved. Mapping loci impor[illegible] blood pressure regulation would facilitate identification of the functional genes that [illegible] cause the disorder.

Monozygotic (MZ) and dizygotic (DZ) twins pro[illegible] a classic model for determination of the influence of heredity and environment on various t[illegible]s, including the risk for CHD and

detrimental serum lipid levels. Results of twin and family studies have shown a substantial genetic component of the hypertension, with a heritable variation of 30 to 60%.

Aims and Plans of Experiments

We recruited monozygotic (MZ) and dizygotic (DZ) twins. The inclusion of the MZ twins enabled us to estimate heritability of blood lipids and blood pressure for the studied population. Furthermore, we performed a sib-pair analysis in the DZ twins, to look for loci related to the quantitative traits, serum lipoprotein concentrations and blood pressure. We did not conduct a total genome scan in the DZ twins, but rather directed our attention to candidate gene loci.

1. **We planned to study healthy twin pairs to determine the influence of heredity and environment on TC, LDL-C, HDL-C, TG levels and blood pressure values.**
2. **We planned to examine the linkage between LDLR and LPL genes and lipoprotein levels in healthy persons.**
3. **When we found no linkage at the LPL locus, we directed our attention to the nearby macrophage scavenger receptor gene locus.**
4. **We planned to investigate also the relationship of LPL gene and blood pressure.** The LPL gene locus has been identified not only as a susceptibility gene locus for hypertriglyceridemia, but it may be relevant as so called "familial dyslipidemic" hypertension as well.
5. **As candidate gene loci for blood pressure, we selected also the components of the renin-angiotensin system.** The renin gene itself has been identified as a quantitative trait locus (QTL) in the rat, hypertension (as a qualitative trait) has been linked previously to

the angiotensinogen gene, and the ACE and AT_1 receptor genes have been associated with increased cardiovascular risk.

6. **We planned to examine the role of gene loci of monogenic hypertension forms in the regulation of normal blood pressure.** Liddle syndrome is a monogenic disorder, which closely resembles to "low renin" hypertension. The condition is caused by hyperactivity of the epithelial amiloride-sensitive sodium channel in the distal renal tubule, leading to salt and volume retention. Autosomal-dominant hypertension with brachydactyly resembles essential hypertension. The responsible gene has been mapped to chromosome 12p; however, the gene has not yet been identified.
7. **We planned to investigate the PLA2 locus on chromosome 12 as a candidate locus for blood pressure**, as described in one previous association study.
8. **When we found linkage with the markers about 20 cM centromeric from the PLA2 gene, we directed our attention to the IGF-1 gene.**
9. **We then coupled our attention on blood pressure to echocardiographically determined cardiac dimensions.**

Materials and Methods

Patients and Clinical Investigation

We recruited 166 pairs of twins (MZ 100 and DZ 66) by advertisement to participate in studies involving blood pressure regulation and cardiovascular phenotypes. The subjects were all German Caucasians with mean age of 30±12 years. They were recruited from various parts of Germany. A written informed consent was obtained from all participants. Each participant underwent a medical history and physical examination. None of them had hypertension or any other chronic medical illness. Persons with history of familial lipid disorders were excluded. Women who were using oral contraceptives or estrogen preparations, women >50 years old, and individuals of both sexes who were ingesting lipid influencing medication were also excluded from this analysis. It was no significant difference between the age, sex, height, weight and body mass index (BMI) of the MZ and DZ twins. The BMI was normal in both groups of twins.

Blood pressure was measured after 5 min (2 measurements, 1 min apart) with a standardized mercury sphygmomanometer in the sitting position by an experienced physician. Two measurements were obtained 2 minutes apart. The same procedure was performed after the subjects were supine for 5 minutes and after 5 minutes of upright posture. The mean of the 2 measurements was used as the blood pressure.

M-mode and two-dimensional echocardiograms were recorded with patients in the left-lateral supine position. Interventricular septal thickness and posterior wall thickness were measured in all patients.

Blood was obtained from all twins after a 12-hour fast. TC, [illegible], and [illegible] levels were determined by automated methods. LDL-C concen[illegible]ns were calculated by the Friedewald equation.

For the determination of zygosity and other [illegible]cular genetic studies, blood was obtained from all the twins and the parents of the DZ twi[illegible]

Molecular Genetical Methods

From the extracted DNA the zygosity was verified with the use of five highly polymorphic short tandem repeat loci.

We examined five highly polymorphic microsatellite markers at LPL - macrophage scavenger receptor (MSR) locus, one at the LDLR locus, three at the AT_1 receptor, two at the angiotensinogen, ACE, and renin loci, three at the Liddle syndrome locus and four at the autosomal dominant hypertension with brachydactyly locus and sex at the locus of PLA2-IGF-1 on chromosome 12.

The oligonucleotides were synthesized, and fluorescent dyes FAM, TET and HEX were attached. The microsatellite loci were amplified using standard PCR conditions with a final volume of 15 μl. The samples were processed in a Gene Amp 9600 thermal cycler. The PCR products were pooled before electrophoresis.

A 24-cm, 6% polyacrilamide gele was used for vertical electrophoresis in a 377 DNA Sequencer (Applied Biosystems). Thirty-six samples were electrophoresed and detected simultaneously by laser. The PCR products were loaded per gel, and electrophoresis was performed for eight hours. The 672 Genescan and Genotyper software (Applied Biosystems) was used for detection, sizing and allele determination of PCR amplified DNA fragments according standard DNA fragments.

Linkage Analysis

For this linkage study, the DZ pairs were selected and used as ordinary sib-pairs, but with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. The MZ twins were used to estimate allele frequencies for the markers tested.

We assessed linkage for continuous traits, such as blood pressure, LDL-C, HDL-C, TC and TG levels, against candidate gene loci. We accepted $p<0.05$ to test for significance.

Sib-pair analysis to determine linkage does not require the specification of a genetic model. The underlying trait can follow either Mendelian or non-Mendelian modes of inheritance. From the four alleles harboured by the parents for a given locus, each child randomly inherits two. Thus, a pair of sibs may have inherited either the same or different alleles. More specifically, they may share zero, one, or two alleles identical by descent (IBD). If the locus under study is a quantitative trait locus (QTL), phenotypic similarity of sibs (measured by the covariance) should increase with the number of alleles they share.

We used the single-point Haseman-Elston (H/E) approach as implemented in the SIBPAL program of the statistical analysis for genetic epidemiology (SAGE, 1994) package. To test for linkage, a linear regression analysis was carried out with the squared trait difference as the dependent variable and IBD as the independent variable. A power calculation is available for this test.

Analysis of the blood pressure QTLs was done also by a new method using a structural equation modelling (SEM) approach as implemented in the MX-package. This approach is based on variance (Var) - covariance (Cov) matrics o[illegible] the probability of sharing 0, 1, or 2 alleles IBD. Assuming [illegible] dominance effects, the total variance of the trait is due to the genetic effect of the Q[illegible] remaining additive genetic effects (A), and environmental influences (E): Var = Q^2 + A^2 + E^2. For the 3 possible IBD states (sharing 0, 1, or 2 alleles) covariance (Cov) of a sib pair was then defined by: Cov_{IBD0} = 0.5 A^2, Cov_{IBD1} = 0.5 Q^2 + 0.5 A^2 and Cov_{IBD2} = Q^2 + 0.5 [illegible]. For linkage analysis, models as

specified estimating Q, A, and E so that the likelihood of the empirical variance-covariance matrix of the sibs, weighted by the probability of sharing zero, one, or two alleles identical by descent, is maximized. For each sib pair and each locus, the proportion of alleles IBD, based on parental genotypes, is calculated using a multipoint approach as implemented in MAPMAKER/SIBS. To test for a QTL effect, the difference in model fit for models with and without a QTL effect is calculated as a χ^2 statistic. Since we used a candidate gene approach, we accepted $p<0.01$ to test for significant linkage.

Parameters of the quantitative genetic models were estimated by structural equation modelling using the MX program. The variability of any given phenotype (p) within a population can be decomposed into genetic influences (A), environmental influences shared by the twins within a family (C) and effects of random environment (E): $p = aA + cC + eE$, with a, c and e as the estimated relative influence. For MZ and DZ, the covariance (r) of their phenotype is given by: $r_{MZ}= a^2 + c^2$ and $r_{DZ}=0.5\ a^2 + c^2$.

Heritability analysis in twin studies can estimate additive components of genetic variability (estimated as a^2) as well as two environmental influences, shared (c^2) and nonshared (e^2). These values estimate the relative amount of the variable's influence on interindividual differences up to a sum of one. Genetic as well environmental effects were estimated by the best fitting model as selected by the χ^2 value. Statistical analysis was conducted using the SPSS program. Adjustment of blood pressure values, TC, HDL-C, LDL-C and TG levels for sex and age was done by multiple linear regression with the unstandardized residuals as the corrected phenotypes.

Results

Both the blood pressure and lipoprotein levels were normal in the MY and DY twins and there were no significant differences between MZ and DZ twins in these parameters. Systolic and diastolic blood pressure were heritable. The echocardiographic parameters also demonstrated strong evidence for heritability. A major genetic effect was demonstrated for all lipid parameters.

A significant linkage relationship was found for HDL-C and TGs with D8S261. Similarly, linkage was found for HDL-C and markers near to macrophage scavenger receptor locus. On the other hand, no linkage was found between any of the lipid variables and the markers nearer to LPL gene locus or the marker of LDLR gene locus. The macrophage scavenger receptor (MSR) gene locus and the LPL gene locus are ≈9 cM (in OMIM database ≈11 cM) apart from each other.

Markers at the IGF-1, Liddle syndrome, AT_1 receptor, and renin gene loci gave statistically significant evidence for linkage with systolic blood pressure in the SEM analysis. Using Haseman-Elston regression, the same loci were either significant or suggestive ($p<0.05$) for systolic blood pressure only in the sitting position. The ACE and LPL loci showed a significant relationship to only one of the blood pressure measurements. Thus, the results are only suggestive and not definitive. For diastolic blood pressure, the locus for autosomal-dominant hypertension and brachydactyly and the renin gene were significant linked.

Linkage for the markers at the IGF-1 locus and echocardiographically with the Penn formula determined cardiac mass was significant. For posterior wall and septal thickness, the results were highly suggestive. This relationship remained significant even after correcting for possible influences of blood pressure on cardiac dimensions. Microsatellite within the IGF-1 gene gave evidence of linkage with the phenotype posterior wall thickness phenotype.

Summary of New Results

1. Important finding of this study are that TC, HDL-C, LDL-C, and TGs are all equally influenced by both genetic and environmental influences. The heritability estimates for systolic and diastolic blood pressure were high. The echocardiographic parameters also demonstrated strong evidence for heritability.
2. Evaluation of the markers suggested lack of linkage between the serum lipid concentrations and the LPL gene locus.
3. We were unable to find any linkage between the LDLR gene locus and LDL-C concentrations.
4. Our results are the first demonstration of linkage between any serum lipid concentration and the macrophage scavenger receptor gene locus.
5. Polymorphic microsatellite markers at the renin gene, the AT_1 receptor gene, the Liddle syndrome gene, and IGF-1 gene loci were linked to the phenotype systolic blood pressure. We identified the IGF-1 gene locus and the Liddle syndrome locus as new QTLs for blood pressure.
6. Linkage with diastolic blood pressure was found with the renin locus and with a new QTL, the locus for autosomal dominant hypertension and brachydactyly.
7. Linking the IGF-1 gene locus to posterior wall thickness suggests that the gene for IGF-1 is an important candidate gene for susceptibility to cardiac hypertrophy.

Conclusions

We examined healthy MZ and DZ twins to test for linkage between the LDLR gene locus, LPL gene locus, and the macrophage scavenger receptor gene locus and serum lipid concentrations. We found evidence for linkage between the macrophage scavenger receptor gene locus and serum HDL-C values, as well as a weaker one to TG concentrations, but could not find linkage between the LDLR gene locus and serum LDL-C concentrations or between the LPL gene locus and the various lipid fractions. The latter observation in no way detracts from the results of earlier association studies but may instead be explained by the difference in susceptibility gene loci and those loci necessary for disease expression. We suggest that the macrophage scavenger receptor gene locus should receive increased attention in terms of atherosclerotic risk.

We examined our healthy twins to test for linkage between the seven QTLs, and systolic and diastolic blood pressure. We found consistent linkage with the gene loci of renin, AT1 receptor, Liddle syndrome and IGF-1 for systolic blood pressure. Interestingly, linkage with diastolic blood pressure was found with the renin locus and the locus for autosomal-dominant hypertension and brachydactyly. Both measurements confer the same degree of risk in terms of complications. Furthermore, systolic blood pressure can be more consistently and accurately measured. The difference of QTLs for systolic and diastolic blood pressure is not necessarily inconsistent and may instead merely underscore differences in systolic and diastolic blood pressure regulation.

We suggest that our new findings, namely that QTLs for blood pressure exist at the Liddle syndrome and IGF-1 loci, may be important to elucidating mechanism related to these genes or stimulate the search for new blood pressure-relevant genes at these locations. The

observation that the IGF-1 locus is also a QTL for cardiac dimensions suggests that particular attention should be directed at the IGF-1 gene.

Because of the high mortality of CVD and hypertension in Hungary it would be very important to continue the investigation of the genetical background of these diseases. It would be also useful to organize again the twin studies and to get a possibility to continue our study in Hungary.

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