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IMMUNOLOGICAL AND GENETIC FACTORS ASSOCIATED WITH TYPE 1 DIABETIC NEPHROPATHY

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Academic dissertation

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ABBREVIATIONS

ACE	angiotensin converting enzyme
AER	albumin excretion rate
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AGE	advanced glycation end product
ARI	aldose reductase inhibitor
ATG	angiotensinogen
AT1	angiotensin II type 1 receptor
FGF	fibroblastic growth factor
CML	carboxymethyl lysine
CV	coefficients of variation
DAG	diacylglycerol
DCCT	Diabetes Control and Complications Trial
DN	diabetic nephropathy
ELISA	enzyme-linked immunosorbent assay
ESRD	end-stage renal disease
GAP	glyceraldehyde 3-phosphate
GFR	glomerular filtration rate
GBM	glomerular basement membrane
HbA_1, $HbA1_c$	glycated hemoglobin
HDL	high density lipoprotein
HPLC	high-performance liquid chromatography
HSPG	heparan sulphate proteoglycan
IGF-1	insulin-like growth factor-1
IL-1	interleukin-1
LAP	latency-associated peptide
LDL	low density lipoprotein
LTBP	latent TGF-β1 binding protein
MDA	malonedialdehyde
NAG	N-Acetyl-β-D-glucosaminidase
Na+/Li+CT	sodium-lithium countertransport
NAT1	*N*-acetyltransferase-1
NAT2	*N*-acetyltransferase-2
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PHA	phytohemagglutinin
RAS	renin-angiotensin system
TGF-β1	transforming growth factor-β1
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
1X	1-methylxanthine

INTRODUCTION

Diabetic nephropathy plays an increasing role in industrialized countries as a reason for dialysis and kidney transplantation. It is a complication of both type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus. The pathogenic mechanisms of diabetic nephropathy are not fully understood, but the affecting mechanisms are probably multiple. Although hyperglycemia is the most important cause for diabetic nephropathy, today many unresolved questions still remain. Why do only one-third of type 1 diabetic patients develop diabetic nephropathy? Would this complication be avoidable with perfect life-long diabetes control? Does one major gene or do many genes predispose to diabetic nephropathy? Why are atherosclerotic vascular diseases so common in association with diabetic nephropathy?

This work has been carried out at the Hospital for Children and Adolescents, University of Helsinki, and in the Department of Biochemistry, National Public Health Institute, from 1994 to 1999. During this period, many new ideas for the prediction and prevention of type 1 diabetic nephropathy have arisen. One established concept is that intensive treatment of diabetes with good glycemic control postpones and diminishes all diabetic complications (DCCT Research Group 1993). The second trend is a movement from separate candidate gene investigations to large family studies with transmission disequilibrium tests and genome-wide searches (Imperatore et al. 1998; Frayling et al. 1999), or to large-scale association studies (Risch and Merikangas, 1996). Third, an increasing number of studies on the possible role of immune mechanisms in the development of type 1 diabetic nephropathy have been published (Furuta et al. 1993; Hirata et al. 1998; Shibayama et al. 1999). Type 1 diabetes is an autoimmune disease. Could the same disturbed immune mechanisms be involved in the development of type 1 diabetic nephropathy? Diabetic nephropathy has traditionally not been considered an immunological disease, although immunological cells have been found in kidney biopsies. During recent years the participation of cytokines and growth factors in the pathogenesis of diabetic nephropathy has been shown by numerous studies (see Parving et. al. 1996a), but the exact role of the immune response in the pathogenesis of nephropathy remains unknown. This study concerns some of the immunological and genetic factors associated with type 1 diabetic nephropathy.

REVIEW OF THE LITERATURE

EPIDEMIOLOGY OF DIABETIC COMPLICATIONS

DIABETIC MICROVASCULAR COMPLICATIONS

Diabetic nephropathy

The incidence of diabetic nephropathy has declined during the last decade. In the 1980's 35 to 45% of type 1 diabetic patients were estimated to develop persistent proteinuria after 40 years of diabetes (Andersen et al. 1983; Krolewski et al. 1985). In the 1990's, the risk of diabetic nephropathy was considerably lower: The prevalence of proteinuria was 27% in the US population (Warram et al. 1996), and 15% in the European population after 30 years of diabetes (The EURODIAB IDDM Complications Study Group 1994). The risk of diabetic nephropathy seems to be higher in the USA than in the European population, a risk also demostrated in the figures for incipient nephropathy (microalbuminuria). In the US, the prevalences of microalbuminuria at 10 and 30 years of diabetes were 25% and 52%, respectively (Warram et al. 1996), whereas in the European population, the cumulative incidence of microalbuminuria was held steady between 18 and 25% after 1 to 30 years' duration (The EURODIAB IDDM Complications Study Group 1994).

One reason for this drop in the incidence of diabetic nephropathy is improvement in the clinical practice of insulin treatment and therefore in glycemic control. For example, in a Swedish center, the cumulative incidence of proteinuria was only 9% after 25 years of diabetes. These patients had had excellent mean glycated hemoglobin for many years (Bojestig et al. 1994).

Microalbuminuria is considered a strong predictor for overt nephropathy (Viberti et al. 1982; Mogensen and Christensen 1984). The rate of the development of diabetic nephropathy varies in individual patients (Viberti et al. 1982), and improvement in glycemic control may result in the disappearance of microalbuminuria (Bojestig et al. 1996). It has been estimated that approximately half the patients with microalbuminuria will progress to overt nephropathy (Krolewski et al. 1996). A relative annual increase in urinary AER over 5% has been used to distinguish progressors from non-progressors (Almdal et al. 1994). Among progressors the median time between microalbuminuria and overt nephropathy is about nine years (Krolewski et al. 1996).

Microalbuminuria in prepubertal children has been rare (Kostraba et al. 1989; Mortensen et al. 1990). In a nationwide cross-sectional study of children and adolescents (aged 1-19 years), the prevalence of persistent microalbuminuria was 4% and of overt nephropathy 0.7% (Mortensen et al. 1990). Of the microalbuminuric patients, only a few were prepubertal. However, in a recent population-based longitudinal study of children diagnosed with diabetes at <5 years of age, five of 11 developed microalbuminuria before puberty (Schultz et al. 1999). Since younger age groups are tending to develop diabetes (Karvonen et al. 1999), prepubertal screening for microalbuminuria may become important, due to the longer prepubertal duration of diabetes.

Risk factors for diabetic nephropathy include postpubertal duration of diabetes (Krolewski et al. 1996), hyperglycemia (DCCT Research Group 1996), hypertension (Parving et al. 1988; Microalbuminuria Collaborative Study Group 1992), male gender (Andersen et al. 1983), cigarette smoking (Microalbuminuria Collaborative Study Group 1993), elevated levels of serum lipids (Coonrod et al. 1993), and familial genetic factors (DCCT Research Group 1997). The differences in the incidences between different populations can in part be explained by differences in these risk factors. To achieve a further decline in the incidence figures, the presence of these risk factors should be carefully estimated and proper treatment for each should be introduced early.

Diabetic retinopathy

Background retinopathy in diabetic patients is common. Its prevalence increases most steeply between 5 to 15 years of diabetes duration, being about 60% after 20 years in the European population. Proliferative retinopathy means pathological development of new vessels in the retina (neovascularization) (Aiello et al. 1998). The cumulative incidence of proliferative retinopathy rises steadily after 10 years of diabetes, being 37% at 30 years of diabetes duration (The EURODIAB IDDM Complications Study Group 1994). The risk of proliferative retinopathy increases twofold after onset of proteinuria (Klein et al. 1993), but its risk can be even higher. Compared to 14% in Danish diabetic patients without proteinuria, proliferative retinopathy had developed in 74% of matched diabetic patients who had had proteinuria for 5 years (Kofoed-Enevoldsen et al. 1987).

The impact of hyperglycemia on the development of diabetic retinopathy was confirmed in the Berlin Retinopathy Study in which the eyes of diabetic children and adolescents were followed regularly from the diagnosis of diabetes by retinal fluorescein angiography. Background retinopathy appeared after a median

duration of 12 to 13 years when glycated hemoglobin (HbA_{1c}) was over 9%, but later than 25 years when HbA_{1c} was under 8% (Danne et al. 1994).

DIABETIC MACROVASCULAR COMPLICATIONS

Diabetes is associated with a 2 to 3-fold increased risk for atherosclerotic disease (Kannel and McGee 1979). In type 1 diabetes, cardiovascular disease has been linked to nephropathy in many studies (Borch-Johnsen and Kreiner 1987; Jensen et al. 1987; Krolewski et al. 1987). In a recent nation-wide Finnish study based on hospital discharge diagnoses, the relative risk for coronary heart disease was 10-fold in type 1 diabetic patients with diabetic nephropathy compared to patients without nephropathy. The risk for stroke was increased 11-fold in the same study population (Tuomilehto et al. 1998).

PATHOGENESIS OF DIABETIC NEPHROPATHY

NATURAL HISTORY OF DIABETIC NEPHROPATHY

Albumin excretion rate (AER) is defined as normal at a urinary albumin excretion of ≤20 µg/min. Microalbuminuria is defined as urinary AER >20 µg/min and ≤200 µg/min. Persistent microalbuminuria (incipient diabetic nephropathy) is present if microalbuminuria is found in two of three consecutive urine samples, collected preferably within 6 months (Parving et al. 1988). The urine sample can be collected overnight or for 24 hours. Macroalbuminuria or persistent proteinuria or overt nephropathy is defined as urinary AER >200 µg/min, or protein excretion >0.5 g during 24-hour urine collection (Mogensen 1997).

The progression of diabetic nephropathy can be divided into five stages from the onset of diabetes until the end-stage renal failure (Mogensen et al. 1983). Stage 1 is early hyperfunction and hypertrophy, which decline after the start of insulin treatment. Stage 2 is progression of silent morphologic lesions without signs of clinical disease; glomerular filtration rate (GFR) is normal or increased. Stage 3 is incipient diabetic nephropathy with persistent microalbuminuria and rising blood pressure. Stage 4, overt diabetic nephropathy, is characterized by persistent proteinuria and decreasing GFR. This fall in GFR can be retarded by antihypertensive medication. Stage 5 is end-stage renal failure with uremia.

STRUCTURAL KIDNEY CHANGES AND THEIR RELATION TO FUNCTION

Diabetes results in glomerular and interstitial structural changes demonstrated by light and electron microscopy. Typical glomerular changes include capillary basement thickening, and expansion of the mesangial region and matrix (∅sterby 1992). In addition, arteriolar hyalinosis and sclerosis of glomeruli are demonstrated (Harris et al. 1991), as well as interstitial expansion (Lane et al. 1993). Structural indexes, such as basement membrane thickness, mesangial volume fraction per glomerulus, matrix volume fraction per glomerulus (∅sterby 1992), interstitial volume fraction for total renal cortex, percentage of globally sclerosed glomeruli, and index of arteriolar hyalinosis (Lane et al. 1993) indicate degree of structural changes.

Structural parameters correlate with functional parameters, but large variation exists in the structural parameters inside the functional groups (normal AER, microalbuminuria, and overt nephropathy), as well as great overlapping between the groups (∅sterby 1992). Structural indexes were significantly higher in patients with microalbuminuria than in those with normal AER (Walker et al. 1992). A parallel course of mesangial and basement membrane changes occurred, and the structural indexes correlated with urinary AER (Walker et al. 1992). Chavers et al. (1989) found a wide range of variation in structural indexes, including normal values, in normoalbuminuric or microalbuminuric patients without hypertension or without decreased creatinine clearance. In a 6-year follow-up study, the change in mesangial volume fraction per glomerulus was the only structural parameter which correlated with increase in AER (Fioretto et al. 1995). In another 6-year prospective study of young adults with persistent microalbuminuria, long-term AER was predicted by the degree of glomerular structural changes (basement membrane thickness or mesangial volume fraction) (Bangstad et al. 1999).

Glomerular basement membrane

Glomerular basement membrane (GBM) of the glomerular capillary wall is thought to provide a major barrier against the filtration of macromolecules. The function of this barrier is based on electrical charges. Its key component, heparan sulphate proteoglycan (HSPG), is negatively charged through anionic sites. Thus, negatively charged macromolecules such as albumin do not undergo filtration, due to repelling electrostatic forces (see Vehaskari and Robson 1992). A classical view of the increased albumin leakage in diabetic nephropathy is based on this theory of electrical charges in GBM. In experimental diabetes, reduction in the number of anionic sites in heparan sulphate has been shown to correlate with increased urinary albumin excretion (van den Born et al. 1995). Anionic sites are

reduced in diabetic patients with clinical proteinuria but not in patients with microalbuminuria (Vernier et al. 1992).

A completely new concept challenges this theory of charge-selectivity. According to a recent view, glomerular capillary wall is not as charge selective for albumin as was thought, and albumin is largely filtered through this barrier (Osicka and Comper 1998). The major part of filtered albumin is returned to the blood circulation rapidly by what is called the high capacity pathway. The exact location of this pathway is unknown (Eppel et al. 1999). A small amount of filtered albumin is degraded and subsequently excreted in the urine under normal conditions, through a pathway called the degradation pathway (Osicka et al. 2000). In early experimental diabetes, the proportion of native albumin in urine increased, and less degraded albumin was present (Burne et al. 1998). It remains to be seen whether other research groups confirm this theory. These findings seem to be reasonable when related to the epidemiological finding that besides being a marker of diabetic nephropathy, proteinuria itself may contribute to renal damage (Parving 1998). Increased filtered protein results in an increased protein concentration in the proximal tubular fluid, which in turn may cause tubular cell damage (Bruzzi et al. 1997).

IMPACT OF HYPERGLYCEMIA ON THE PATHOGENESIS OF DIABETIC NEPHROPATHY

Hyperglycemia is the major cause of diabetic nephropathy. The Diabetes Control and Complications Trial (DCCT) showed that intensive therapy for diabetes can reduce the risk of developing microalbuminuria and clinical albuminuria. Intensive therapy delays the onset of microalbuminuria and albuminuria when compared to results of conventional therapy. This treatment effect appears after 3 years for microalbuminuria, and after 5 years for albuminuria (DCCT Research Group 1995). The effect of hyperglycemia may be mediated by several routes; of these, activation of protein kinase C (PKC), the aldose reductase pathway (polyol pathway), and glycoxidation (advanced glycation endproducts, AGEs) seem to be the most important (Fig 1). Activation of PKC and the polyol pathway are intracellular processes, whereas AGEs affect both the extracellular and intracellular environment.

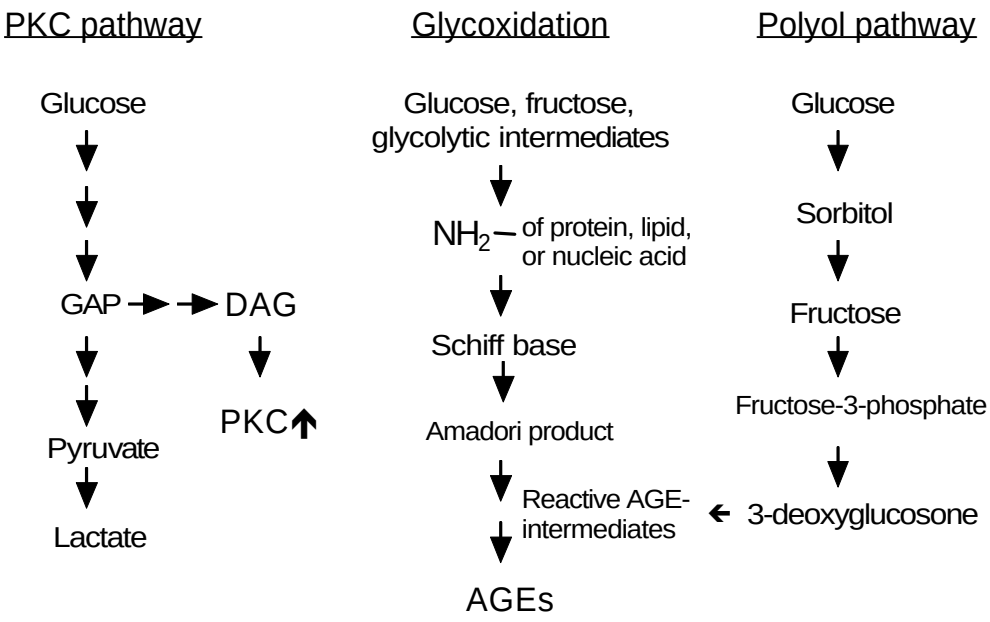

Figure 1. *Central pathways in the pathogenesis of diabetic nephropathy. GAP, glyceraldehyde 3-phosphate; DAG, diacylglycerol; PKC, protein kinase C; AGEs, advanced glycation end products.*

PROTEIN KINASE C

Protein kinase C (PKC) is a family of serine-threonine kinases that plays an important role in signal transduction mechanisms (Ron and Kazanietz 1999). PKC is the cellular receptor for the lipid second messenger diacylglycerol (DAG), which is one of the activators of PKC, in addition to $Ca^{2++}$and phorbol esters (Ron and Kazanietz 1999). The DAG-PKC pathway is activated in diabetes as a result of hyperglycemia, because increased amounts of diacylglycerol (DAG) are synthesized directly from glycolytic intermediates (King et al. 1997). It is possible that also AGEs and oxidants increase formation of DAG and activate PKC (Koya and King 1998).

PKC regulates many important factors related to the vascular system, such as permeability, contractility, cellular proliferation, basement membrane synthesis, and signal transduction for hormones and growth factors (King et al. 1997). In diabetes, increased DAG-PKC activation has been found in the retina, aorta, heart, and glomeruli, but not in the brain or peripheral nerves (King et al. 1997). In diabetes, the effects of PKC are multiple. PKC decreases retinal blood flow. It regulates renal nitric oxide production. It enhances the production of extracellular matrix and TGF-β1 (Koya and King 1998). Evidence for the central role of PKC in the pathogenesis of diabetic vascular complications has been obtained by study

of PKC antagonists. In diabetic rats, a specific inhibitor of PKC β improves GFR, AER, and retinal circulation (Ishii et al. 1996). Moreover, PKC antagonist prevents in diabetic rats the increased mRNA expression of TGF-β1 and extracellular matrix components in glomeruli (Koya et al. 1997). That AGEs and angiotensin II are involved in the activation of PKC is suggested by a study performed in streptozotocin-induced diabetic rats in which both an AGE inhibitor and ramipril were able to inhibit an increase in glomerular PKC activity (Osicka et al. 2000).

POLYOL PATHWAY

During hyperglycemia, the excess glucose is metabolized by the aldose reductase pathway, also called the polyol pathway. First, glucose is converted to sorbitol by aldose reductase. Thereafter, sorbitol is converted to fructose, and further to fructose-3-phosphate (Kinoshita 1990). Aldose reductase inhibitors (ARI), such as epalrestat, tolrestat, or sorbinil, inhibit the first step in the pathway. In animal models, an ARI has been successful in diminishing proteinuria, and glomerular hyperfiltration (Tilton et al. 1989; Bank et al. 1989; Donnelly et al. 1996). In a 6-month study in patients with nephropathy (mean AER 291 μg/min), glomerular filtration rate and urinary AER decreased significantly (mean AER 158 μg/min) during ARI administration (Passariello et al. 1993); both regarded as beneficial effects in early diabetic nephropathy.

GLYCOXIDATION

Advanced glycation end products

Advanced glycation end products (AGEs) form as a result of a complicated series of reactions between glucose, fructose, or glycolytic intermediates and amino groups of proteins, lipids, or nucleic acids (Brownlee 1994; Vlassara et al. 1994). The reaction begins with glucose attachment to amino groups, thus forming a reversible Schiff base adduct. This undergoes a slow intramolecular rearrangement to form an Amadori product. Glycated hemoglobin, which is clinically used to measure glycemic control is an Amadori product (de Rosa et al. 1998). The Schiff base reflects the immediate ambient glucose level, whereas an Amadori reaction reaches the steady state level in approximately 4 weeks. This means that in diabetes after achievement of the steady state these early glycosylation products do not accumulate. In diabetic tissue Amadori levels are 2.5-fold compared to those in non-diabetic tissue (Bucala and Vlassara 1995). The AGEs are formed when Amadori products react further with each other or with other intermediates by rearrangement, condensation, and dehydration

reactions. AGEs form stable cross-linked structures which accumulate in tissues, especially in tissues containing proteins with a long half-life such as eye lens, nervous and vascular tissues, and kidney (Bucala and Vlassara 1995). The AGEs are produced even after hyperglycemia has been corrected (Vlassara et al. 1994). Several AGEs have been characterized, such as pyrraline, pentosidine, and carboxymethyl lysine (CML) (Bucala and Vlassara 1995). CML seems to be the major epitope of AGEs which is recognized by experimentally induced polyclonal AGE-antibodies, but other epitopes exist as well (Ikeda et al. 1996).

Recently, the concept of AGEs has widened considerably. Wolff et al. (1991) proposed that metal-catalyzed oxidation of glucose itself was the major route for AGE formation, and that other carbohydrates besides glucose, and their metabolic intermediates, are important sources of AGEs. Baynes (1991) emphasized the role of oxidation in the formation of AGEs. Bucala et al. (1993) described AGE lipids. After this, a new term, "carbonyl product", was introduced. The carbonyl product and AGE mean the same thing, and both consist of glycoxidation and lipoxidation products (Baynes and Thorpe 1999). The term "carbonyl stress" describes an increase in the concentration of reactive carbonyl precursors of AGEs. These carbonyl precursors may be derived both from oxidative and from nonoxidative reactions, and from carbohydrates and lipids.

AGEs have profound effects on cells and tissues; they can upregulate gene expression associated with diabetic glomerular disease, expression prevented by an AGE inhibitor, aminoguanidine (Yang et al. 1994; Pugliese et al. 1997). AGEs have been found in advanced glomerular lesions in human beings (Makino et al. 1995), and in proximal tubules in rats (Youssef et al. 1999). Receptors for AGEs have been found in monocyte-macrophages, fibroblasts, and renal mesangial cells (Vlassara 1992), in T-lymphocytes (Imani et al. 1993), in vascular smooth muscle cells (Higashi et al. 1997), vascular endothelial cells (Stitt et al. 1999), and in human neurons and glial cells (Li et al. 1998). AGE-receptor interaction has been shown to mediate secretion of several cytokines and growth factors relevant to the pathogenesis of diabetic nephropathy (Vlassara 1992).

In the glomeruli of patients with diabetic nephropathy, both early and advanced glycation products occur (Sakai et al. 1996), and both have been considered important in the pathogenesis of nephropathy (Cohen and Ziyadeh 1996). Glycoxidation and peroxidation products have been found in the glomerular lesions of patients with diabetic nephropathy (Horie et al. 1997; Suzuki et al. 1999).

A complex interaction occurs between three metabolic pathways: the DAG-PKC pathway, polyol pathway, and glycoxidation. For example, the breakdown product of fructose-3-phosphate, 3-deoxyglucosone, is also an AGE intermediate, and as such an extremely potent glycating agent (Hamada et al. 1996). In diabetic patients with hemodialysis, one ARI was able to decrease both erythrocyte 3-deoxyglucosone and AGE concentration (imidazolone) (Tsukushi et al. 1999). Furthermore, polyol and DAG-PKC pathways must be associated, because an inhibitor of aldose reductase was able to decrease PKC activity (Keogh et al. 1997).

AGEs, PKC, and the polyol pathway seem all to be important in the pathogenesis of diabetic nephropathy. As to prevention of diabetic nephropathy, all the studies aiming to prevent diabetic nephropathy by use of a specific inhibitor of these pathways have been performed in experimental diabetes. When an aldose reductase inhibitor, an AGE inhibitor, and an antioxidant were compared in streptozotocin-induced diabetic rats, elevation of AER was prevented by an AGE inhibitor, aminoguanidine, but not by an aldose reductase inhibitor or by an antioxidant (Soulis-Liparota et al. 1995). However, nothing is known about the benefit of these inhibitors in the prevention of human diabetic nephropathy, and it remains to be seen whether an AGE inhibitor is the most beneficial or whether a combination of many drugs would be preferable.

Oxidation and glycation of low density lipoprotein

Low density lipoprotein (LDL) has a special place in studies of glycation and oxidation in diabetes because it is a strong risk factor for atherosclerotic vascular diseases. LDL is the major cholesterol and cholesteryl ester carrier in plasma. It consists of a lipid portion, including phospholipids, free cholesterol, cholesteryl ester, and triglycerides, and a carrier protein, apoprotein (apo B-100) (Illingworth 1993). In the atherosclerotic process, circulating LDL moves into the subendothelial space, where it is taken up by macrophages through a scavenger receptor. In LDL overload, macrophages transform themselves into foam cells which further change to a fatty streak (Ross 1993). Important in this respect is, that modified LDL is taken up faster by macrophages than is native LDL. Oxidized LDL in particular is thought to be a key molecule in atherogenesis (Ross 1993; Witztum 1994).

In diabetic patients, glycated LDL comprises up to 21% of total LDL compared to 5% in normal subjects (Klein et al. 1995), and the degree of glycation correlates with glycemic control (Lyons et al. 1986). LDL of diabetic patients with poor glycemic control is more susceptible to oxidation than is LDL of

normal subjects (Tsai et al. 1994), but in diabetic patients with good glycemic control the degree of LDL glycation and the susceptibility of LDL to oxidation do not differ from those in healthy subjects (Jenkins et al. 1996).

Similarly to LDL, high density lipoprotein (HDL) is susceptible to oxidation and glycation. In vitro oxidation of HDL has been shown to decrease its capacity to mediate the cholesterol efflux from macrophage-derived foam cells (Nagano et al. 1996), but in vitro glycation of HDL did not increase its susceptibility to oxidation, or decrease its capacity to mediate cholesterol efflux (Rashduni et al. 1999).

GENETIC FACTORS IN THE DEVELOPMENT OF DIABETIC NEPHROPATHY

General

The basis for genetic studies of risk factors for diabetic nephropathy is the clinical fact that two-thirds of diabetic patients do not develop diabetic nephropathy despite long-term hyperglycemia. Familial clustering of diabetic nephropathy has been observed (Seaquist et al. 1989; Quinn et al. 1996; DCCT Research Group 1997). Also a single pathogenetically important factor such as hypertension clusters in families with probands with diabetic nephropathy (Krolewski et al. 1988; Fagerudd et al. 1998). Parental history of type 2 diabetes has also been associated with type 1 diabetic nephropathy in probands (Fagerudd et al. 1999). Strong concordance in structural parameters of the kidney has been apparent in siblings with type 1 diabetes (Fioretto et al. 1999).

Brownlee et al. (1986) were the first to show that an AGE-inhibitor, aminoguanidine, could prevent diabetic complications. Later on, they formulated a hypothesis that genetic differences in the pathways dealing with formation and degradation of AGEs, or in the effects of AGEs on cell matrix turnover, on cytokine and growth factor production, or on signal transduction, may be important in susceptibility to diabetic complications (Brownlee et al. 1988).

Although Quinn et al. (1996) suggested that diabetic nephropathy may be due to single genetic factor, more commonly diabetic nephropathy is viewed as a multigenic disease. Several possible approaches to multigenic disorders exist: Candidate gene studies are based on knowledge of the pathogenetic mechanisms behind the disease. Cases and controls are compared with each other with respect to the specific candidate gene. In type 1 diabetic nephropathy, cases are typically patients with overt diabetic nephropathy, and controls are diabetic patients with

normal albumin excretion despite long-duration diabetes. Genetic polymorphisms in the candidate genes may increase or decrease susceptibility to the nephropathy. Another approach is to study pathogenetically interesting genes occurring in families with parents and offspring with or without diabetic nephropathy (transmission/disequilibrium tests) (Rogus et al. 1998), and in concordant and discordant siblings (Moczulski et al. 1998). A genome-wide search is an interesting new method already applied in type 2 diabetic patients, affected sib-pairs of Pima Indian origin. Scanning showed the strongest evidence for linkage on chromosome 7q, followed by tentative evidence for linkage on chromosomes 3, 9, and 20 (Imperatore et al. 1998).

Table 1. *Central polymorphisms of the renin-angiotensin system (RAS) with respect to type 1 diabetic nephropathy.*

Gene	Polymorphism	Alleles	Genotypes
Angiotensin converting enzyme (ACE)	Insertion/deletion (I/D)	I and D	II, ID, DD
Angiotensinogen (ATG)	M235→T	M and T	MM, MT, TT
Angiotensin II type 1 receptor (AT1)	A1166→C	A and C	AA, AC, CC

Evidence for polymorphisms in the renin-angiotensin system in pathogenesis of diabetic nephropathy

The renin-angiotensin system (RAS) consists of renin, angiotensinogen (ATG), angiotensin I, angiotensin II, and the angiotensin II type 1 (AT1) receptor. Renin cleaves ATG to form angiotensin I. Angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II, which is a potent vasoconstrictor. RAS consists of several genetic polymorphisms. The three most investigated are presented in Table 1. An insertion/deletion (I/D) polymorphism of the ACE gene was first found to associate with myocardial infarction (Cambien et al. 1992), and later on with type 1 diabetic nephropathy (Marre et al. 1994). Based on the numerous positive (Barnas et al. 1997; Marre et al. 1997) and negative (Tarnow et al. 1995a; Schmidt et al. 1995) studies on ACE polymorphism and diabetic nephropathy, two meta-analyses have been appeared, one showing an association between the D allele and diabetic nephropathy (Fujisawa et al. 1998), and the other none (Kunz et al. 1998). An important finding is that patients with an II genotype seem to have better response to ACE-inhibitor treatment in terms of a

greater reduction in urinary AER than patients with an ID or DD genotype (Penno et al. 1998; Jacobsen et al. 1998). Moreover, the D allele remained a risk allele for cardiovascular disease in type 1 diabetic patients with nephropathy (Tarnow et al. 1995b). In Caucasian patients with type 2 diabetes, ACE polymorphism was not a genetic marker of diabetic nephropathy (Huang et al. 1998a) but was associated with coronary heart disease (Huang et al. 1998b).

The cleavage of ATG is the rate-limiting step in the renin-angiotensin system (Campbell et al. 1991; Sealey and Laragh 1990). No association was found between angiotensinogen M235T polymorphism and diabetic nephropathy (Schmidt et al. 1996; Tarnow et al. 1996), but again contradictory studies have been published in a case-control setting (Fogarty et al. 1996), and in a family-based study (Rogus et al. 1998). In a non-diabetic population, the T235 allele was associated with cardiovascular disease and hypertension (Winkelmann et al. 1999).

The polymorphic sites of an angiotensin II type 1 receptor (AT1) have also been under investigation with both negative (Tarnow et al. 1996) and positive results, the latter in patients with severe hyperglycemia during the first decade of diabetes (Doria et al. 1997). In a discordant sib-pair analysis screening genes for the renin-angiotensin system, the region containing angiotensin II type 1 receptor showed linkage with diabetic nephropathy. This finding implicates region surrounding AT1 on chromosome 3q as a major locus for susceptibility to diabetic nephropathy (Moczulski et al. 1998).

Most of the studies of RAS and diabetic nephropathy are association studies in which the central methodological problem is reliably to characterize cases and controls (Corvol et al. 1999); if cases and controls are not reliable, false negative results may follow. The problem is to know what appropriate factors need to be taken into account. In the studies mentioned selection criteria for cases and controls seem to vary considerably. In the original finding (Marre et al. 1994), patients with microalbuminuria and overt nephropathy were compared to patients with normal AER. The two groups were matched for retinopathy severity, which meant that 26% of the cases showed no retinopathy. The mean duration of diabetes was rather low, 22 years for controls and 20 years for cases. In contrast, in studies performed at the Steno Diabetes Center in Denmark (Tarnow et al. 1995; Tarnow et al. 1996a; Tarnow et al. 1996b), only those patients with overt nephropathy and concominant retinopathy were accepted into the case group, and the duration of diabetes was higher, 27 years for cases and 26 for controls. These Danish studies included hypertensive patients in both groups, while another study excluded patients on antihypertensive medication from the control group due to

possible effect of this medication on AER (Fogarty et al. 1996). In a later study by Marre et al. (1997), only diabetic patients with proliferative retinopathy were included, to ensure that the controls no longer had a risk for nephropathy. These examples show that the criteria for patient selection for genetic studies on diabetic nephropathy have not yet been firmly established, and thus the interpretation and comparison of the results is difficult.

Polymorphisms in the polyol pathway and diabetic nephropathy

A gene coding for aldose reductase is located on chromosome 7q35 (Graham et al. 1991). The aldose reductase gene contains at least two polymorphic sites, a dinucleotide repeat polymorphism $(A\text{-}C)_n$ (Ko et al. 1995) and a single substitution C106T polymorphism (Kao et al. 1999). A strong association was found between the Z-2 allele [$(A\text{-}C)_{23}$ allele] of the repeat polymorphism and type 1 diabetic nephropathy (Heesom et al. 1997), which was confirmed by Shah et al. (1998) and Moczulski et al. (1999a). The Z-2 allele was also associated with an increased expression of aldose reductase mRNA in peripheral blood mononuclear cells (Shah et al. 1998). In contrast, patients with type 2 diabetes with normal albumin excretion, microalbuminuria, and proteinuria in regard to dinucleotide repeat polymorphism and nephropathy showed a negative result (Moczulski et al. 1999b).

Interleukin-1 and diabetic nephropathy

Interleukin-1 (IL-1) forms a family of three proteins, IL-1α, IL-1β, and the IL-1 receptor antagonist (IL-1ra) produced by mononuclear cells, endothelial cells, and many other cells. IL-1α and IL-1β are potent enhancers of immune responses such as T-cell activation and proliferation and cytokine secretion. IL-1α and IL-1β also induce acute inflammatory response, including fever, anorexia, myalgia, and synthesis of C-reactive peptide. IL-1ra is a natural antagonist of IL-1α and IL-1β which regulates the course of this inflammation. (Rosenwasser 1998). IL-1 stimulates mesangial cell proliferation and extracellular matrix production (Melcion et al. 1982). Type 1 diabetic patients with overt nephropathy showed significant difference in frequency of interleukin 1B*2 allele as compared to patients with normal AER (Loughrey et al. 1998). However, an earlier Danish study was negative with respect to the IL-1 family and diabetic nephropathy (Tarnow et al. 1997). A notable difference between the two study populations was that 48% cases of the former study had end-stage renal failure, and 36% impaired renal function, whereas in the latter, the highest creatinine was 684 μmol/L, suggesting that patients in the former study had more advanced diabetic nephropathy.

Transforming growth factor-b1 and diabetic nephropathy

Transforming growth factor-β1 (TGF-β1) is a central growth factor in fibrotic diseases (see pages 25-26). A gene encoding transforming growth factor-β1 (TGF-β1) is located on chromosome 19q13 (Lawrence 1995) contains several polymorphic sites. A Pro25Arg polymorphism was linked to blood pressure in healthy European males (Cambien et al. 1996). A Thr263Ile mutation was associated with diabetic nephropathy in type 1 diabetic patients (Pociot et al. 1998). TGF-β1 plasma levels were associated with blood pressure in patients with end stage renal disease (Li et al. 1999).

N-acetyltransferase-2 and diabetic nephropathy

N-acetyltransferases 1 and 2 are cytosolic enzymes which transfer acetyl groups. Both are located at the same gene locus on chromosome 8 (Blum et al. 1990). Each metabolizes distinct arylamine drugs and carcinogens. NAT2 is operative in liver cell cytosols and is also expressed in the small intestine, colon, esophagus, bladder, ureter, stomach, and lung (Windmill et al. 1997), whereas the distribution of NAT1 is more widespread in the body. NAT1 was thought to be a monomorphic enzyme until 1993, when a genetic polymorphism of NAT1 was found (Vatsis and Weber 1993). The polymorphism of NAT2 has been known for over 40 years (Evans et al. 1960). Drugs such as hydrazine, hydralazine, salazosulfapyridine, procainamide, isonizid, and caffeine are metabolized either slowly or rapidly depending on the inherited NAT2 genotype (Evans 1989). At least 12 different alleles of the NAT2 gene are known, which are mainly slow allele mutations of the dominant fast allele (Vatsis et al. 1995). Foreign substances metabolized by NAT2 include benzidine, and β-naphtylamine, a tobacco-smoke derivative.

Traditionally, the NAT2 phenotype has been determined from a urine or a plasma sample after a single oral dose of sulfametazine, dapsone, or caffeine, and the metabolic ratio of unacetylated and acetylated drug calculated (Evans 1989). In the past decade, genotyping has become the principal test method to detect fast and slow acetylators (Doll et al. 1995; Agúndez et al. 1996). A marked interethnic variation is found in the distribution of fast and slow acetylators. For example, among Eskimos and Arabs, slow acetylators predominate (80-90%), whereas in Japan over 90% are fast acetylators. Among Caucasians, slow acetylators are 50 to 60% of the population (Evans 1989). This interethnic variation is due to differing frequencies of mutations in the NAT2 gene (Lin et al. 1993; Lin et al. 1994).

In early studies an increased prevalence of fast acetylators was observed in patients with type 1 diabetes (Evans 1984). However, the series were small, and the conclusions were based on the combination of several studies (Bodansky et al. 1981). It is possible that in these studies, the diabetic state has influenced the metabolic ratio used for the distinction between slow and fast acetylators. Recently, in patients with type 2 diabetes, the onset of insulin treatment decreased the acetylation capacity of sulfamethazine, suggesting that the poor metabolic control before the start of insulin treatment had increased NAT2 activity by enzyme induction (Lautenschlager et al. 1996). In contrast, a study of type 1 diabetic patients on two occasions with different HbA_{1c} levels suggested that the improvements in glycemic control increased NAT2 activity (Bechtel et al. 1988). In patients with type 2 diabetes, fast acetylators showed higher fasting insulin and first-phase insulin peak values during intravenous glucose tolerance test than did slow acetylators (Burrows et al. 1978). In young patients with type 1 diabetes, 15 patients of 16 with early microalbuminuria were slow acetylators (Madácsy et al. 1992). From the above studies, it can thus be concluded that a link may exist between NAT2 and diabetes. NAT2 gene alleles have not previously been studied in patients with established type 1 diabetic nephropathy.

HEMODYNAMIC FACTORS IN THE PATHOGENESIS OF DIABETIC NEPHROPATHY

The overall prevalence of 15% for systemic hypertension in type 1 diabetes is mainly associated with incipient and overt nephropathy, because in patients with normal AER the prevalence of essential hypertension corresponds to that of the general population (4%) (Norgaard et al. 1990). During the transition phase from normal AER to microalbuminuria, early changes in blood pressure are already possible to find by ambulatory blood pressure monitoring (Poulsen et al. 1994). Hypertensive diabetic patients have increased total body sodium content and vascular resistance. During incipient nephropathy sodium retention and extracellular fluid volume load both increase, which further elevates the blood pressure (Arauz-Pacheco and Raskin 1996).

Diabetes causes intraglomerular hemodynamic changes (hyperfiltration and increased intraglomerular pressure and renal plasma flow) which can be stronger when systemic hypertension is present (Hostetter et al. 1982). High intraglomerular pressure induces mesangial stretch which during hyperglycemia increases production of TGF-β1 and collagen (Riser et al. 1998).

During overt nephropathy, the rate of decline in GFR can be effectively retarded by antihypertensive drugs (Parving et al. 1987). Urinary albumin excretion, as

well, decreases with antihypertensives (Parving and Rossing 1994). Angiontensin-converting enzyme (ACE) inhibitors may have other renoprotective effects in addition to the effect resulting from blood pressure lowering (Björck et al. 1992; Rumble et al. 1995), probably through the inhibition of the growth-promoting effect of angiotensin II (Wolf 1995). In the future, calcium antagonists may prove as useful as ACE inhibitors in renoprotection (Parving et al. 1996b). Even beta-blockers may be useful, since in young microalbuminuric patients glomerular structural changes did not progress after the start of enalapril or metoprolol treatment (Rudberg et al. 1999).

ENDOTHELIAL DYSFUNCTION AND INSULIN RESISTANCE IN ASSOCIATION WITH DIABETIC NEPHROPATHY

The endothelium covers the inner surface of all blood vessels. It regulates the adherence of leukocytes and platelets to the vessel wall, controls permeability of the vessel wall to macromolecules and regulates vascular tone (Haller 1997). Vascular tone is regulated by secretion of vasodilatating agents such as nitric oxide (NO) and prostaglandins. Damaged endothelial cells can secrete vasoconstricting agents such as endothelin-1 (ET-1) and thromboxane (Haller 1997) with disturbed balance between vasodilating and vasoconstricting factors being one marker of endothelial dysfunction. Degree of endothelial dysfunction can be estimated by plasma markers such as von Willebrand factor, endothelin-1, and plasminogen activator inhibitor type 1 (PAI-1) (Vischer et al. 1998; Huvers et al. 1999), or by use of a forearm vasodilatation test. One feature of diabetic microangiopathy is endothelial dysfunction (La Selva et al. 1993), which in type 1 diabetes precedes the onset of microalbuminuria (Stehouwer et al. 1995). Endothelial dysfunction is associated with insulin resistance, and hyperglycemia contibutes to both via differing mechanisms (Mäkimattila et al. 1996a). Glycated LDL may indirectly contribute to endothelial dysfunction, because it reduces NO synthesis and bioactivity and consequently the vasodilatory response seen in diabetes (Posch et al. 1999).

A variable degree of insulin resistance is typical of patients with type 1 diabetes (Yki-Järvinen and Koivisto 1986), resistance induced by hyperglycemia (Vuorinen-Markkola et al. 1992), and causing difficulties in glucose extraction (Mäkimattila et al. 1996b). Insulin resistance can be normalized by physical training (Yki-Järvinen et al. 1984). In type 1 diabetes, insulin resistance is associated with microalbuminuria (Yip et al. 1993) and precedes the latter (Ekstrand et al. 1998).

GROWTH FACTORS IN THE PATHOGENESIS OF DIABETIC NEPHROPATHY

Transforming growth factor-b1

With respect to diabetic nephropathy, transforming growth factor-β (TGF-β) is one important growth factor. Several reviews emphasize its position as a key mediator in diabetic nephropathy (Sharma and Ziyadeh 1995; Border et al. 1996; Sharma and Ziyadeh 1997a; Gilbert and Cooper 1999). The three mammalian isoforms of TGF-β, TGF-β1, TGF-β2, and TGF-β3 show a 70 to 80% sequence identity. Mature TGF-βs are all 25 kD homodimers with 112 amino acids in each monomer (Lawrence 1995). Since most research performed on diabetic nephropathy deals with TGF-β1, the focus here is on this isoform.

TGF-β1 is synthesized as a large precursor molecule which is cleaved by furin convertase into two parts, TGF-β1 and the precursor peptide; these two associate non-covalently with each other (Dubois et al. 1995). The precursor peptide is called a latency-associated peptide (LAP) and has an important function in maintaing latency of TGF-β1. Another protein, latent TGF-β1-binding protein (LTBP) usually associates with LAP. Thus, TGF-β1 is secreted from the cells as a large inactive complex which contains both LAP and LTBP. The activity of TGF-β1 is controlled extracellularly (Crawford et al. 1998). Most cultured cells secrete TGF-β1, including fibroblasts, lymphocytes, macrophages, and endothelial cells. Platelets contain a large reservoir of TGF-β1, and it is also found in the kidney (Roberts and Sporn, 1992).

The biological actions of TGF-β1 are multiple. TGF-β1 displays an antiproliferative action by lengthening or arresting the cell-cycle G1 phase. Through an antiproliferative action on B- and T-lymphocytes, TGF-β1 shows immunosuppressive activity. TGF-β1 enhances synthesis and deposition of extracellular matrix, as well as decreases its proteolysis. It controls cell adhesion receptor expression and cell differentiation (Massague 1990). The ability of TGF-β1 to induce progressive tissue fibrosis is explained by an exaggerated prolonged response to an injury which is caused by sustained production of TGF-β1 (Border and Noble 1994).

High ambient glucose concentration enhances TGF-β1 production through stimulation of the transcription rate of mRNA, the total TGF-β1 protein production and its bioactivity (Hoffman et al. 1998). In kidney cell cultures, high glucose stimulates TGF-β1 production in proximal tubular epithelial cells (Rocco et al. 1992), in cortical fibroblasts (Han et al. 1999), in mesangial cells (Ziyadeh et al. 1994), and in glomerular visceral epithelial cells (podocytes) (van Det et al.

1997). In addition, glomerular endothelial cells are capable of TGF-β1 production (Wolf et al. 1999). TGF-β1 production is also stimulated by angiotensin II (Wolf et al. 1993), mesangial stretch (Riser et al. 1998), and AGEs (Pugliese et al. 1997).

In rat experimental diabetes, increased glomerular staining for TGF-β1 protein correlated with albuminuria (Bertoluci et al. 1996). Increased expression of TGF-β1 protein and mRNA was found in renal tubules, and this tubular expression of TGF-β1 associated with signs of tubulointerstitial injury (Gilbert et al. 1998a). In human beings, strong staining for TGF-β1 was observed in glomeruli from six patients with advanced diabetic nephropathy (Yamamoto et al. 1993). In addition, in five patients with diabetic nephropathy, significant increases occurred in the immunoreactivity of all three isoforms of TGF-β both in the glomeruli and tubulointerstitium (Yamamoto et al. 1996). In 29 patients with type 2 diabetic nephropathy, intraglomerular TGF-β1 mRNA was increased and correlated with HbA_{1c} (Iwano et al. 1996).

Studies in man are few, because kidney biopsies are rarely performed in type 1 diabetic patients with normal AER or microalbuminuria, or even with overt diabetic nephropathy. For diabetic nephropathy urinary excretion of TGF-β1 can be regarded as a potential non-invasive marker (see also page 33). Urinary TGF-β1 seems to reflect TGF-β1 production in the kidney (Sharma et al. 1997b). In various studies of human glomerular diseases, urinary excretion of TGF-β1 has been associated with interstitial fibrosis (Murakami et al. 1997), progression of glomerular disease (Honkanen et al. 1997), and mesangial expansion (Sato et al. 1998). By performance of regular kidney biopsies in the early phases of human diabetic nephropathy with simultaneous urinary TGF-β1 measurements, important information about the role of TGF-β1 in the pathogenesis of nephropathy is likely to be obtained. The amount and localization of TGF-β1, whether in the tubulointerstitium, mesangium, or basement membrane, when compared to urinary excretion of TGF-β1, may provide valuable new information.

Insulin-like growth factor

Insulin-like growth factor-1 (IGF-1) shows a 50% sequence homology with proinsulin. IGF-1 contributes to the regulation of glomerular filtration rate and in diabetes to the renal hypertrophy (Hirschberg and Adler 1998). Mesangial cells from diabetic NOD mice secrete an increased amount of IGF-1 (Elliot et al. 1993). In the same cells, collagen degradation is decreased by IGF-1 (Lupia et al. 1999). Moreover, incubation of human or rat mesangial cells with one AGE,

bovine serum albumin-AGE, resulted in markedly increased production of IGF-1 (Pugliese et al. 1997). In young patients with type 1 diabetes, urinary IGF-1 has been associated with kidney volume. Levels of urinary IGF-1 and growth hormone were higher in patients with microalbuminuria than in patients with normal AER (Cummings et al. 1998). Growth hormone is an important regulator of IGF-1. A new growth hormone antagonist has prevented the rise in kidney IGF-1, kidney weight and glomerular volume in experimental diabetes (Flyvbjerg et al. 1999).

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a key regulator of connective tissue cells. PDGFs are dimers of A- and/or B-chains with PDGF-B up-regulated in atherosclerotic lesions (Betsholtz and Raines 1997). In mice deficient for PDGF-B, kidney mesangial cells were completely lacking, with only a single or just a few dilatated capillary loops and practically no urinary space present (Betsholtz and Raines 1997). PDGF has been shown to increase extracellular matrix production in the glomerulus (Johnson et al. 1992). However, in a model of a hyperglycemic GK rat which does not spontaneously develop nephropathy, PDGF treatment did not cause diabetic nephropathy (Riley et al. 1999). In cultured human mesangial cells, high glucose induced increased expression of PDGF, which further activated TGF-β1 gene expression (Di Paolo et al. 1996). In rats with one kidney removed, increased glomerular capillary pressure led to increased mRNA levels for PDGF and TGF-β1 (Shankland et al. 1994). Urinary PDGF was increased in patients with microalbuminuria or overt diabetic nephropathy when compared to patients with normal AER (Fagerudd et al. 1997). No other PDGF studies are available concerning humans with diabetic nephropathy.

Fibroblastic growth factor

Fibroblastic growth factor (FGF-2 or basic FGF) is a multifunctional growth factor thought to be involved in vascular homeostasis, atherosclerosis, wound healing, and angiogenesis (Gospodarowicz 1991). In normal human kidney it is expressed most consistently in glomerular parietal epithelial cells, distal tubular cells, and arterial endothelial cells (Floege et al. 1999). The expression of FGF-2 in diabetic kidney has not been studied. FGF-2 and TGF-β1 can interact with each other. FGF-2 stimulates the release of preformed, latent TGF-β1 in cultured proximal tubular cells (Phillips et al. 1997). In the same way, TGF-β1 upregulates the production of FGF-2 in cultured proximal tubular cells (Jones et al. 1999). Daily intravenous FGF-2 increases podocyte damage, proteinuria, and

glomerulosclerosis in rats with membranous nephropathy (Floege et al. 1995), suggesting a significant role for FGF-2 in damage to the filtration barrier, but in a hyperglycemic rat model, FGF-2 treatment has caused no diabetic nephropathy (Riley et al. 1999).

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is an important growth factor in the pathogenesis of diabetic retinopathy (Gilbert et al. 1998; Hammes et al. 1998; Smith et al. 1999), but few studies have been performed in diabetic nephropathy. A rat model of type 2 diabetic nephropathy demonstrated overproduction of VEGF (Tsuchida et al. 1999). Streptozotocin-induced diabetes was also associated with increased renal VEGF gene expression (Cooper et al. 1999).

IMMUNOLOGICAL FACTORS AND DIABETIC NEPHROPATHY

Several studies suggest the involvement of immunological cells in the pathogenesis of diabetic nephropathy. Bending et al. (1988) studied DR-positive T-lymphocytes from peripheral blood in patients with and without diabetic nephropathy. In patients with non-nephrotic-range proteinuria, a higher percentage of T-lymphocytes were activated than in patients without proteinuria but of same diabetes duration. T-lymphocytes have been found in the juxtaglomerular bodies of type 1 diabetic patients (Paulsen et al. 1994). In experimental diabetic nephropathy, an early increase of platelets appeared in glomeruli of diabetic rats, followed by a prominent macrophage infiltration from day 3 to a peak at day 30 (Young et al. 1995). In patients with type 2 diabetes, a transient infiltration of macrophages was observed during moderate diabetic glomerulosclerosis (Furuta et al. 1993). Furthermore, increased expression of P- and E-selectins was found in the glomeruli and interstitium of patients with type 2 diabetic nephropathy (Hirata et al. 1998). In a large series of patients with type 1 diabetic nephropathy, kidney biopsies often showed interstitial inflammation with infiltrated monocytes, macrophages, T-lymphocytes, fibroblasts, and fibrocytes in the interstitium (Bohle et al. 1991). The 5- and 10-year survival rates were best in patients with a normal appearance of the cortical interstitium (Bohle et al. 1991). Only a few studies have been published on this issue, and the role of the kidney-infiltrating T-cells remains open in diabetic nephropathy. The antigen-specificity of these T cells is unknown.

Two cytokines, secreted by immunological cells and relevant to diabetic nephropathy, are interleukin-1 (IL-1, see also page 20) and tumor necrosis factor (TNF). These cytokines have been studied in connection with AGEs. The

removal of AGEs by macrophages was associated with the release of interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Vlassara et al. 1988). In addition, TNF has enhanced several-fold the removal of AGEs by monocytes and macrophages by upregulating the receptor for AGEs (Vlassara et al. 1989). An AGE-induced effect is also suggested by the fact that GBM material isolated from diabetic rats induced elevated TNF and IL-1 secretion from cultured rat peritoneal macrophages, in contrast to GBM material of control rats or aminoguanidine-treated diabetic rats (Hasegawa et al. 1991). Recently, plasma TNF levels have correlated with glycemic control in type 1 diabetic patients (Lechleitner et al. 1999), and with proteinuria in type 1 diabetic patients with advanced diabetic nephropathy (Navarro et al. 1999). Both these cytokines have been found to act together with TGF-β1. IL-1β has induced TGF-β1 production in cultured proximal tubular cells when the cells were primed in hyperglycemic medium (Phillips et al. 1996). The combination of IL-1β and TGF-β1, or TNF-α and TGF-β1 had a synergistic effect on rat mesangial-cell fibronectin production (Pawluczyk and Harris 1998). These studies suggest that these cytokines produced by immunological cells may interact with growth factors produced by kidney cells.

The significance of autoantibodies in the pathogenesis of diabetic nephropathy is unclear. However, analogous mechanisms between atherosclerosis and glomerulosclerosis suggest the involvement of an autoimmune process (Diamond 1991). It has been observed that any simple modification of a protein can evoke autoantibody production (Steinbrecher et al. 1984). Thus, early glycation of a protein (Amadori formation) may cause an immune response (Witztum et al. 1983). Antibodies to glycated proteins have been found in diabetic patients. These antibodies bind to a common glucitol-lysine epitope (Witztum et al. 1984). In rats, preimmunization with glycated albumin led to an acceleration of diabetic nephropathy (Bassiouny et al. 1985).

In type 1 diabetic patients with microangiopathy, antibodies to oxidized LDL were lower than in patients without microangiopathy (Festa et al. 1998). In type 1 diabetes, antibodies to oxidized LDL were inversely related to coronary artery disease (Orchard et al. 1999). These studies raise the issue of the possible importance of oxidized LDL-containing immune complexes in the pathogenesis of diabetes-related vascular complications rather than free autoantibodies (Lopes-Virella et al. 1999). Recently an autoantibody to an AGE (CML) was found which was associated with end-stage diabetic nephropathy or non-diabetic kidney failure (Shibayama et al. 1999).

ENVIRONMENTAL FACTORS IN THE PATHOGENESIS OF DIABETIC NEPHROPATHY

Diet

Increased protein content of the diet causes increased protein traffic across the glomerular capillary, especially during microalbuminuria and overt nephropathy, traffic which induces damage in proximal tubular cells (Remuzzi et al. 1997; Bruzzi et al. 1997). A low-protein diet retards the progression of diabetic nephropathy by decreasing proteinuria and diminishing the decline in glomerular filtration rate (Hansen et al. 1999; Pedrini et al. 1996). Foods contain dietary glycotoxins, which readily react with AGE intermediates in the body to form AGEs. Browned, protein-rich food prepared with sugar are especially harmful in this respect. A roasted duck skin and a cake made in the oven at a high temperature have very high AGE contents (Koschinsky et al. 1997). Food with excessive fat may increase cholesterol and LDL which are associated with diabetic nephropathy and atherosclerosis (Groop et al. 1996). A vegetarian diet may be beneficial in preventing LDL oxidation (Korpela et al. 1999). Phenolic compounds of olive oil, and purple grape juice may decrease the susceptibility of LDL to oxidation (Caruso et al. 1999; Stein et al. 1999). Vitamins E and C may be useful in the prevention of diabetic nephropathy (Craven et al. 1997; Jain et al. 1999). Reduced sodium content of the diet may also be beneficial to the kidneys (Allen et al. 1997; Mülhauser et al. 1996a), especially after onset of microalbuminuria (Trevisan et al. 1998).

Smoking

In patients with type 1 diabetes, smoking increases all-cause and cardiovascular mortality (Moy et al. 1990; Rossing et al. 1996), but its significance as a risk factor for diabetic nephropathy is more complex. In some studies, smoking associates with the progression of type 1 diabetic nephropathy (Sawicki et al. 1994; Mühlhauser et al. 1996b), or type 2 nephropathy (Biesenbach et al. 1997; Forsblom et al. 1998). In other studies, smoking did not associate with the progression of nephropathy (Orchard et al. 1990; Gall et al. 1997). Since smoking is an established risk factor for atherosclerosis, and diabetic nephropathy is associated with macrovascular atherosclerotic disease, one would expect smoking to promote diabetic nephropathy. One possible explanation for the discrepancy is individual variation in the elimination of tobacco smoke substances. Tobacco smoke contains thousands of substances (Hoffmann and Wynder 1986; Marques and Beland 1994; Fischer et al. 1991), and several polymorphically distributed enzymes, such as NAT2 and NAT1 and cytocrom P 450 enzymes, participate in the metabolism of these substances. Tobacco smoke also contains reactive glycation products which can rapidly react with proteins to form AGEs (Cerami

et al. 1997). Increased levels of AGEs have been found in the lenses and blood vessels of smokers (Nicholl et al. 1998). Smoking does not, however, seem to worsen endothelial sensitivity (Helve et al. 1986).

PREDICTION OF DIABETIC NEPHROPATHY

At present it is impossible reliably to predict who of the young diabetic patients with normal AER will progress to diabetic nephropathy during their lifespan. Although intensive treatment and good metabolic control reduces the risk for diabetic nephropathy significantly (DCCT Research Group 1995), good metabolic control is not always achievable. It would be of considerable value if risk for diabetic nephropathy could be predicted at an early stage, and a special effort could be focused on those especially at risk.

URINARY MARKERS FOR DIABETIC NEPHROPATHY

Several urinary proteins and enzymes supplement urinary albumin as markers for diabetic nephropathy, a topic reviewed recently. These markers can be divided into glomerular, tubular, and other types of markers (Hong and Chia 1998). Albumin is traditionally considered a marker for glomerular dysfunction, and α1-microglobulin a marker for tubular dysfunction.

Albumin

After onset of microalbuminuria, the risk of progressing to diabetic nephropathy is about 50% (Krolewski et al. 1996). This risk may, however, vary greatly between different age groups (Couper et al. 1997), or at different levels of glycemic control (Bojestig et al. 1996), or after differing durations of diabetes (Forsblom et al. 1992). To identify those who will develop microalbuminuria is even more difficult. Baseline AER and glycated hemoglobin were the best independent predictors for microalbuminuria during a 10-year follow-up (Mathiesen et al. 1995). The rate of annual increase in AER seems to be important for prognosis (Almdal et al. 1994), as are borderline or intermittent increases in AER (Couper et al. 1997). As a common laboratory test, urinary albumin remains thus far the most useful predictive marker for diabetic nephropathy.

a1-microglobulin

α1-microglobulin is a low-molecular-weight plasma protein easily filtered in the glomerulus and reabsorbed in the proximal tubulus. Its amount in urine reflects tubular reabsorptive capacity of small plasma proteins. Its measurement in urine is useful for diagnosing tubular proteinuria in various clinical conditions (Itoh

and Kawai 1990). Simultaneous measurement in serum and urine allows detection of decreased GFR at early stages (Itoh and Kawai 1990). Urinary α1-microglobulin is increased in children with type 1 diabetes and correlates with glycosylated haemoglobin (Walton et al. 1988). A 7-year prospective study showed urinary α1-microglobulin not to be predictive for microalbuminuria in young patients with type 1 diabetes (Kordonouri et al. 1998).

N-Acetyl-b-D-glucosaminidase

N-Acetyl-β-D-glucosaminidase (NAG) is a tubular lysosomal enzyme. Urinary NAG excretion developed before microalbuminuria in a 7-year follow-up study of type 1 diabetic children and adolescents with normal AER. If NAG levels remained low, none of the patients progressed to microalbuminuria, but all patients with increased NAG developed later microalbuminuria (Kordonouri et al. 1998).

TGF-b1

Urinary TGF-β1 has been studied in experimental kidney diseases and in patients with non-diabetic and diabetic kidney diseases. Urinary excretion of TGF-β1 correlates with degree of glomerulosclerosis and interstitial fibrosis in rats with subtotal renal ablation (Monteiro et al. 1998). In patients with glomerulonephritis, urinary TGF-β1 activity associated with structural and functional renal changes (Dominguez et al. 1998). In patients with type 2 diabetes, urinary TGF-β1 excretion was higher in patients with severe mesangial expansion than in patients with low mesangial expansion (Sato et al. 1998). Urinary TGF-β1 was elevated in adult type 1 diabetic patients but could not distinguish between patients with normal AER, microalbuminuria, or overt nephropathy (Fagerudd et al. 1997). No studies have been performed in children and adolescents with type 1 diabetes in regard to urinary TGF-β1.

BLOOD MARKERS FOR DIABETIC NEPHROPATHY

Serum AGE and carboxymethyl lysine levels were increased in children and adolescents with type 1 diabetes (Berg et al. 1997a; Berg et al. 1998a). In a study by Chiarelli et al. (1999a), serum AGEs were markedly increased in adolescents and young adults with early microvascular complications. In addition, serum AGE levels predicted morphological changes in kidney during a 2.5-year follow-up (Berg et al. 1997b).

Erythrocyte sodium-lithium countertransport (Na+/Li+ CT) activity is associated with hypertension and hyperlipidemia in the general population (van Norren et al. 1998). When diabetic patients have been divided into two groups based on their

individual Na+/Li+ CT activity, elevated sodium-lithium countertransport levels have predicted microalbuminuria with a sensitivity of 85% and a specificity of 55% (Monciotti et al. 1997). Elevated Na+/Li+ CT activity may help to identify children and adolescents at risk for microalbuminuria (Chiarelli et al. 1999b).

These urinary or blood markers do not yet compete with urinary albumin in predicting diabetic nephropathy, because prospective studies thus far are few. In the future, a combination of genetic, urinary, and blood markers may serve together for risk profile estimation for diabetic nephropathy analogous to risk profile estimation for coronary artery disease (Wood et al. 1998).

AIMS OF THE STUDY

The aims of the study were

(I) to investigate the role of *N*-acetyltransferase-2 (NAT2) polymorphism as a candidate gene for type 1 diabetic nephropathy,

(II) to study the occurrence of antibodies to oxidized and glycated LDL in adult type 1 patients with and without diabetic nephropathy and their role as a marker for nephropathy-related atherosclerosis,

(III) to study the activation of transforming growth factor-β1 (TGF-β1) in peripheral blood mononuclear cells from type 1 diabetic patients with and without diabetic nephropathy,

(IV) to study urinary transforming growth factor-β1 and α1-microglobulin excretion in children and adolescents with type 1 diabetes.

PATIENTS AND CONTROLS

The four Studies included adult patients with and without type 1 diabetic nephropathy (I, II, III), children and adolescents with type 1 diabetes (I, IV), and healthy control subjects (I-IV) (Table 2). Studies I and II included British and Finnish adult type 1 diabetic patients and controls (Guy's Hospital, London and Department of Medicine, University of Helsinki, Helsinki), and Study I also young type 1 diabetic patients (Hospital for Children and Adolescents, University of Helsinki). Study III included Finnish type 1 diabetic patients and controls from the Department of Medicine, University of Helsinki, and Study IV type 1 diabetic children and adolescents from the Hospital for Children and Adolescents, University of Helsinki, and from the Aurora Hospital, Helsinki.

Table 2. *Study populations of Studies I-IV.*

Study		Normo	Micro	DN	Control	ACE inhibitor Normo/ Micro/DN
I	n (m/f) B/F	83 (42/41) 26/57	58 (35/23) 29/29	73 (41/32) 17/56	53 (29/24) 24/29	0/3/8
I	n (m/f)	70 (36/34)	5 (3/2)	-	-	0/2/-
II*	n (m/f) B/F	64 (31/33) 27/37	52 (35/17) 30/22	37 (17/20) 18/19	54 (30/24) 25/29	0/3/10
III	n (m/f)	30 (13/17)	12 (6/6)	10 (6/4)	13 (6/7)	3/10/8
IV	n (m/f)	105 (50/55)	8 (6/2)	-	39 (17/22)	0/2/-

B, British; F, Finnish

Normo, normal albumin excretion rate; Micro, microalbuminuria; DN, overt diabetic nephropathy

* Includes also 52 microalbuminuric patients

The clinical characteristics of patients and controls are presented in Table 3. The current stage of diabetic nephropathy was defined according to the median AER

of two to three overnight urine collections. The normoalbuminuric patients had a median AER less than 20 µg/min. The microalbuminuric patients had a median AER between 20 and 200 µg/min, and the nephropathic patients over 200 µg/min, or they were being treated by dialysis for end-stage renal disease.

Table 3*. Clinical characteristics of patients and controls. Data presented as means±SD except for AER median (range).*

Study	Subjects	Age (years)	Duration of diabetes (years)	Glycated hemoglobin (%)	AER rate (µg/min)
I	Diabetic patients with:				
	Normal AER	37.5±10.5	21.6±8.8	9.4±1.6 [a]	6 (0-19)
	Microalbuminuria	39.3±11.7	23.7±8.9	9.8±1.5 [a]	53 (6-192)
	Diabetic nephropathy	39.7±8.5	26.4±7.6	9.9±1.6 [a]	537 (48-3160)
	Healthy controls	38.6±10.3	-	6.1±0.5 [a]	4 (0-9)
I	Diabetic patients with:				
	Normal AER	13.1±3.2	6.3±2.3	9.3±1.5 [b]	4 (1-17)
	Microalbuminuria	15.4±2.8	10.5±2.8	10.3±2.1 [b]	52 (20-196)
II	Diabetic patients with:				
	Normal AER	37.6±10.1	22.5±8.6	9.4±1.6 [a]	7 (0-19)
	Microalbuminuria	40.3±11.8	24.2±9.2	9.8±1.5 [a]	53 (20-192)
	Diabetic nephropathy	37.8±8.6	24.4±7.1	9.7±1.5 [a]	433 (48-1756)
	Healthy controls	38.6±10.2	-	6.0±0.6 [a]	4 (0-9)
III	Diabetic patients with:				
	Normal AER	40.5±9.7	28.0± 7.3	8.3±1.3 [b]	5 (2-19)
	Microalbuminuria	39.5±9.0	28.3±8.6	8.6±1.6 [b]	75 (22-198)
	Diabetic nephropathy	36.6±8.9	28.0±7.7	8.9±1.5 [b]	308 (217-682)
	Healthy controls	33.9±8.7	-	-	-
IV	Diabetic patients with:				
	Normal AER	13.9±2.8	7.2±2.8	9.3±1.4 [b]	5 (2-19)
	Microalbuminuria	16.8±3	10.1±3.0	9.9±1.9 [b]	35 (20-196)
	Healthy controls	13.8±2.8	-	-	3 (1-29)

[a] HbA_1

[b] HbA_{1c}

For Study I NAT2 genotype was analyzed from the adult diabetic patients with and without nephropathy, while young Finnish type 1 diabetic patients were recruited for the parallel NAT2 genotype and phenotype analysis. Twenty-nine nephropathic patients were on dialysis. Smoking habits were asked by the study physician during the clinical investigation. Non-smokers were either never-smokers, or had a short smoking history (less than 12 years). Current smokers as well as those responding as non-smokers but having smoked over 12 years were classified as smokers. Of the adult normoalbuminuric patients 23% were smokers, of the microalbuminuric 42%, of the nephropathic 34%, and of the control subjects 28% (no difference between the groups). Six young patients (8%) smoked at least five cigarettes per day.

Study II included the bulk of adult diabetic patients from Study I. All participants had normal renal function with a serum creatinine concentration below 110 µmol/l. Patients taking diuretics or betablockers were excluded. None of the normoalbuminuric patients nor any of the healthy subjects took antihypertensive medication. Three microalbuminuric patients were on ACE inhibition, and one was taking a Ca-channel blocker. Seven nephropathic patients were being treated with ACE-inhibitors, one with a Ca-channel blocker, and three with a combination of ACE-inhibitor and Ca-channel blocker for hypertension. The presence of macroangiopathy was defined as a positive history for cardiovascular events (angina, myocardial infarction), or intermittent claudication associated with one or more absent foot pulses.

For Study III 52 adult type 1 diabetic patients were recruited in a consecutive manner. Of them, 21 were on ACE inhibitor medication. One patients was on dialysis. Seven normoalbuminuric, eleven microalbuminuric, and nine nephropathic patients had undergone retinal laser treatment. Eleven patients were smokers (21%). Blood pressure was measured in the right arm in a sitting position by use of a calibrated mercury sphygmomanometer after a 5-minute rest. Systolic (Korotkoff I) and diastolic (Korotkoff V) blood pressure was measured twice and the mean value used in analyses. The control individuals were recruited from the hospital and laboratory staff. None of them showed any signs of acute or chronic illness.

The recruitment criteria for Study IV were a minimum age of 7 years at the Hospital for Children and Adolescents, and 10 years at the Aurora Hospital, and diabetes duration of at least 3 years. Thirty-nine healthy subjects were recruited for a single timed overnight urinary sample for AER, TGF-β1 and α1-microglobulin measurements. Altogether 113 patients (53 from the Hospital for

Children and Adolescents and 60 from the Aurora Hospital), were included in the study. Exclusions numbered 73, because either no sample for TGF-β1 measurement or an inadequate number of overnight urine collections for AER measurement were avaílable. Neither glycemic control, duration of diabetes, insulin dose, nor serum creatinine differed between the included and excluded patients. At the Hospital for Children and Adolescents blood pressure was recorded at three consecutive visits at 3-month intervals in a sitting position by an automatic device (Dinamap Critikon, Tampa, FL). TGF-β1 and α1-microglobulin were analyzed from a single urine sample.

ETHICS

The study protocols were approved by the Ethics Committees of the Hospital for Children and Adolescents, of the Aurora Hospital, Helsinki, of the Department of Medicine, University of Helsinki, Helsinki, Finland, and of Guy's Hospital, London, UK. An informed consent was obtained from the participants.

METHODS

NAT2 GENOTYPING (I)

The fast acetylator allele *NAT2*4* and three slow acetylator alleles *NAT2*7B*, *NAT2*6A*, and *NAT2*5B* were detected by a method developed earlier (Abe et al. 1993). DNA samples were either dried EDTA-blood on blotting paper (Lin et al. 1993), or DNA extracted according to standard procedures. The 559-basepair *NAT2* genomic sequence was amplified by two oligonucleotide primers: 5' TGACGGCAGGAATTACATTGTC and 3'ACACAAGGGTTTATTTTGTTCC. The PCR mixture contained a DNA template, 10 pmol of each primer, dNTPs 200 µM, 10 mM Tris-HCl, pH 8.8, $MgCl_2$ 1.5 mM, KCl 50 mM, 0.1% Triton X-100, and 1.0 unit Taq DNA polymerase (Dynazyme, Espoo, Finland) in a final volume of 50 µl. Negative controls, either without a template or primers, were present after every 12 samples. The mixture was incubated for 5 minutes at 95°C, and then at 80°C for as long as Taq DNA polymerase was being added to the reaction tubes. The "Touch down" PCR method (Don et al. 1991) was applied to the blotting paper samples, where the following conditions were used: denaturation at 94 °C for 1 min, annealing for 1 min at decreasing temperatures from 65 until 55 °C, and extension at 72 °C for 1 min followed by a final extension period at 72°C for 7 min. For amplification, 35 cycles were performed by a programmable heat block (Perkin Elmer Cetus, Norwalk, CO, USA). For extracted DNA, amplification conditions were: denaturation at 94° for 50 seconds, annealing at 55°C for 50 seconds, extension at 65°C for 50 seconds, with 30 cycles until the final extension period.

After amplification, a mixture of 5 units of Asp 718, BamHI and TaqI (Boehringer Mannheim, Mannheim, Germany) was added in 10 µl aliquots of samples. The tubes were incubated at 37 °C for 3 hours and at 65 °C for 1 hour. The digestion was halted with 3 µl loading buffer in each tube. The digested PCR products were electrophoresed at 80 V for 2 hours on 3% Nusieve GTG agarose (FMC BioProducts, Rockland, ME, USA) mini gel containing 0.5 X TBE and 1:20,000 ethidium bromide (10 mg/ml). The DNA marker was ø174 DNA/Hae III marker (Promega, Madison, WI, USA), and alleles were recognized by distinctive allele fragments.

NAT2 PHENOTYPING (I)

Urinary 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1X) were measured by high-performance liquid chromatography

(HPLC) after caffeine ingestion. After emptying the bladder, each patient ingested caffeine 1-2 mg/kg. After 2 hours the bladder was emptied again, and urine collected for the following 2 hours. Urine sample pH was lowered to 3.5 with acetic acid (Lloyd et al. 1992), and the aliquots were stored at -20°C until analyzed.

Seventy µl (7 µg) of internal standard (4-acetamidophenol, Sigma, St. Louis, MO, USA) stock solution and 120 mg of ammonium sulphate were added to 200 µl of urine in a 15 ml centrifuge tube. The tube was mixed for 15 s. The metabolites were extracted with 6 ml of chloroform-isopropanol (95:5, v/v) by mixing for 30 s. Following centrifugation at 2,400 rpm for 5 minutes, the organic phase was transferred to another tube and evaporated under N_2 in a 40°C water bath. The dry residue was resuspended in 400 µl of 0.1% acetic acid, and 20 µl was injected into the HPLC column. The extracts were analyzed by the isocratic HPLC method (Grant et al. 1984) by an automatic Shimadzu LC-6A HPLC chromatography system with a UV-detector (Shimadzu Corporation, Kyoto, Japan). The reverse-phase column was Superspher 60 RP-select B (244 × 4 mm I.D., 4 µm) with a precolumn (10 × 5 mm I.D., 5 µm), both from Merck, Darmstadt, Germany. The mobile phase was made up of 0.05% acetic acid-methanol (88:12) at a flow-rate of 0.8 ml/min and monitored by ultraviolet absorbance at 280 nm. The stock solutions were 100 µg/ml AFMU in 0.1% acetic acid, 100 µg/ml 1X in distilled water, and 100 µg/ml internal standard in chloroform (Klebovich et al. 1993). The molar ratios AFMU to 1X were measured, and any ratio under 0.3 was classified as slow acetylation. Intra-assay coefficients of variation (n=9) were 5.9% for AFMU and 1.7% for 1X, and interassay coefficients of variation (n=5) were 2.9% and 1.8%, respectively. One chromatographic run was excluded because of its wide AFMU peak. AFMU was generously provided by Dr. G. Philippossian of Nestle, Vevey, Switzerland.

ANTIBODIES TO OXIDIZED AND GLYCATED LDL (II)

Antibodies to oxidized LDL were measured by ELISA with MDA-modified LDL as an antigen and native LDL as control antigen as previously described (Vaarala et al. 1993). MDA-LDL represents a prominent epitope of oxidized LDL (Palinski et al. 1989). Antibodies to glycated LDL were measured by ELISA in a manner otherwise similar to the ELISA for antibodies to oxidized LDL, but glucose-modified LDL served as the antigen. Glycated LDL was prepared by incubating native LDL in PBS containing 80 mM glucose in a nitrogen-saturated atmosphere for 7 days at room temperature. The glycated LDL subfractions were separated from the glucose-incubated LDL preparation by Sephadex-G25

(Pharmacia) desalting columns. Native LDL was treated in the same manner in the absence of glucose.

For ELISA, one half of a polystyrene plate (Nunc) was coated with native LDL and the other half with either MDA-LDL or glycated LDL (0.5 µg/well in PBS). The plates were incubated for 2 h at 37°C and then overnight at 4°C. After being washed four times with PBS containing 0.05% Tween and 0.001% Aprotinin (Sigma), plates were blocked with 2% human serum albumin in PBS for 2 h at room temperature. Serum samples were studied as duplicates, and diluted 1:50 in PBS containing 0.05% Tween and 0.2% human serum albumin. Samples were incubated for 2 h at room temperature. After washing, alkaline-phosphatase-conjugated rabbit anti-human IgG $F(ab)_2$ (Jackson ImmunoResearch), diluted 1:3000 in PBS-Tween-human serum albumin buffer, was added for 1 h at room temperature; 1 mg/mL p-nitrophenyl-phosphate (Sigma) in 50 mmol/L carbonate buffer containing 1 mmol/L $MgCl_2$, pH 9.8, served as the substrate. The reaction was stopped with 1 mol/L NaOH after 30 min and the color was read at 405 nm. The results were expressed as optical density (OD) values, and binding to oxidized or glycated LDL was calculated by subtracting OD values for native LDL from those for MDA-modified or glycated antigen, respectively. In both assays OD values for the modified antigen correlated with the final calculated values (for oxidized LDL; r =0.862, and for glycated LDL; r =0.861).

PBMC CULTURE (III)

Fresh heparinized venous blood was collected from each subject. PBMC were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation (3,400 rpm for initial Ficoll centrifugation and 2,000 rpm for washing). Mononuclear cells were washed three times in PBS and diluted to 1 x 10^6 cells/ml in RPMI-1640 supplemented with 5% pooled human AB+ serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and 2 mmol/l glutamine. Two-ml tissue culture wells (Nunc, Roskilde, Denmark) were used to culture 2 x10^6 PBMC at 37°C in a 5% CO_2 humidified incubator for 48 hours with and without phytohemagglutinin (PHA) at a concentration of 5 µg/ml. Cells and supernatants were collected after 24 hours and 48 hours and stored at -70°C, cells in Ultraspec™ solution (Biotecx, Houston, TX, USA).

For the lymphocyte proliferation test, 10^5 PBMC/well in 200 µl of medium were cultured in quadruplicates with and without PHA at 5 µg/ml for 3 days with [^{3}H]-thymidine (µCi/ml) present for the last 16 to 20 hours of the culture time. [^{3}H]-thymidine uptake was assayed by a liquid scintillation counter. The stimulation index (SI) was calculated by dividing the median value of PHA-stimulated cells by the median value of unstimulated ones.

REVERSE TRANSCRIPTASE-PCR FOR TGF-β1 mRNA (III)

Total RNA was extracted according to the Ultraspec™ procedure. The reverse transcriptase (RT) reaction was performed from 8 µl of total RNA by the First Strand cDNA Synthesis Kit (Pharmacia). cDNA samples were diluted serially at 1:5, 1:50, 1:500, and 1:5,000 (Halminen et al. 1997). β-actin served as a positive RNA control with dilutions at 1:5 and 1:50. For TGF-β1, primers 5´GCCCTGGACACCAACTATTGCT and 3´AGGCTCCAAATGTAGGGGCAGG (Leung et al. 1992), and for β-actin, primers TGA AGT CTG ACG TGG ACA TC and ACT CGT CAT ACT CCT GCT TG (Melby et al. 1993), were used. Each set of samples had two negative controls, one without TGF-ß1 primers, the other without a sample. The PCR conditions were as follows: hot start at 94°C for 2 minutes, 30 cycles with denaturation at 94 °C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute, and the last cycle with a final extension period for 10 minutes at 72°C. The PCR products were run on a 3% agarose gel (NuSieve GTG, FMC Bioproducts, Rockland, ME, USA), stained with ethidium bromide and photographed. Samples with at least one β-actin band were accepted for the final analysis. Intra-assay variation was 11% for sample dilutions at 1:500 and 1:5,000, and 17% for dilutions at 1:5 and 1:50. Inter-assay variations were 21% and 36%, respectively.

ASSAY FOR TGF-β1 (III-IV)

The concentration of TGF-β1 in the cell culture medium and in the urine was measured by enzyme-linked immunoassay (Honkanen et al. 1997). The microtiter plates were coated with monoclonal mouse antibody to TGF-β1-3 at 0.1 µg/well (Genzyme Diagnostics, Cambridge, MA, USA). To activate latent TGF-β1, diluted samples were incubated with 100 mmol/l HCl at 4°C for 2 h. Activated samples were added to the wells for overnight. The detection antibody was a chicken IgG-antibody reacting with human TGF-β1 (R&D Systems, Minneapolis, MN, USA), followed by a sheep antibody to chicken IgG (Serotec, Oxford, UK), and by an alkaline phosphatase-conjugated donkey antibody to sheep IgG (Serotec). Natural human TGF-β1 (R&D Systems, Abingdon, UK) served as a standard. The detection limit was 10 ng/L, but the lower limit for exact concentration was kept at 70 ng/L. All assays were performed as duplicates. The intra-assay and interassay coefficients of variation (CV) were 5.9 and 8.1%, respectively.

ASSAY FOR α1-MICROGLOBULIN (IV)

A double antibody radioimmunoassay was developed for the determination of α1-microglobulin. First, α1-microglobulin in urine competed with ^{125}I-labelled α1-microglobulin (Fitzgerald Ind., Concord, MA, USA) for binding to 500-fold

diluted rabbit antibodies to human α1-microglobulin (Dako, Copenhagen, Denmark). The antibody-bound α1-microglobulin was then precipitated by adding Sepharose-anti-rabbit IgG (Pharmacia, Uppsala, Sweden). The samples were centrifuged, and radioactivity of the pellets was measured. Standards were purchased from Orion Diagnostica (Espoo, Finland). The sensitivity of the assay was 0.05 mg/L, within-assay CV 3.9%, from day-to-day 5.1%.

CLINICAL LABORATORY TESTS (I-IV)

In Studies I and II, HbA_1 was assayed by microcolumn chromatography (Isolab Inc., Akron, OH, USA) in Helsinki, and by electroendosmosis (Corning Chemical, Palo Alto, CA, USA) in London. In Studies I and IV, HbA_{1c} was measured by an instant immunological device (DCA-2000, Bayer Diagnostics, Leverkusen, Germany) at the Hospital for Children and Adolescents, and by HPLC (Diamat analyzer, Bio-Rad, Anaheim, CA, USA) at the Aurora Hospital (normal range 4-6% with an interassay CV of 2-4% at both hospitals). In Study III, HbA_{1c} was determined by HPLC (Diamat Analyzer, Biorad Laboratories, Germany, normal range 4.0-6.0%).

In Studies I and II, urinary albumin was measured by radioimmunoassay (Keen and Chlouverakis 1963). In Study III, urinary albumin was measured by radioimmunoassay (Albumin-RIA, Pharmacia-Upjohn, Uppsala, Sweden) with an interassay CV of 4%. In Studies I and IV, urinary albumin was measured by immunoturbidometry (Hitachi 911 Analyzer, Hitachi, Tokyo, Japan, at the Hospital for Children and Adolescents, and Cobas Mira Analyzer, Roche Diagnostics, Basel, Switzerland at the Aurora Hospital). Interassay CV was 9.7 % at 43 mg/L at the Hospital for Children and Adolescents, and 5.6% in the range of 13-50 mg/L at the Aurora Hospital.

In Study III, serum glucose was measured by an automated hexokinase method with a reference range of 4.0-6.4 mmol/L and an interassay CV of 2% (Hitachi 917, Hitachi, Tokyo, Japan). In Study IV, urinary creatinine was measured by the Jaffe kinetic method (Hitachi 911, Hitachi, Tokyo, Japan). Urinary glucose was measured by an automated hexokinase method with a normal value of <0.1 g/L and an interassay CV of 2% (Hitachi 917, Hitachi, Tokyo, Japan).

STATISTICAL ANALYSIS

Differences in continuous parameters were tested by the Kruskal-Wallis analysis of variance when more than two groups were compared, and by the Mann Whitney U-test when two groups were compared (Studies I-IV). Differences in genotype and acetylator frequencies and percentages between groups were tested

by the Chi-Square test (Study I and III). Correlations were performed by the Spearman test (Studies I-II), or by the Pearson test (Studies III-IV). Logistic regression was used for calculating odds ratios and 95% confidence intervals for nephropathy. Regression analysis for repeated measurements was used for comparisons between the groups at two time-points (Study III). Multiple linear regression analysis was used to explore relationships among multiple variables (Study IV). For the Pearson correlation test and regression analyses those parameters not normally distributed were logarithmically transformed. P-value <0.05 was considered statistically significant.

RESULTS

NAT2 POLYMORPHISM, SMOKING, AND TYPE 1 DIABETIC NEPHROPATHY (I)

NAT2 allele frequencies in adult type 1 diabetic patiens

No difference was seen between adult type 1 diabetic patients and control subjects in NAT2 allele frequencies (p=0.82), neither were there any significant differences between normoalbuminuric, microalbuminuric, and nephropathic patients, and control subjects (p=0.90). Allele frequencies did not differ significantly between British and Finnish diabetic patients, respectively: the frequency of NAT2*4 was 0.26 and 0.31, NAT2*7B 0.01 and 0.04, NAT2*6A 0.25 and 0.26 , and NAT2*5B 0.49 and 0.39 (p=0.084).

NAT2 genotype and acetylator type distribution

The NAT2 genotype distribution was also similar in type 1 diabetic patients and control subjects (p=0.33), as well as in the normoalbuminuric, microalbuminuric, and nephropathic patients (p=0.86). No significant differences existed in the proportions of fast and slow acetylators between the diabetic subgroups and control subjects (p=0.71). The odds ratio of fast acetylators for microalbuminuria and diabetic nephropathy was 0.94 (95% CI 0.54-1.64), p=0.84 by logistic regression. British and Finnish subjects did not differ.

Effect of smoking

When the non-smoking and smoking diabetic patients were analyzed separately, NAT2 allele frequencies differed significantly between the non-smoking normoalbuminuric, microalbuminuric, and nephropathic patients, p=0.013 (Table 4). In addition, the proportion of fast acetylators differed between non-smoking and smoking subgroups (Fig.2). In the normoalbuminuric group, the frequency of fast acetylators was 33% among non-smokers, and 71% among smokers. The corresponding figures for the microalbuminuric group were 68% and 45%, and for the nephropathy group 52% and 42% (p=0.025). In non-smokers with a fast acetylation capacity, the odds ratio for microalbuminuria and diabetic nephropathy was 3.1 (95% CI 1.36-7.05), p=0.007. In smokers, the corresponding odds ratio was 0.31 (95% CI 0.08-1.2), p=0.09. The odds ratios for non-smoking British and Finnish patients with fast acetylation capacity were 3.0 (95% CI 0.90-9.75) and 3.0 (95% CI 0.92-9.53), and in smokers 0.34 (95% CI 0.05-2.13) and 0.21 (95% CI 0.02-2.22), respectively. In non-smoking patients with long-duration diabetes (≥23 years, median for these diabetic patients) the

risk for nephropathy in fast acetylators was even higher 6.33 (95% CI 1.87-21.4); *p*=0.003, but 1.53 (0.48-4.83); *p*=0.47 in patients with diabetes duration less than 23 years. Due to the small number of patients in the smoking groups, the effect of the duration of diabetes was not possible to study.

Table 4. *NAT2 allele frequencies in non-smoking (N) and smoking (S) diabetic patients with subgroups of normal urinary albumin excretion (Normo), microalbuminuria (Micro) and overt diabetic nephropathy (DN). Number indicates allele numbers in each group.*

NAT2 allele (N/S)	Diabetic patients (99/46)	Normo (48/14)	Micro (28/20)	DN (23/12)	Control (37/15)
NAT2*4	0.27/0.29	0.18 [a]/0.39	0.41 [a]/0.25	0.30 [a]/0.25	0.28/0.17
Number	54/27	17/11	23/10	14/6	21/5
NAT2*7B	0.03/-	0.04 [a]/-	- [a]/-	0.02 [a]/-	0.05/-
Number	5/-	4/-	-/-	1/-	4/-
NAT2*6A	0.26/0.28	0.35 [a]/0.21	0.18 [a]/0.33	0.17 [a]/0.29	0.26/0.33
Number	52/26	34/6	10/13	8/7	19/10
NAT2*5B	0.44/0.42	0.43 [a]/0.39	0.41 [a]/0.43	0.50 [a]/0.46	0.41/0.50
Number	87/39	41/11	23/17	23/11	30/15

[a] *p*=0.013 by Chi Square test

NAT2 phenotyping in young type 1 diabetic patients

The AFMU/1X molar ratio (mean±SD) in 70 young type 1 diabetic patients was 0.54±0.04 for fast acetylators, and 0.13±0.006 for slow acetylators ($p<0.0001$), with a cut-off point of 0.30. The caffeine test revealed 41% to be fast and 59% slow acetylators. The concordance rate between phenotyping and genotyping tests was 97%. Two patients were slow acetylators according to the phenotyping test but had only one recognizable slow acetylator allele: molar ratios 0.14 (NAT2*4/NAT2*5B) and 0.25 (NAT2*4/NAT2*6A). Fast and slow acetylator genotype frequencies were 0.41 and 0.59 for normoalbuminuric patients (n=70), and 0.80 and 0.20 for microalbuminuric patients (n=5), respectively. Fast and slow acetylator frequencies did not differ between young type 1 diabetic patients and adult Finnish control subjects ($p=0.7$). Neither age, duration of diabetes, HbA_{1c}, nor AER differed between fast and slow acetylators in this group of young diabetic patients.

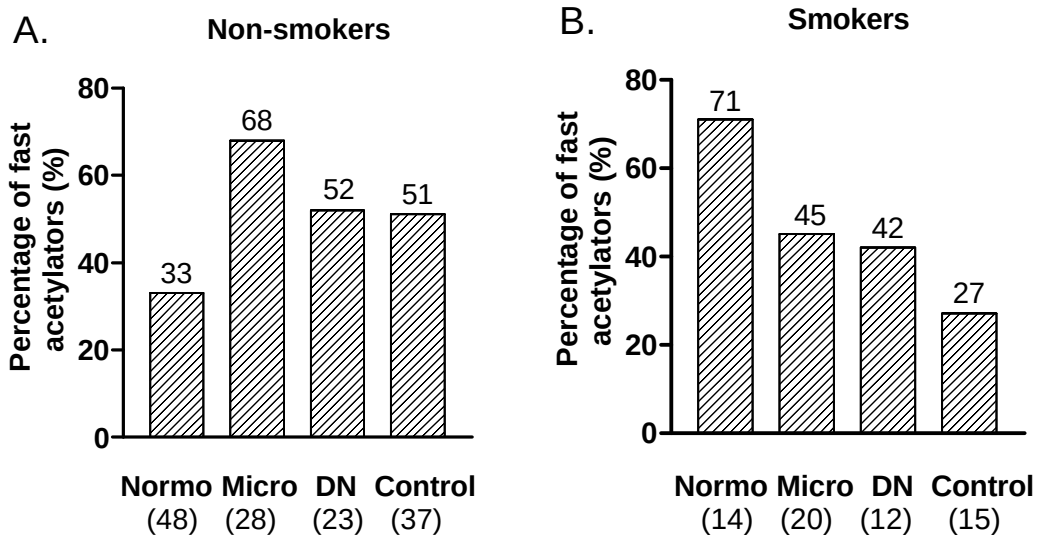

Figure 2. A*: Fast acetylators in the non-smoking subgroups of 99 type 1 diabetic patients and 37 controls. Chi Square p=0.031.* ***B****: Fast acetylators in the smoking subgroups of 46 type 1 diabetic patients and 15 controls. Normo, normal urinary albumin excretion; Micro, microalbuminuria; DN, overt diabetic nephropathy; Control, control subjects. Above the bars, percentages of fast acetylators, under the bars, numbers of subjects in each group.*

IMMUNE RESPONSE TO GLYCATED AND OXIDIZED LDL IN TYPE 1 DIABETIC PATIENTS WITH AND WITHOUT RENAL DISEASE (II)

The results regarding microalbuminuric patients have not been published earlier.

Antibodies to glycated LDL

The mean antibody level against glycated LDL was higher in diabetic patients than in healthy subjects, but the difference was not significant (0.242±0.343 vs. 0.165 ± 0.22; p=0.14). Nephropathic patients had higher antibody levels to glycated LDL (0.388 ± 0.459) than normoalbuminuric patients (0.258 ± 0.354), or microalbuminuric patients (0.116±0.125), p=0.007 for the diabetic groups and p=0.008 for all groups (Fig 3A). Microalbuminuric patients had significantly lower antibody levels than nephropathic patients (p=0.004), but normoalbuminuric and nephropathic patients did not differ from each other (p=0.195). Antibody levels to glycated LDL did not correlate with HbA_1 (r =-0.033), or with AER (r =-0.012) in diabetic patients.

Antibodies to oxidized LDL

Antibody levels to oxidized LDL did not differ between the three groups, but diabetic patients as a group had lower antibody levels to oxidized LDL than did

healthy controls (0.150 ± 0.094 vs 0.180 ± 0.105 OD, p=0.04) (Fig. 3B). Antibodies to oxidized LDL correlated inversely with HbA_1 in diabetic patients (r =-0.193, p=0.02), and even more strongly in nephropathic patients (r=-0.460; p=0.005). Antibodies to oxidized LDL did not correlate with AER (r=0.137; p=0.092). Antibodies to oxidized LDL did not correlate with glycated LDL in any of the groups.

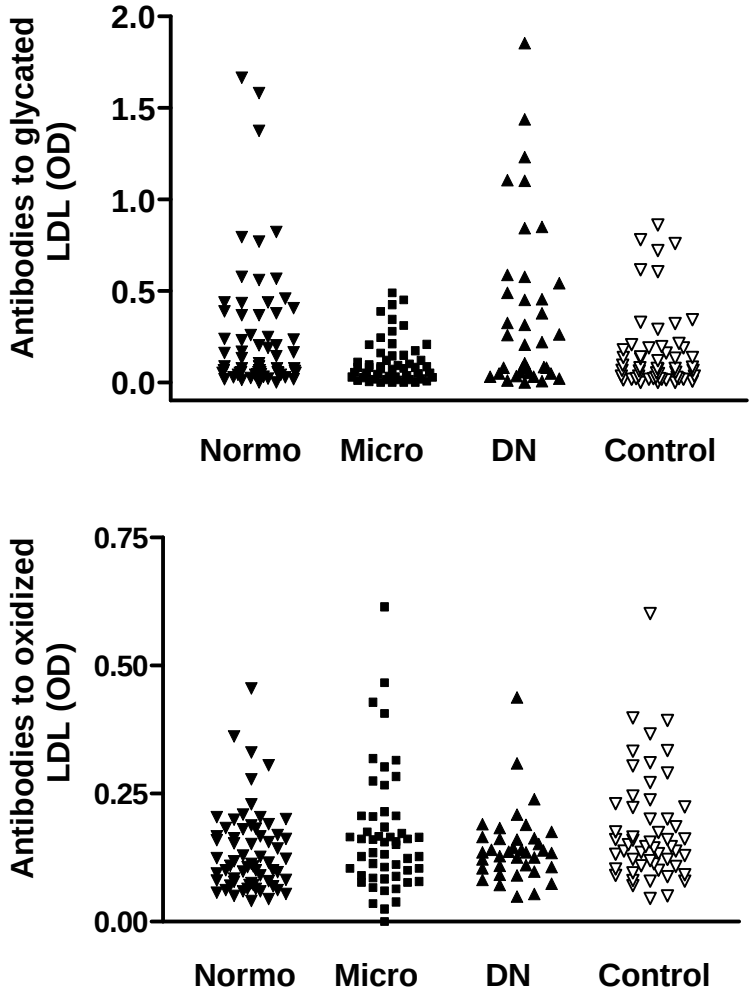

***Fig 3A and B**. Antibodies to glycated and oxidized LDL as optical density units (OD) in normoalbuminuric, microalbuminuric, and nephropathic type 1 diabetic patients and in healthy control subjects.*

Normoalbuminuric patients had lower LDL cholesterol concentrations (2.7±0.8 mmol/L) than microalbuminuric patients (3.2±0.9), nephropathic patients (3.2±0.9 mmol/L) or healthy subjects (3.1±0.7 mmol/L, p=0.003). LDL cholesterol concentrations did not correlate with antibodies to oxidized LDL in diabetic patients (r=0.010, p=0.4), but an inverse correlation was found between LDL cholesterol and antibodies to glycated LDL (r=-0.194, p=0.016).

In the nephropathic group, no significant difference was found in antibody levels or in other laboratory or clinical parameters between patients with and without macroangiopathy (Table 4). One patient in the normoalbuminuric group (2%), 13 patients in the microalbuminuric group (25%), and 18 patients in the nephropathic group (49%) had clinical macroangiopathy.

Table 4*. Presence of macroangiopathy in nephropathic patients, and its relation to clinical and laboratory parameters.*

	Macroangiopathy		
	Yes	No	*p*-value
n	18	19	
Sex (m/f)	8/10	9/10	
Age (years)	39.9±9.2	35.8±7.7	0.153
Duration of diabetes (years)	25.5±7.6	23.4±6.7	0.316
HbA_1 (%)	9.6±1.7	9.8±1.4	0.557
LDL cholesterol (mmol/l)	3.1±0.9	3.2±0.9	0.090
Antibodies to glycated LDL (OD)	0.353±0.506	0.421±0.421	0.438
Antibodies to oxidized LDL (OD)	0.141±0.057	0.150±0.083	0.843

Data are means ± SD

OD, optical density units

TGF-β1 PRODUCED BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM TYPE 1 DIABETIC PATIENTS WITH AND WITHOUT DIABETIC NEPHROPATHY (III)

TGF-b1 mRNA expression

No differences were found in PBMC TGF-β1 mRNA expression between the diabetic subgroups at either time-point. After 24 h culture, the unstimulated and PHA-stimulated mRNA expression titers correlated significantly (r=0.31, p=0.04). No correlation existed between TGF-β1 mRNA expression and HbA_{1c} nor between TGF-β1 supernatant and mRNA levels in the diabetic patients.

TGF-b1 secreted by PBMC

After 48 h culture, the highest TGF-β1 levels secreted by unstimulated PBMC were found in patients with nephropathy (6.2 (0.9-20.0) ng/ml) when compared to levels in patients with normal albumin excretion (4.1 (0.2-11.3) ng/ml), with microalbuminuria (1.8 (0.2-6.4) ng/ml), and healthy controls (1.0 (0.2-7.0) ng/ml); p=0.02 for the three diabetic groups and 0.006 for all groups (Fig. 4).

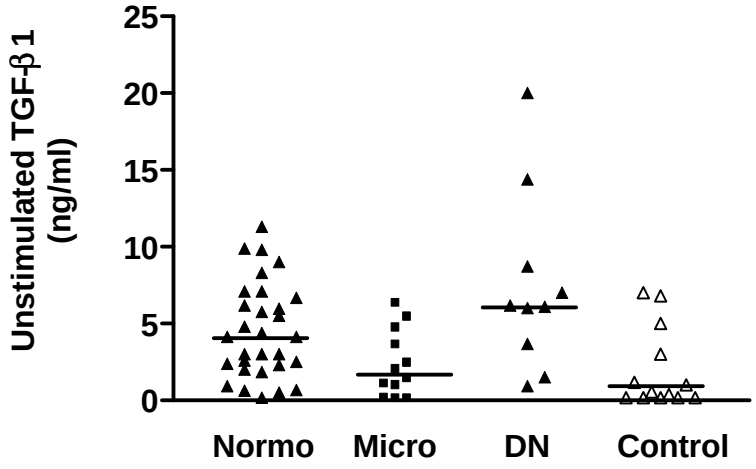

Fig. 4. *TGF-b1 levels secreted by unstimulated peripheral blood mononuclear cells after 48 h culture in type 1 diabetic patients with normal albumin excretion (Normo), microalbuminuria (Micro), overt nephropathy (DN), and healthy controls (Control) (p=0.006 for all groups and 0.02 for the diabetic groups).*

The presence of PHA in the culture medium resulted in an increase in TGF-β1 protein secretion. The PHA-stimulated TGF-β1 levels of PBMC were 12.4 (2.9-30.0) ng/ml in nephropathic, 7.3 (0.5-21.2) ng/ml in normoalbuminuric, 5.5 (0.5-27.6) ng/ml in microalbuminuric patients, and 2.0 (0.4-17.5) ng/ml in controls (p<0.05 for all groups and 0.05 for the diabetic groups, Fig. 5). Patients with overt nephropathy differed from patients with microalbuminuria with respect to their PBMC TGF-β1 secretion in both unstimulated and PHA-stimulated cultures (p<0.05).

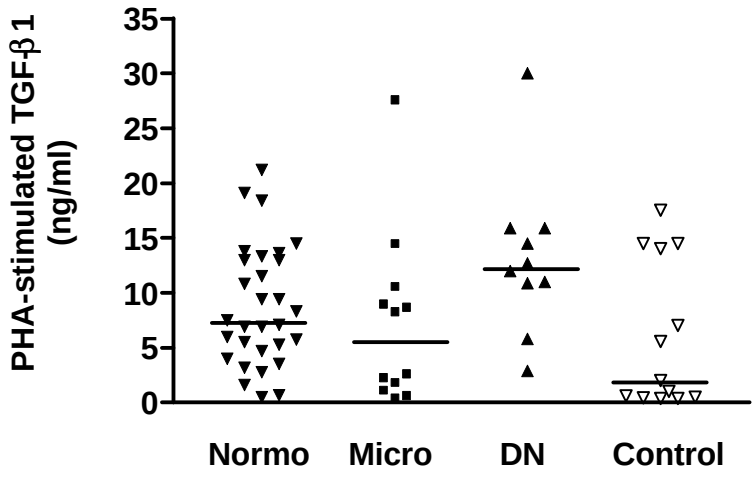

Fig 5. *TGF-b1 levels secreted by PHA-stimulated PBMC after 48 h culture in type 1 diabetic patients with normal albumin excretion (Normo), microalbuminuria (Micro), overt nephropathy (DN), and healthy controls (Control) (p<0.05 for all groups and 0.05 for the diabetic groups).*

When the unstimulated TGF-β1 levels at 24 and 48 h culture were analyzed by regression analysis, PBMC from patients with overt nephropathy secreted higher levels of TGF-β1 than PBMC from patients with microalbuminuria and normal albumin excretion (p=0.004 and p=0.03, respectively), or from controls (p=0.003) (Fig. 6). The unstimulated TGF-β1 levels were higher after 48-hour than after 24-hour culture in 22 out of 52 diabetic patients compared to none of the healthy subjects (p=0.004).

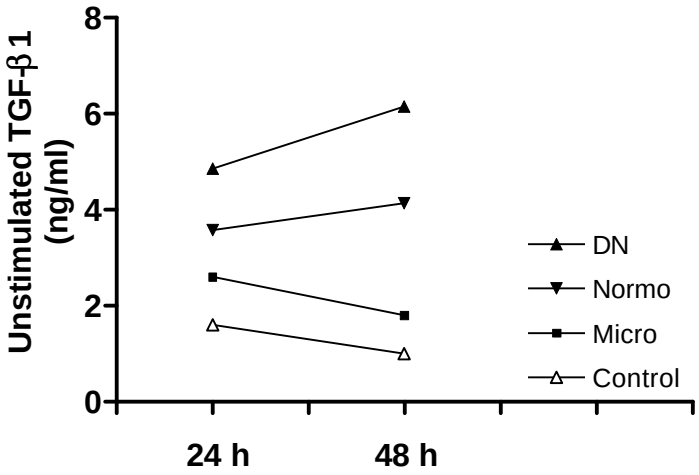

Fig 6. *The median levels of TGF-b1 secreted by unstimulated PBMC after 24 and 48 h in type 1 diabetic patients with normal albumin excretion (Normo), microalbuminuria (Micro), overt nephropathy (DN), and healthy controls (Control); (DN vs. Micro, p=0.004; DN vs. Normo, P=0.03; DN vs. Control, p=0.003).*

Correlation between TGF-b1 and clinical parameters

A correlation was observed between PHA-stimulated and unstimulated TGF-β1 levels and diastolic blood pressure in diabetic patients (r=0.3, p=0.03 for both), and between unstimulated TGF-β1 levels and diastolic blood pressure in a combined patient group with incipient and overt nephropathy (r=0.45, p=0.04). PHA-stimulated or unstimulated TGF-β1 levels did not correlate with HbA_{1c} (r=-0.09, p=0.5; r=-0.12, p=0.4, respectively), or with AER (r=0.07, p=0.6; r=-0.04, p=0.8, respectively) in diabetic patients.

Proliferation responses

No difference was observed in proliferation response of PBMC to PHA between the groups (p=0.7 for all groups and p=0.6 for the diabetic groups).

URINARY TGF-β1 AND α1-MICROGLOBULIN IN CHILDREN AND ADOLESCENTS WITH TYPE 1 DIABETES (IV)

Urinary TGF-b1

Type 1 diabetic patients had higher urinary TGF-β1 concentrations than control subjects: 0.9 (0.05-122.3) vs. 0.3 (0.05-2.2) ng/mg creatinine (median (range)), p=0.003. Microalbuminuric patients did not differ from normoalbuminuric patients in TGF-β1 levels (p=0.4, Fig. 7). Urinary TGF-β1 correlated with the concurrent urinary glucose concentration (r=0.2, p=0.03), and α1-microglobulin concentration (r=0.2, p=0.02), but urinary TGF-β1 showed no correlation with

HbA_{1c} in patients with type 1 diabetes (r=-0.03, p=0.7), nor with age in diabetic patients or controls. The mean systolic and diastolic pressure of three consecutive visits, available in 51 diabetic patients, did not correlate with TGF-β1 (r=0.2, p=0.1 for diastolic pressure and 0.2 for systolic pressure). AER or duration of diabetes did not correlate with urinary TGF-β1. In the multiple linear regression model containing α1-microglobulin and urinary glucose, neither of these variables showed any independent association with urinary TGF-β1 with an adjusted R^2 of 0.04.

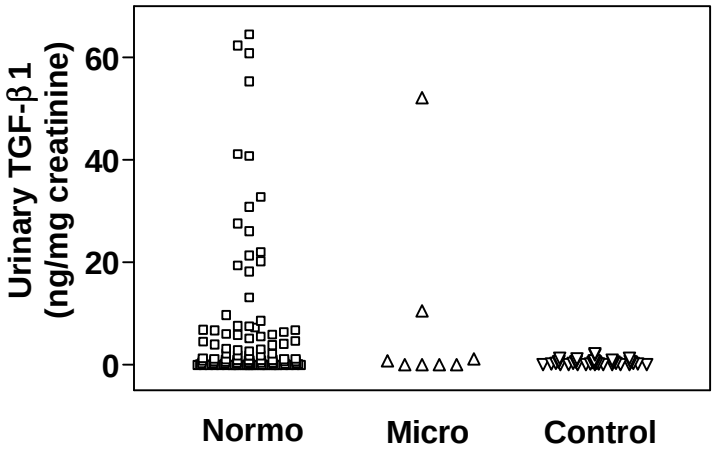

Fig 7*. Urinary TGF-b1 levels in children and adolescents with normal AER (Normo) or microalbuminuria (Micro), and in healthy subjects (Control). A single value of 122 ng/mg creatinine in the microalbuminuric group is not visualized. Diabetic patients vs. controls, p=0.003.*

Of the diabetic patients, 43 (38%) had urinary TGF-β1 concentrations exceeding the levels seen in the controls (over 2.25 ng/mg creatinine). In these 43 patients, urinary TGF-β1 correlated with urinary glucose (r=0.6, p<0.001, Fig. 8), and α1-microglobulin (r=0.6, p<0.001). The correlation between urinary TGF-β1 and HbA_{1c} was not significant (r=0.3, p=0.07). The correlation between urinary TGF-β1 and mean systolic pressure was not significant (r=0.4, p=0.11). Systolic pressure values were available in only 15 of 43 patients. In the multiple linear regression model including α1-microglobulin and urinary glucose, α1-microglobulin independently associated with urinary TGF-β1 (p=0.02) with adjusted R^2 of 0.35.

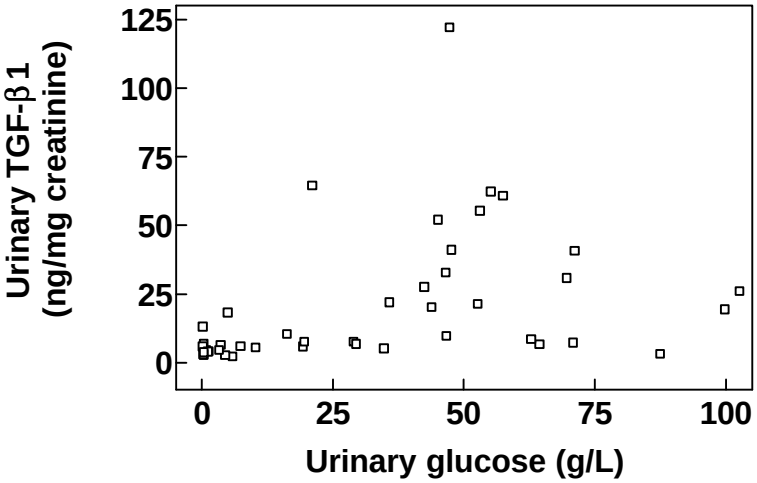

Fig 8. *Urinary TGF-b1 and urinary glucose in a subgroup of children and adolescents (43/113) with type 1 diabetes and urinary TGF-b1 exceeding levels in healthy subjects. r=0.6, p<0.001.*

Urinary a1-microglobulin

Type 1 diabetic patients had higher urinary α1-microbglobulin levels than control subjects: 4.8 (0.6-48.8) vs. 2.7 (0.8-11.6) µg/mg creatinine, $p<0.001$. In diabetic patients, urinary α1-microglobulin correlated with urinary AER ($r=0.2$, $p=0.02$), HbA_{1c} ($r=0.3$, $p<0.001$), and with urinary glucose ($r=0.5$, $p<0.001$) in addition to urinary TGF-β1. Multiple linear regression analysis of these four variables showed that urinary glucose ($p<0.001$) and TGF-β1 ($p=0.046$) independently influenced urinary α1-microglobulin levels (adjusted R^2 0.28). HbA_{1c} and AER had no significant effect ($p=0.07$ and 0.08, respectively).

Urinary AER

In diabetic patients, urinary AER correlated with age ($r=0.4$, $p<0.001$), duration of diabetes ($r=0.3$, $p=0.001$), systolic blood pressure ($r=0.4$, $p=0.01$), and urinary α1-microglobulin ($r=0.2$, $p=0.02$). In the linear regression model including these variables, duration of diabetes independently associated with AER ($p=0.04$).

DISCUSSION

GENETIC FACTORS ASSOCIATED WITH TYPE 1 DIABETIC NEPHROPATHY

The genes which carry the risk for diabetic nephropathy are thus far poorly identified, but it seems evident that the genetic background of diabetic nephropathy is multifactorial. The candidate gene studies provide a confusing picture with contradictory results partly due to a great variation in the criteria for cases and controls. The present thesis presents a new candidate gene for diabetic nephropathy. Non-smokers with the fast NAT2 genotype had an increased risk for incipient and overt diabetic nephropathy (odds ratio 3.1). Since the odds ratio for smokers was the opposite (0.3), the overall result regarding NAT2 genotype and diabetic nephropathy was negative.

None of the earlier studies regarding NAT2 polymorphism and diabetic complications comprised separate analyses between the non-smoking and smoking subjects (Madácsy et al. 1992; Neugebauer et al. 1994; Agúndez et al. 1996; Gawronska-Szklarz et al. 1997). As for smokers, the small number of smokers limited the analyses, but results suggested that the risk for diabetic nephropathy is decreased in smokers with the fast NAT2 genotype. This is a logical finding, since the slow acetylation capacity may lead to slower metabolism of tobacco-derived toxic substances and possibly to a higher risk for diabetic nephropathy. NAT2 polymorphism may in part explain why the results concerning smoking as a risk factor for diabetic nephropathy are inconsistent. More difficult to explain is the metabolic mechanism of the association of fast acetylation genotype with diabetic nephropathy in non-smokers.

A clue is provided by a study regarding insulin levels during an intravenous glucose tolerance test in fast and slow acetylators with diabetes. In fast acetylators the higher fasting insulin and first-phase insulin peak levels observed (Burrows et al. 1978) may reflect a higher prevalence of insulin resistance among fast acetylators. Since insulin resistance is linked with diabetic nephropathy (Yip et al. 1993; Kikkawa and Kojima 1996), it is possible that our finding can be explained by the association of fast acetylation capacity and insulin resistance. An even more speculative explanation could be the possible role of NAT2 in the metabolism of nephrotoxic agents in diabetes, such as advanced glycation end products (AGEs). Formation of AGEs produces highly reactive toxic

intermediates which remain partly uncharacterized (Bucala and Vlassara 1995). Serum and tissue AGE levels correlated with severity of diabetic nephropathy (Makita et al. 1991).

The mechanism behind the association of NAT2 gene polymorphism and diabetic nephropathy may not rely on acetylation capacity. Linkage with a causal gene near the NAT2 locus on chromosome 8p22 is possible. No genome-wide searches have thus far been published suggesting susceptibility loci for type 1 diabetic nephropathy. In type 2 diabetic nephropathy, a recent genome-wide study in Pima Indians pointed out chromosomes 7, 3, 9, and 20 as containing loci potentially linked to nephropathy (Imperatore et al. 1998). These do not include the NAT2 gene on chromosome 8. However, the Pima Indian population differs genetically from the Caucasian population. Although the distribution of NAT2 alleles among Pima Indians is unknown, it can be anticipated that it differs from that in Caucasians. For example, prevalence of the D allele of an ACE polymorphism is lower in Pima Indians than in Caucasians (8% versus 27.5%) (Foy et al. 1996). The distribution of NAT2 alleles varies significantly in different populations. Two distinct ethnic origins, Japan and Polynesia, produce high percentages (88-93%) of fast acetylators (Clark 1985), whereas among Egyptians and Israelis, percentages of fast acetylators are only 18 to 33%. An interesting feature is that Japanese and Polynesians show an increased prevalence of diabetic nephropathy not fully explained by conventional risk factors (Simmons et al. 1994; Matsushima et al. 1995). Genes relevant to the pathogenesis of diabetic nephropathy may differ in different populations. Therefore, genome-wide searches in the Caucasian population with and without type 1 diabetic nephropathy would be valuable, and could clarify the position of NAT2 among other candidate genes.

The study of NAT2 polymorphism and diabetic nephropathy emphasizes an important aspect of gene studies: the interaction of genes and environmental factors. We showed that an environmental factor, smoking, confounded the gene effect to the extent that no association could be seen without analyzing data separately taking this factor into account. The question is whether these kinds of confounding environmental factors have been taken into account in the gene analyses regarding diabetic nephropathy. One example is diet, which can vary with respect to dietary glycotoxins, lipid composition, sodium content, and protein intake (Pinelli et al. 1999; Koschinsky et al. 1997; Ying and Sanders 1998; Toeller et al. 1997). All these factors play a role in the pathogenesis of diabetic nephropathy. In addition, physical exercise and BMI may prove to be important environmental factors associated with insulin resistance. It is not even known whether poor but stable metabolic control is more beneficial than

fluctuation of metabolic control. Therefore in genetic studies it would be useful to characterize patients very carefully with respect to their diets, lipid levels, exercise habits, BMI, and smoking. In addition to these patient characteristics, the rate of nephropathy progression would be valuable to know.

IMMUNOLOGICAL FACTORS ASSOCIATED WITH TYPE 1 DIABETIC NEPHROPATHY

Antibodies to glycated and oxidized LDL

An altered immune response to glycated and oxidized LDL was found in diabetic patients when compared to that in healthy individuals. However, antibody responses to glycated and oxidized LDL were quite different, and these two antibodies did not correlate with each other in diabetic patients. Normoalbuminuric and nephropathic patients showed an enhanced immune response to glycated LDL, but microalbuminuric patients resembled healthy individuals. In contrast, antibodies to oxidized LDL were decreased in all diabetic patients compared to levels in healthy individuals, but the diabetic groups did not differ from each other.

Antibodies to modified LDL may reflect the extent of LDL modification in vivo (Lopes-Virella et al. 1996). The percentage of glycated LDL in type 1 diabetic patients is elevated (Klein et al. 1995; Posch et al.1999), and correlates with glycemic control (Lyons et al 1986). In patients with poorly controlled diabetes, LDL was more susceptible to oxidation than LDL from control subjects (Tsai et al. 1994), whereas in patients with well-controlled type 1 diabetes, the opposite was true (Jenkins et al. 1996). When oxidation of LDL was studied in type 2 diabetic patients, the percentage of glycated ApoB in LDL correlated with the susceptibility of LDL to oxidation (Moro et al. 1999).

Antibody levels to oxidized LDL were decreased in diabetic patients in the present study. In contrast, in patients of comparable age, antibodies to oxidized LDL were 1.5-fold increased (Mäkimattila et al. 1999). Another study also found increased antibodies to oxidized LDL in type 1 patients, but the patients were younger and their duration of diabetes was shorter than in the present study. Levels of antibodies to oxidized LDL correlated inversely with age in that study (Festa et al. 1998). The two latter studies used copper-induced oxidation compared to our MDA-induced oxidation in the preparation of oxidized LDL antigen. MDA is one of the epitopes present in copper-oxidized LDL which contains several other epitopes as well. This difference cannot explain the discrepancy between findings of our study and the two other studies, since

antibody levels to MDA-modified LDL are usually higher than antibody levels to copper-oxidized LDL (Bellomo et al. 1995; Ahmed et al. 1999a).

We did not find a difference in antibody levels to oxidized LDL in nephropathic patients with and without clinical signs of macroangiopathy. It is possible that the small number of patients in each group, and the cross-sectional design of the study has led to a false negative finding. In type 2 diabetic patients of 10 years follow-up, antibody levels to oxidized LDL were not predictive of cardiovascular events, but the authors speculated that the use of copper-induced oxidation of LDL may have reduced the sensitivity of the study (Uusitupa et al. 1996). In non-diabetic subjects, antibodies to MDA-modified LDL have been predictive for the progression of carotid atherosclerosis (Salonen et al. 1992), and myocardial infarction (Puurunen et al. 1994), but not for stroke (Ahmed et al. 1999a). Alternatively, the mechanisms of atherosclerosis may differ at least in part between diabetic atherosclerosis and common atherosclerosis.

An inverse correlation between HbA_1 and antibodies to oxidized LDL was found in our type 1 diabetic patients. This finding was repeated later by Festa et al. (1998), who also found that antibody levels to oxidized LDL were lower in patients with microvascular complications. The latter finding may in part result from a difference in age and duration of diabetes between their patients with and without microvascular complications (duration 22 vs.11 years), a difference absent from our patients. However, immune complexes were found only in patients with low antibody titers. Recently, antibodies to oxidized LDL were found to be inversely related to coronary artery disease in the subsequent 8 years of follow-up in type 1 diabetes (Orchard et al. 1999). In the same patients circulating immune complexes (oxidized LDL *vs.* antibody to oxidized LDL) were present, suggesting consumption of free antibodies (Lopes-Virella et al. 1999). Other circulating immune complexes, those containing antibodies to cardiolipin were found in 83% of type 1 diabetic patients compared to 5% of healthy controls. In patients with vascular complications, the prevalence of circulating immune complexes was as high as 91% (Ahmed et al. 1999b). Immune complexes may prove to be more important than free autoantibodies in the pathogenesis of vascular complications. It is possible that in our study the decreased antibody level in diabetic patients compared to that in healthy individuals reflected the presence of immune complexes. An inverse correlation between antibodies to oxidized LDL and HbA_1 suggests that poor glycemic control increases the formation of immune complexes and increases the risk for diabetic complications.

Few studies are available regarding antibodies to glycated LDL. Similarly to the situation in normoalbuminuric and nephropathic patients in the present study, antibodies to glycated LDL were increased in type 2 diabetic patients compared to levels in healthy controls. However, antibodies to glycated LDL were not associated with vascular complications (Bellomo et al. 1995). Neither could we see any association of these antibodies with diabetic nephropathy or macrovascular complications. Because the clinical characteristics of the type 2 patients differed from our type 1 diabetic patients, the results are not totally comparable; the short mean duration of diabetes (8 years) resulted in a low proportion of patients with incipient or overt diabetic nephropathy. It is, however, evident that glycated LDL is immunogenic and may lead to autoantibody production and formation of immune complexes (Witztum et al. 1983). Glycated LDL enhances cholesteryl ester synthesis in human monocyte-derived macrophages (Lopes-Virella et al. 1988). These two factors may be related to atherogenesis in diabetes.

PBMC-secreted and urinary TGF-b1 in type 1 diabetic patients with and without diabetic nephropathy

Two non-invasive means were used to study activation of TGF-β1 in diabetes. PBMC isolated from patients with overt type 1 diabetic nephropathy secreted higher levels of TGF-β1 than did PBMC from patients with normal AER or microalbuminuria, or from healthy subjects. Urinary TGF-β1 was elevated in one-third of young diabetic patients with normal AER. Neither PBMC-secreted nor urinary TGF-β1 correlated with AER or glycemic control.

According to several studies, glucose is an important activator of TGF-β1. High ambient glucose stimulates TGF-β1 bioactivity and gene transcription in cultured glomerular epithelial, mesangial, and proximal tubular cells (Nakamura et al. 1992; Rocco et al. 1992; van Det et al. 1997; Hoffman et al. 1998, Han et al. 1999). Nothing is known about interindividual variation in response to ambient glucose. In diabetes, plasma glucose varies according to diet, exercise, and insulin treatment. This variation and its effect on TGF-β1 secretion could not be studied in *ex vivo* isolated PBMC which were cultured in a constant 11 mmol/L glucose concentration. In urinary TGF-β1 excretion, we observed an association with simultaneous urinary glucose. However, this association seemed more to reflect tubular dysfunction than a direct effect of glucose, because a marker of tubular dysfunction, α1-microglobulin, and urinary glucose did not independently predict urinary TGF-β1. In addition, the association between urinary TGF-β1 and urinary glucose was rather low.

An interesting feature was the presence of low urinary TGF-β1 excretion in the majority of young type 1 diabetic patients (62%), despite high HbA_{1c} and urinary glucose concentrations. Thus, in a substantial group of the diabetic patients, hyperglycemia did not lead to increased urinary TGF-β1 excretion. Another interesting feature was that PBMC from 22 of 52 (42%) diabetic patients secreted increasing levels of TGF-β1 when secretion was measured at two time points, after 24 and 48 hours culture. In contrast, PBMC isolated from healthy controls did not increase their TGF-β1 secretion between the two time-points. Thus, PBMC from over half the diabetic patients resembled that of healthy controls in this respect. The reasons for these differences are not known, but genetic regulation may play a role (Grainger et al. 1999).

Large interindividual variation in TGF-β1 secretion by PBMC was observed both in patients and controls, as recently reported in healthy individuals at mRNA level (Pociot et al. 1998). In diabetic patients these large *in vitro* differences in TGF-β1 secretion cannot be explained by glycemic control, since HbA_{1c} did not correlate with TGF-β1 levels. The variation in urinary TGF-β1 excretion was also high, and was not associated with glycemic control. This finding was in accordance with a study performed in adult type 1 diabetic patients (Ellis et al. 1998). It is noteworthy that TGF-β1 is an important growth factor in many non-diabetic fibrotic diseases such as cirrhosis of the liver, lung fibrosis, and inflammatory kidney diseases (Border and Noble 1994). Recently it was found to be involved in human atherosclerosis (Bobik et al. 1999). Perhaps the fact that TGF-β1 is such a wide-spread growth factor can explain the lack of correlation between TGF-β1 and the two key factors in the pathogenesis of diabetic nephropathy (AER, glycemic control). Moreover, various growth factors and cytokines may act together, boosting the combined effect, as observed concerning IL-1β and TGF-β1 (Phillips et al. 1996), and TNF and TGF-β1 (Pawluczyk and Harris 1998). Since other growth factors are also activated in the diabetic milieu (Elliot et al. 1993; Di Paolo et al. 1996), have effects similar to those of TGF-β1 (Lupia et al. 1999; Johnson et al. 1992), and interact with TGF-β1 (Di Paolo et al. 1996; Phillips et al. 1997), the relative importance of a single growth factor may decrease.

One genetic polymorphism of TGF-β1, C509T in the promoter region of the gene, has been shown to contribute to plasma TGF-β1-levels (Grainger et al. 1999). Another polymorphism of TGF-β1 has been shown to associate with blood pressure. In a study by Cambien et al. (1996), one of the polymorphic alleles, Arg25, when present homozygously, was associated with higher blood pressure than was the Pro25 allele. This was confirmed by Li et al. (1999) in hypertensive and normotensive patients. In addition, a correlation has been

observed between circulating TGF-β1 levels and blood pressure (Li et al. 1999). In the present study, a correlation was found in type 1 diabetic patients between PBMC TGF-β1 secretion and diastolic blood pressure. The correlation between systolic pressure and urinary TGF-β1 in a subgroup of patients with urinary TGF-β1 above control levels was also rather high (r=0.4), although not significantly (p=0.11), probably due to the small number of patients. The association of TGF-β1 and blood pressure is interesting, because patients with overt nephropathy have a familial predisposition to essential hypertension (Fagerudd et al. 1998). TGF-β1 production in these patients may be related to their susceptibility to hypertension.

Hypertension is associated with increased intraglomerular pressure, which is an important pathogenetic factor in diabetic nephropathy (Zatz et al. 1985; Hostetter 1994). Cyclic mechanical force causing mesangial stretching and capillary expansion, which mimic glomerular hypertension, has been shown to stimulate production of extracellular matrix in rat mesangial cells (Riser et al. 1992), and TGF-β1 is involved in this process (Hirakata et al. 1997; Yasuda et al. 1996). In type 2 diabetic patients urinary TGF-β1 correlated with degree of mesangial expansion (Sato et al. 1998). The present study in young diabetic patients showed an early rise in urinary TGF-β1 in one-third of diabetic patients with normal AER, suggesting that an accumulation of extracellular matrix in kidney mesangium may take place. Increased levels of PBMC-secreted and urinary TGF-β1 can be seen as a marker for the capacity of an individual to produce TGF-β1, a potentially harmful growth factor in relation to diabetic nephropathy.

Diabetic patients with microalbuminuria

Microalbuminuric patients showed lower TGF-β1 secretion by PBMC and lower antibody levels to glycated LDL than did normoalbuminuric or nephropathic patients. The main risk factors for diabetic nephropathy include duration of diabetes, glycemic control, and hypertension. In both studies, duration of diabetes was the same for microalbuminuric and for nephropathic patients. As shown in Table 3, differences in glycated hemoglobin cannot quite explain the slower development of nephropathy in microalbuminuric patients. Unfortunately, blood pressure levels were not available in Study II. In Study III, nephropathic patients had higher blood pressure than did microalbuminuric patients. The reasons for the various rates of progression of diabetic nephropathy have not been fully elucidated. Type 1 diabetic patients with microalbuminuria are generally considered high-risk patients for overt diabetic nephropathy (Viberti et al. 1982; Mogensen and Christensen 1984; Krolewski et al. 1985), although recently it has been estimated that only about half the microalbuminuric patients will ever

proceed to diabetic nephropathy (Krolewski et al. 1996). The risk for proceeding to overt diabetic nephropathy is relatively low after long-duration diabetes (Forsblom et al. 1992). Our results for microalbuminuric patients regarding PBMC TGF-β1 secretion and antibodies to glycated LDL suggest that immunological factors may modify the progression of diabetic nephropathy. These factors can be regarded as indirect contributors rather than direct causal factors.

PATIENTS AND METHODS

Patient and control selection and design of the studies

In Study I, patients and controls were either British (36%) or Finnish (64%) Caucasians. This was a disadvantage in our study, because in candidate gene studies, cases and controls should have similar genetic backgrounds (Witte et al. 1999). To ensure that our results were not due to a confounding effect caused by population stratification (Witte et al. 1999), all the analyses were made separately as well as combined for British and Finnish patients. No differences in the results were evident.

Smoking history obtained from the patients and controls in Study I included the current or >12-year smoking history in the past but not the amount of cigarettes per day, duration of smoking, or pack-years (20 cigarettes per day equals one pack-year; Benbow et al. 1997). It is difficult to evaluate whether this kind of detailed information would have improved the reliability of the results. The group of smokers was too small to make any dose-response analyses even had the pack-years been available. A further disadvantage was that smoking data came from only 197 individuals of 267 (74%).

Study II included 207 patients and controls of British and Finnish origin. The levels of antibodies are known to be regulated by several factors including environmental factors. Antibody levels to glycated LDL were higher in Finnish than in British individuals, although the trends between the subgroups were similar between the two populations. A uniform British or Finnish population would have been preferable to the combined one in this study as well.

All the four studies were cross-sectional. Especially in Studies III and IV, several follow-up samples and especially samples taken before and after the start of ACE inhibitors would have furnished valuable new information.

Laboratory methods

In Study I one three slow NAT2 alleles were analyzed out of the at least 12 currently identified (Vatsis et al. 1995). At the beginning of the study, however, knowledge of the alleles was limited. The four alleles were reported to cover approximately 95% of acetylation types (Hickman and Sim 1991), which was confirmed by our own caffeine test performed in children and adolescents. Therefore, the genotyping can be considered rather reliable to separate fast and slow acetylators.

In Study II, antibodies to oxidized LDL were analyzed according to a method developed earlier in our laboratory. Oxidized LDL was prepared by incubating LDL with malondialdehyde, an end-product of lipid peroxidation. The preparation of glycated LDL for antibody measurement was a difficult task. The reason for the variation in the quality of prepared antigen after incubation of LDL with glucose in a nitrogen atmosphere remains unclear. The antigenic epitopes are poorly characterized for both oxidized and glycated LDL. No international standards are available for these methods, and the comparisons of the results between different laboratories are difficult to perform.

The measurement of TGF-β1 secretion by PBMC (Study III) cultured for 48 hour is a biological assay which is influenced by multiple factors. PBMC were isolated by Ficoll Hypaque gradient centrifugation which distinguishes red blood cells under the Ficoll Hypaque layer, and mononuclear cells above it, and plasma including thrombocytes is supposed to stay in the topmost layer. It has been estimated that the yield of thrombocytes is under 0.5% of the total thrombocyte count of the original blood sample when blood is obtained from a healthy donor (Kanof and Smith, 1991). We performed no special procedures except washes by PBS to purify the mononuclear cells. It is therefore possible that TGF-β1 released from thrombocytes has affected our results to some extent, since TGF-β1 enhances its own mRNA production (van Obberghen-Schilling et al. 1988). No correlation was observed between current thrombocyte levels and PBMC TGFβ1 secretion.

SUMMARY AND CONCLUSIONS

Sustained hyperglycemia is a central predisposing factor for long-term microvascular complications in patients with type 1 diabetes mellitus. Because the level of long-standing glycemia does not fully explain the presence of diabetic complications, it is possible that genetic or immunological factors can protect against or predispose to microvascular complications. Our aim was to characterize some genetic or immmunological features in type 1 diabetic patients with and without early (microalbuminuria) or fully developed (overt) diabetic nephropathy.

The methods included PCR with restriction fragment length polymorphism for *N*-asetyltransferase (NAT2) genotyping, measurements of two caffeine metabolites in urine by HPLC for NAT2 phenotyping, ELISA assays for measuring antibodies to oxidized and glycated LDL and for measuring urinary transforming growth factor-β1 (TGF-β1) from cell supernatants and urine. Peripheral blood mononuclear cells were isolated and cultured to determine mRNA activation of TGF-β1 and to quantitate TGF-β1 secreted by cells. Urinary α1-microglobulin was measured by radioimmunoassay.

NAT2 gene polymorphism was investigated in 214 adult patients with and without diabetic nephropathy. NAT2 is a cytosolic enzyme which participates in the metabolism of certain drugs and foreign compounds in the liver. Several polymorphic alleles of the NAT2 gene are known, and individuals are fast or slow acetylators depending on their NAT2 allele combination. We found that fast acetylators had an increased risk for early and overt diabetic nephropathy (odds ratio 3.1; 95% confidence interval 1.4-7.1). This phenomenon was seen only in non-smokers, which can be explained by the fact that smoking as such is a strong risk factor for nephropathy and thus a confounding factor for NAT2.

Equal levels of antibodies to oxidized (MDA-modified) LDL were found in 153 diabetic patients with normal AER, microalbuminuria, and overt nephropathy. Antibody levels for oxidized LDL correlated inversely with glycemic control, a finding which was later repeated by Festa et al. (1998). The mechanism behind this correlation may be related to immune complex formation leading to low levels of free antibodies in patients with high levels of the substrate (e.g., glycoxidized LDL) for these antibodies. Antibodies to glycated LDL when compared to other diabetic patients or controls, were lowest in patients with microalbuminuria.

To explore a possible genetic predisposition to high TGF-β1 production, we studied TGF-β1 activation in peripheral blood mononuclear cells (PBMC) from 52 adult type 1 diabetic patients with and without diabetic nephropathy. TGF-β1 is considered a key cytokine mediator in various kidney diseases. It enhances mesangial matrix expansion, which is a central event in diabetic glomerulosclerosis. TGF-β1 secretion by PBMC was highest in diabetic patients with overt nephropathy when compared to patients with normal AER and with microalbuminuria, and healthy individuals. There was, however, considerable overlapping between the groups.

Young type 1 diabetic patients (n=113) were studied for two urinary markers for diabetic nephropathy: TGF-β1 and α1-microglobulin. α1-microglobulin is a marker for tubular dysfunction. Young diabetic patients excreted elevated levels of both TGF-β1 and α 1-microglobulin. One-third of diabetic patients with normal AER had increased TGF-β1 excretion. Urinary TGF-β1 levels did not correlate with any conventional risk factors for nephropathy, such as HbA_{1c} or duration of diabetes.

The following conclusions can be drawn from the Studies I to IV:
(I) In non-smokers, the fast NAT2 genotype may be a new risk factor for type 1 diabetic nephropathy. Smoking is a confounding factor in NAT2 analyses regarding diabetic nephropathy.
(II) Elevated levels of antibodies to glycated LDL found in diabetic patients do not seem to be associated with nephropathy or nephropathy-related atherogenesis. Antibodies to oxidized LDL correlated inversely with glycated HbA_1-levels in type 1 diabetic patients. This suggests that hyperglycemia may modify the circulating levels of these antibodies.
(III) PBMC from type 1 diabetic patients with overt nephropathy showed increased TGF-β1 secretion, but in diabetic patients PBMC TGF-β1 secretion did not associate with AER. PBMC TGF-β1 secretion correlated in diabetic patients with diastolic blood pressure. These findings suggest that the TGF-β1 secretion measured from PBMC is not directly related to the pathogenesis of diabetic nephropathy, but may reflect risk for vascular disease in diabetes.
(IV) Urinary TGF-β1 is increased in young type 1 diabetic patients. Urinary TGF-β1 associated with two interrelated tubular markers: α1-microglobulin and urinary glucose.

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