Cellular basis of diabetic nephropathy: IV Antioxidant enzyme mRNA expression levels in skin fibroblasts of type 1 diabetic sibling pairs

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Abstract

Background. Blunted cultured skin fibroblast (SF) antioxidant enzyme responses to hyperglycaemia are associated with diabetic nephropathy risk. The present study explores whether this association is, at least in part, genetically determined.

Methods. We measured glomerular structure and SF mRNA expression for catalase and glutathione peroxidase in 21 sibling pairs concordant for type 1 diabetes. All patients had four or more (mean 21.5) years of diabetes and glomerular filtration rate >40 ml/min/1.73 m². Thirty-four patients were normoalbuminuric, four were microalbuminuric, three were proteinuric and one was not classifiable. Heritability of patient characteristics was assessed by intra-class correlation and by a genetic variance component model.

Results. Mesangial fractional volume, mesangial matrix fractional volume, glomerular basement membrane width and surface density of peripheral glomerular basement membrane per glomerulus were significantly correlated in these sibling pairs. Catalase mRNA expression levels were also related and highly heritable in these sibling pairs. The association between sibship and glutathione peroxidase mRNA expression levels did not reach statistical significance.

Conclusions. This study suggests that SF catalase mRNA expression levels, known to be associated with diabetic nephropathy risk, are in part genetically determined.

Keywords: genetics of diabetic nephropathy; glomerular structure; type 1 diabetes

Introduction

Family studies have shown strong concordance for diabetic nephropathy (DN) risk in type 1 and type 2 diabetes [1–5]. This suggests that DN risk, although related to glycaemia [6], is substantially genetically determined [3,7,8]. Skin fibroblast (SF) behaviours after multiple in vitro passages are associated with DN risk [9–11]. Moreover, sodium/hydrogen exchanger isoform 1 (NHE-1) activity [12] is concordant in type 1 diabetic sibling pairs who are concordant for DN lesions [13].

Oxidative stress has been linked to the pathogenesis of the β-cell destruction involved in the initiation of type 1 diabetes [14–17]. The idea tested here that antioxidant responses are, at least in part, inherited, derive from studies showing increased oxidative stress in non-diabetic relatives of type 1 diabetic patients [18]. It is also known that there are differences in antioxidant enzyme activity among different races. Both lower activity [19] and increased DN risk [20,21] are present in African patients. Oxidative stress has also been implicated in the pathogenesis of DN [22]. When grown in high as compared with normal glucose conditions, SF from type 1 diabetic patients with DN showed a marked impairment in the expected increases in mRNA expression, protein and activity levels for catalase and glutathione peroxidase [23].

Thus, SF of type 1 diabetic subjects with DN have a blunted antioxidant enzyme response and increased oxidative stress on exposure to high glucose [23]. These results were largely confirmed by Chiarelli et al. [24]. Moreover, the parallel findings for catalase and glutathione peroxidase mRNA expression levels in monocytes from type 1 diabetic patients [25] and in erythrocytes from type 2 diabetic patients [26] suggest that these phenomena might be widespread among different cell types.
Antioxidant enzymes gene expression in type 1 diabetic sibling pairs

We reported strong familial concordance for DN lesions and patterns of lesions among largely normoalbuminuric type 1 diabetic sibling pairs [13]. We have also used the type 1 diabetic sibling pair model to study the familial relationships of SF sodium-hydrogen (Na⁺/H⁺) antiport activity [12], a marker associated with DN risk [9,10,27]. Using this model, we demonstrated concordance in this SF behaviour between sibling pairs concordant for type 1 diabetes and concluded that, there was at least in part, genetic regulation of this cellular activity in association with DN risk [12]. Herein we studied mRNA expression for catalase and glutathione peroxidase in type 1 diabetic sibling pairs to determine whether variations in antioxidant enzyme mRNA levels are genetically regulated.

Subjects and methods

Patients

Twenty-one type 1 diabetic sibling pairs had research renal biopsies and renal function studies in the University of Minnesota (U of MN) General Clinical Research Center (GCRC). They all had at least 4 years of diabetes, serum creatinine \( \leq \) 2.0 mg/dL, glomerular filtration rate (GFR) \( >40 \) ml/min/1.73 m² and no other kidney disease. Thirty-four (80%) had no clinical or laboratory evidence of renal disease (see subsequently). Studies were approved by the committee for the use of human subjects in research of the U of MN and prior informed consent was always obtained. Glomerular structural parameters of 17 [13] and SF Na⁺/H⁺ antiport activity of 14 [12] of these sibling pairs have been reported.

Clinical studies

Blood pressure (BP) was measured with an oscillometric monitor as described [28]. Hypertension was defined as BP \( \geq 130/85 \) mmHg [29,30] or use of antihypertensive drugs. Glycated haemoglobin (HbA₁c) was measured by HPLC, serum and urinary creatinine by the Jaffe’s reaction and albumin excretion rate (AER) by a fluorimetric assay [31] in three 24-h urine collections. Patients were classified as normoalbuminuric (AER < 20 \( \mu \)g/min), microalbuminuric (AER 20–200 \( \mu \)g/min) or proteinuric (AER > 200 \( \mu \)g/min) [31]. Patients who were microalbuminuric or proteinuric before antihypertensive treatment were classified according to their pre-treatment AER values. GFR was estimated by the mean of three 24-h creatinine clearances which are highly correlated \( (R = 0.92; \ P < 0.001) \) with inulin clearances performed during the same GCRC admission [32]. Retinopathy was assessed by fundoscopy [31].

Renal morphometric analyses

Glomerular basement membrane (GBM) width [33], mesangial, mesangial matrix and mesangial cell fractional volumes [Vv(Mes/glom), Vv(MM/glom) and Vv(MC/glom), respectively] [31,34,35], and surface density of the peripheral GBM [Sv(PGBM/glom)] [31,33] were measured as detailed elsewhere. Reference ranges for glomerular structural parameters were derived from 76 normal living kidney transplant donors [33 males; age 37.6 \( \pm \) 12.1 (19–64) years].

Cellular studies

Skin biopsy and cell culture. Skin tissue was obtained with a 3 mm punch at the kidney biopsy site and processed as detailed elsewhere [11].

RNA isolation. The modified single-step isolation method [36] was used to extract SF total RNA and the quantity and quality determined as described [11]. No samples required exclusion for RNA degradation. Total RNA was adjusted to 0.05 g/L and aliquots frozen at \(-70^\circ\)C in RNA Storage Solution (Ambion Inc, Austin, Texas) containing 20 U/\muL of SUPERase In² (Ambion Inc, Austin, Texas) until assayed. Pooled total RNA from SF of six normal subjects was the ‘reference’ standard. A set of eight 2-fold serial dilutions of the 0.4 ng/ml RNA standard was prepared and stored in aliquots at \(-70^\circ\)C until used.

Quantitation of unknown samples by real-time reverse transcriptase polymerase chain reaction (RT–PCR). The primers and the Taqman fluorogenic probe were designed using PE Primer Express Version 1.5 software to produce an amplicon spanning an intron. TTCACACTGCCAATGATGAT was the forward primer, CCTCATTCAACGCTTTTCACA was the reverse primer and TTGAAGATGCCGGAGACTCTCCCC was the probe for catalase. ACCCGCTTATGACCGAC, GGCACACGTGGCGACACA and CCAAGCTCATACACCCTGTCCTCGGAG was the forward primer, reverse primer and probe for glutathione peroxidase, respectively. The assay was carried out as described [11], cRNA standards were run in triplicate, on separate days, simultaneously with the unknown samples. The correlation coefficients \( (R) \) of the standard curves were greater than 0.98; within run variability was 9.1%. The non-template RNA controls were run for each gene. No amplicons were detected in these controls. The relative value of target mRNA in 0.1 \( \mu \)g of unknown total mRNA sample was expressed as fold change based on the concentration of target mRNA in 0.1 \( \mu \)g of the reference total mRNA.

Statistical analyses. Data are presented as mean \( \pm \) SD or median (range). AE values were log transformed before analysis. Mixed models were used to estimate the intra-class correlation \( (R) \) for the structural renal parameters and SF catalase and glutathione peroxidase mRNA expression within sibling pairs. Since siblings are often of similar age and diabetes duration, the effects of age, age², duration and duration² were entered into a mixed effects ANOVA.

To determine the contribution of genetic factors on variation in the SF catalase and glutathione peroxidase mRNA expression levels, we constructed nuclear families from the sibling pair data and performed genetic variance component analyses using the SOLAR software package (Southwest Foundation for Biomedical Research, San Antonio, TX) [37]. SOLAR performs an analysis of family data that decomposes the total variance of the phenotype (SF catalase and glutathione peroxidase mRNA expression level) into components that are due to genetic (polygenic) effects.
(estimating the additive genetic variance), measured covariates and random (unmeasured) environmental effects. The relative contribution of genetic factors to each phenotype is then determined by the heritability \((h^2)\), defined by the ratio of additive genetic variance to the residual phenotypic variance (after the removal of covariates). Thus \(h^2\) is presented as the percentage of the variability in mRNA expression levels (mean ± SE) that is explained by genetic factors. A series of models were developed that incorporates covariates in a hierarchical manner. The first model contained no covariates. The final model was adjusted for covariates that were significantly associated with catalase or glutathione peroxidase levels. Significance of the heritability estimates was determined by a likelihood ratio test, in which the likelihood of the models with an additive genetic variance component and covariates was compared with the likelihood of a model with the additive genetic variance component constrained to zero.

### Results

The 42 diabetic patients (25 females) were 38.1 ± 7.9 years old, 15.9 ± 9.0 years old at diabetes onset and had diabetes for 21.5 ± 10.6 years. HbA1c at biopsy was 8.4 ± 1.2%. AER was 6.6 (1.0–860) µg/min; 34 patients were normoalbuminuric, four were microalbuminuric, three were proteinuric and one was unclassifiable. GFR ranged from 46 to 162 ml/min/1.73 m². Retinopathy was present in 13 patients (31%) and eight (19%) had proliferative changes. Hypertension was present in 11 patients (26%), six (14%) were on antihypertensive drugs, three (7%) of them were on angiotensin converting enzyme inhibitors or angiotensin II type 1 receptor blockers.

GBM width, \(Vv(Mes/glom)\) and \(Vv(MM/glom)\) were increased, while \(Sv(PGBM/glom)\) was decreased in these patients compared with controls (Table 1). As expected from our earlier report [13] and the partial overlap in the patient populations, GBM width \((R = 0.50; P = 0.014)\), \(Vv(Mes/glom)\) \((R = 0.62; P = 0.001)\), \(Vv(MM/glom)\) \((R = 0.63; P = 0.001)\) and \(Sv(PGBM/glom)\) \((R = 0.80; P = 0.001)\) were correlated among the sibling pairs, and this was independent of age and diabetes duration (data not shown).

The intraclass correlation between the sibling pairs was \(R = 0.38 (P = 0.076)\) (Figure 1) for catalase mRNA expression levels and \(R = 0.31 (P = 0.140)\) (Figure 2) for glutathione peroxidase. Using the variance component analyses model without covariates, the estimated heritability for catalase mRNA expression levels was \(h^2 = 0.78 ± 0.45\) \((P = 0.06)\). After adjustment for sex and HbA1c, the residual heritability was \(h^2 = 0.69 ± 0.46\) \((P = 0.04)\). Glutathione peroxidase mRNA levels did not show a significant heritability component before \((h^2 = 0.47 ± 0.46; P = 0.16)\) or after \((h^2 = 0.24 ± 0.52; P = 0.32)\) adjustment for age at diabetes onset.

### Discussion

The present work supports the concept that the behaviour of cells studied after several passages in vitro, at least in part, reflects their genetic background. The association between SF behaviours and DN risk was first reported in regards to (NHE-1) activity [9,10]. Subsequently, with similar numbers of subjects, we showed that type 1 diabetic siblings concordant for DN lesions [13] and for DN risk [1,3,4] were also concordant for NHE-1 activity [12] and concluded that this was likely, to a large extent, substantially genetically determined.

Ceriello et al. [23] demonstrated similar directions for SF message, protein and activity levels for catalase and glutathione peroxidase in response to high glucose, and established that lesser increases were related to increased DN risk. The findings for SF antioxidant enzymes mRNA, protein and activity levels were subsequently confirmed by Chiarelli et al. [24]. Parallel findings in antioxidant enzyme gene expression levels were found in cultured monocytes from type 1 diabetic patients with and without DN [25], strongly confirming the original observation and suggesting that this blunted cell response in DN patients could be present in multiple cell types. The present study was not designed to retest these well-established associations between in vitro cellular enzyme systems and DN risk, but rather focused on the question of whether these phenomena could, at least in part, be due to a heritable component in the antioxidant cellular phenotype among type 1 diabetic sibling pairs. The concordance for antioxidant enzyme mRNA levels in sibling pairs is concordant with at least partial genetic determination of these levels.

Oxidative stress may lead to extracellular matrix accumulation, as elegantly hypothesized by

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**Table 1. Glomerular structural parameter in the 42 diabetic patients and in 76 non-diabetic controls**

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Controls</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM width (nm)</td>
<td>482.4 ± 125.2</td>
<td>331.5 ± 45.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(Vv(Mes/glom))</td>
<td>0.33 ± 0.08</td>
<td>0.20 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(Vv(MM/glom))</td>
<td>0.19 ± 0.06</td>
<td>0.09 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(Vv(MC/glom))</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>(Sv(PGBM/glom))</td>
<td>0.106 ± 0.037</td>
<td>0.126 ± 0.018</td>
<td>&lt;0.001</td>
</tr>
</tbody>
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GBM, glomerular basement membrane; \(Vv(Mes/glom)\), fractional volume of mesangium per glomerulus; \(Vv(MM/glom)\), fractional volume of mesangium matrix per glomerulus; \(Vv(MC/glom)\), fractional volume of mesangium cell per glomerulus; \(Sv(PGBM/glom)\), surface density of peripheral glomerular basement membrane per glomerulus; NS, not significant.
unlike that individuals with genetic predisposition to or protection from DN would inherit multiple defects in the antioxidant enzyme system, upstream genes that regulate both catalase and glutathione peroxidase and other enzymes in this system would be good candidates for study. For example, the lower catalase and glutathione peroxidase mRNA expression levels seen in monocytes cultured under high vs normal glucose conditions in patients with DN were associated with aldose reductase genotype and mRNA levels [25], and responsiveness to high glucose was partially normalized by an aldose reductase inhibitor [25]. Thus, further exploration of genes related to the regulation of antioxidant enzymes could lead to new insights in this area.

The current study was limited in its power to evaluate the relationships between mRNA expression levels of antioxidant enzymes and renal function. These siblings, to avoid bias, were not selected for the presence of complications. Thus, since most type 1 diabetic patients are not at risk of DN, sibling pairs in this unselected series were predominantly normoalbuminuric and had normal GFR. This made the range of AER and GFR too narrow to explore the correlations between these parameters and mRNA expression levels for catalase and glutathione peroxidase. It would be of interest to design sibling pair studies where the associations between expression and activity of these enzymes and relevant pathways, renal structure and function could be tested.

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Conflict of interest statement. None declared.

References

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