Serum Heat Shock Protein 27 and Diabetes Complications in the EURODIAB Prospective Complications Study

A Novel Circulating Marker for Diabetic Neuropathy

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OBJECTIVE—Heat shock protein 27 (HSP27) is a member of the small heat shock protein family of proteins. HSP27 expression is enhanced in target tissues of diabetic microvascular complications, and changes in circulating serum HSP27 levels (sHSP27) have been reported in patients with macrovascular disease. We investigated whether sHSP27 levels were associated with micro- and macrovascular complications in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS—A cross-sectional, nested, case-control study from the EURODIAB Prospective Complications Study of 531 type 1 diabetic patients was performed. Case subjects (n = 363) were defined as those with one or more complications of diabetes; control subjects (n = 168) were defined as those with no evidence of any complication. We measured sHSP27 levels and investigated their associations with diabetes complications.

RESULTS—Mean sHSP27 levels were significantly higher in case subjects with distal symmetrical polyneuropathy (DSP) than in control subjects, even after adjustment for age and albumin excretion rate (AER) (785.9 vs. 574.7 pg/ml, P = 0.03). In logistic regression analysis, sHSP27 levels in the upper quartile were associated with a twofold increased odds ratio (OR) of DSP, independently of conventional risk factors, markers of inflammation, and AER (OR 2.41 [95% CI 1.11–5.24]).

CONCLUSIONS—In this large cohort of type 1 diabetic subjects, we found an independent association between sHSP27 and DSP. This suggests that sHSP27 levels may be a novel marker for diabetic neuropathy. ***Diabetes*** **57:1966–1970, 2008**

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Heat shock protein 27 (HSP27), a member of the small heat shock protein family of proteins, is a highly conserved peptide of ~27 kDa associated with cytoskeletal actin (1). In addition to its chaperone activity, HSP27 acts as a filament stabilizer under stress conditions, interferes with apoptotic pathways, and participates in cytoskeletal dynamics by controlling actin polymerization (2). Therefore, HSP27 plays an important role in both cytoprotection and cell motility.

Recent studies in experimental diabetes have shown HSP27 overexpression in glomeruli (3), dorsal root ganglia (4,5), retina (6), and the area adjacent to atherosclerotic plaque (7), indicating HSP27 induction in target tissues of diabetes complications. HSP27 is also released into circulation (8). A pilot study has shown reduced plasma HSP27 levels in patients with carotid stenosis (9), but in a more recent study, HSP27 levels were increased in patients with acute coronary syndromes (7). However, no large study is yet available on circulating HSP27 in vascular disease.

Type 1 diabetes is associated with a greatly increased risk of vascular complications that cannot be completely accounted for by conventional risk factors. The aim of the present study was to assess whether high serum HSP27 (sHSP27) levels increased odds ratios (ORs) of micro- and macrovascular complications in a nested case-control sample of type 1 diabetic individuals from the EURODIAB Prospective Complications Study.

RESEARCH DESIGN AND METHODS

The EURODIAB Prospective Complications Study (1997–1999) is a follow-up of the EURODIAB Type 1 Diabetes Complications Study (1989–1991), which was designed to explore risk factors for diabetes complications in 3,250 randomly selected people with type 1 diabetes, aged 15–60 years, attending 31 diabetes centers in 16 European countries (10,11).

A cross-sectional, nested, case-control study was designed at the 1997–1999 follow-up examination (12–15). The response rate at follow-up examination was 57.8% (16). Case subjects were selected based on the greatest complication burden possible in order to provide sufficient numbers for subgroup analyses. Thus, case subjects were defined as those with cardiovascular disease, proliferative retinopathy, or micro- or macroalbuminuria at follow-up. Control subjects were selected based on being completely free of complications. This design allowed the comparison of individuals with single or multiple complications with individuals free of complications, according to the study question, as efficiently as possible. Applying these criteria, this yielded 363 case and 168 control subjects with full data on complications and samples available for analysis. The sample size provides a power of 95 and 80% (α = 0.05), respectively, to detect a difference in log-HSP27 of at least one-third of an SD between all case and control subjects and between case subjects with single complications and control subjects with no complications.

TABLE 1
Baseline characteristics of the 531 subjects with type 1 diabetes of the EURODIAB Prospective Complications Study

	Case subjects	Control subjects	*P*
n	363	168	
Age (years)	41.4 ± 10.5	35.7 ± 7.7	<0.0001
Diabetes duration (years)	24.4 ± 9.3	15.4 ± 6.7	<0.0001
Males (%)	52.3	48.8	0.45
BMI (kg/m^2)	24.9 ± 3.5	23.6 ± 2.5	<0.0001
Waist-to-hip ratio	0.89 ± 0.12	0.89 ± 0.17	0.64
A1C (%)	8.9 ± 1.6	7.7 ± 1.2	<0.0001
Systolic blood pressure (mmHg)	127.0 ± 21.7	114.9 ± 13.1	<0.0001
Diastolic blood pressure (mmHg)	75.8 ± 11.7	73.7 ± 10.6	0.04
Hypertension (%)	54.6	13.8	<0.0001
Total cholesterol (mmol/l)	5.46 ± 1.18	4.91 ± 1.08	<0.0001
LDL cholesterol (mmol/l)	3.60 ± 1.11	3.06 ± 0.97	<0.0001
HDL cholesterol (mmol/l)	1.61 ± 0.44	1.67 ± 0.42	0.14
Triglycerides (mmol/l)	1.21 (0.83–1.58)	0.84 (0.66–1.09)	<0.0001
AER (μg/min)	51.0 (7.3–347.6)	6.4 (4.5–9.2)	<0.0001
CRP (mg/l)	1.23 (0.52–2.88)	0.75 (0.36–1.69)	<0.0001
IL-6 (pg/ml)	2.48 (1.34–3.91)	1.71 (1.06–2.50)	<0.0001
TNF-α (pg/ml)	3.22 (2.34–4.29)	2.14 (1.67–2.78)	<0.0001
Homocysteine (μmol/l)	7.7 (5.7–9.6)	6.8 (5.6–81)	0.002
Amadori albumin (U/ml)	47.0 ± 13.5	42.2 ± 12.3	0.0001
E-selectin (ng/ml)	33 (26–44)	29 (22–38)	0.0001
sVCAM (ng/ml)	412 (340–500)	368 (516–420)	<0.0001
HSP27 (pg/ml)	658.0 (286.4–1,315.0)	567.6 (250.0–1,136.0)	0.18

Data are means ± SD, percentage, or geometric means (25th–75th centile) for log-transformed data.

Patient evaluation for the presence of cardiovascular risk factors (hypertension, BMI, waist-to-hip ratio, smoking, cholesterol, triglycerides, and A1C) is described elsewhere (12–15). Retinopathy was graded according to the EURODIAB protocol (17). Albumin excretion rate (AER), assessed on two 24-h urine collections by the immunoturbidimetric method, was categorized as normoalbuminuria (<20 μg/min), microalbuminuria (20–200 μg/min), and macroalbuminuria (≥200 μg/min). Cardiovascular disease (CVD) was defined as physician-diagnosed myocardial infarction, angina, coronary artery bypass graft, or stroke and/or ischemic changes on a centrally Minnesota-coded electrocardiogram. Distal symmetrical polyneuropathy (DSP) was diagnosed on the basis of *1*) the presence of one or more neuropathic symptoms, *2*) the absence of two or more ankle or knee reflexes, and *3*) abnormal vibration perception threshold, measured by centrally calibrated biothesiometers (Biomedical, Newbury, OH) on the right big toe and on the right medial malleolus.

Soluble vascular cell adhesion molecule (sVCAM)-1, soluble E-selectin (sE-selectin), interleukin (IL)-6, and tumor necrosis factor (TNF)-α were measured by commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Oxon, U.K.), and plasma levels of C-reactive protein (CRP) and Amadori albumin were measure by in-house ELISA (12,13). Plasma homocysteine was determined with an automated fluorescence polarization immunoassay on an Abbott IMx analyzer (Abbott Laboratories, Abbott Park, IL) (18). sHSP27 levels were measured by ELISA (Calbiochem, San Diego, CA). Briefly, 96-well plates were precoated with a mouse anti-human HSP27, used as capture antibody. Then, both HSP27 standards and samples, together with a rabbit polyclonal detection antibody specific for human HSP27, were simultaneously incubated in the precoated wells. After washing, a goat anti-rabbit IgG conjugated to horseradish peroxidase was added. Reaction was revealed by 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate and stopped with sulfuric acid. The absorbance was read at 450 nm, and HSP27 levels were determined by comparing the absorbance of samples with the values obtained from the standard curve. The range of the assay was 20–1,000 pg/ml and intra- and interassay CVs were 4.4 and 8.5–9%, respectively, for both low (132 pg/ml) and high (993 pg/ml) range HSP27 levels. Samples were coded and tested blind.

Statistical analyses. Variables distributed normally are presented as means ± SD, whereas variables with skewed distribution were analyzed after logarithmic transformation (triglycerides, AER, creatinine, CRP, IL-6, TNF-α, sVCAM, sE-selectin, homocysteine, HSP27) and are presented as geometric means (interquartile range). Logistic regression analyses were used to estimate the ORs of HSP27 for any complication (AER ≥20 μg/min, retinopathy, neuropathy, CVD), independently of confounders and known risk factors. Both backward and forward strategies examining all explanatory variables were used to select models. The likelihood ratio test was used to compare nested models examining the role of age, sex, diabetes duration, BMI, waist-to-hip ratio, A1C, blood pressure, lipids, AER, CRP, IL-6, TNF-α, homocysteine, Amadori albumin, sE-selectin, sVCAM, and smoking. Analyses were hypothesis oriented and did not use stepwise regression (19). Variables were retained in the final model if they added significantly to the likelihood of models or to the estimated coefficients of predictors. In light of the hypothesis of a different role of HSP27 in the pathogenesis of different complications, logistic regression models were also fitted separately for each complication. To assess patterns of ORs across increasing HSP27 values, ORs were categorized by the quartile distribution in control subjects. We tested for linear trends across quartiles by entering a single ordinal term into the models. When ORs in the lower quartiles of HSP27 were similar, they were aggregated as the reference category in the final analysis and compared with the upper quartile.

RESULTS

The study population (n = 531) had a mean age of 39.6 years, a mean diabetes duration of 21.5 years, and an equal proportion of men and women. Case subjects with vascular complications had a more adverse risk factor profile than control subjects (Table 1). Of the 363 case subjects, nephropathy was present in 206 (22.6% micro- and 34.3% macroalbuminuria), retinopathy in 292 (background 39.1% and proliferative 41.3%), DSP in 205 (56.5%), and autonomic neuropathy in 118 (27.6%). Most people, however, had more than one complication; indeed, 187 (51.5%) individuals had both AER ≥20 μg/min and retinopathy, 128 (35.3%) had both AER ≥20 μg/min and DSP, and 123 (33.9%) had AER ≥20 μg/min, DSP, and retinopathy. CVD was present in 146 subjects (40.2%), all of whom also had at least one microvascular complication, apart from 12 individuals who had CVD only.

HSP27 was measurable in all 531 samples, with a right-skewed distribution of values (Table 1). sHSP27 levels were not significantly different in case and control subjects, even after adjustment for age (670.9 vs. 548.8 pg/ml, P = 0.08). With respect to control subjects, however, we found significantly greater age-adjusted HSP27 levels in case subjects with DSP (P = 0.002) and in case subjects with micro-/macroalbuminuria (P = 0.03). Although sHSP27 levels were also slightly higher in case

TABLE 2
OR (95% CI) values for diabetes complications by HSP27 values in the nested case-control study within the EURODIAB Prospective Complications Study

	OR (95% CI)	OR (95% CI)*	OR (95% CI)†
All complications			
logHSP27	1.11 (0.95–1.31)	1.14 (0.95–1.36)	1.15 (0.93–1.43)
HSP27 (pg/ml)			
<250.0	1.00	1.00	1.00
250.0–507.1	1.22 (0.72–2.07)	1.22 (0.66–2.23)	0.94 (0.45–1.97)
507.2–1,135.0	1.13 (0.67–1.92)	1.42 (0.78–2.60)	0.92 (0.44–1.95)
>1,135.0	1.42 (0.85–2.39)	1.55 (0.85–2.82)	1.31 (0.64–2.73)
P for trend	0.24	0.13	0.47
DSP			
logHSP27	1.24 (1.04–1.49)	1.32 (1.06–1.63)	1.53 (1.16–2.02)
HSP27 (pg/ml)			
<250.0	1.00	1.00	1.00
250.0–507.1	1.36 (0.74–2.50)	1.13 (0.54–2.39)	0.65 (0.24–1.74)
507.2–1,135.0	1.39 (0.76–2.54)	1.84 (0.89–3.82)	1.19 (0.46–3.10)
>1,135.0	1.94 (1.08–3.50)	2.13 (1.04–4.33)	2.27 (0.90–5.75)
P for trend	0.03	0.016	0.03
Micro-/macroalbuminuria			
logHSP27	1.19 (1.00–1.42)	1.21 (0.98–1.41)	1.24 (0.92–1.68)
HSP27 (pg/ml)			
<250.0	1.00	1.00	1.00
250.0–507.1	1.20 (0.66–2.18)	1.22 (0.58–2.55)	0.88 (0.31–2.52)
507.2–1,135.0	1.31 (0.72–2.37)	2.10 (1.02–4.32)	1.10 (0.38–3.16)
>1,135.0	1.68 (0.94–2.99)	1.92 (0.95–3.89)	1.56 (0.58–4.20)
P for trend	0.08	0.03	0.31
Retinopathy			
logHSP27	1.13 (0.96–1.34)	1.15 (0.94–1.41)	1.16 (0.90–1.51)
HSP27 (pg/ml)			
<250.0	1.00	1.00	1.00
250.0–507.1	1.15 (0.66–1.99)	1.21 (0.61–2.40)	0.98 (0.41–2.38)
507.2–1,135.0	1.23 (0.71–2.13)	1.94 (0.99–3.82)	1.35 (0.55–3.29)
>1,135.0	1.48 (0.87–2.54)	1.69 (0.86–3.31)	1.39 (0.58–3.34)
P for trend	0.15	0.06	0.34
CVD			
logHSP27	1.13 (0.93–1.37)	1.18 (0.94–1.49)	1.10 (0.83–1.46)
HSP27 (pg/ml)			
<250.0	1.00	1.00	1.00
250.0–507.1	1.43 (0.75–2.72)	1.86 (0.84–4.14)	1.22 (0.47–3.15)
507.2–1,135.0	1.25 (0.65–2.41)	1.83 (0.82–4.08)	1.09 (0.41–2.86)
>1,135.0	1.54 (0.81–2.91)	1.91 (0.86–4.24)	1.23 (0.48–3.16)
P for trend	0.28	0.16	0.74

*Adjusted for age and diabetes duration. †Adjusted for age, diabetes duration, hypertension, A1C, smoking, and logTNF-α.

subjects with retinopathy ($P = 0.06$), this was mainly due to the confounding association with AER, as values became similar after further adjustment for AER ($P = 0.57$). On the contrary, the difference between case subjects with DSP and control subjects was significant, even after further adjustment for AER (785.9 vs. 574.7 pg/ml, $P = 0.03$). No difference was found between case subjects with CVD and control subjects.

We then performed logistic regression analyses to assess whether higher values of HSP27 conferred an increased OR of having any complication, independently of main risk factors. Models performed in all subjects and separately for each complication showed a tendency toward a negative confounding effect of both age and diabetes duration (increasing ORs from model 1 to model 2) and a positive confounding effect of A1C, hypertension, smoking, and TNF-α (decreasing ORs from model 2 to model 3). In the fully adjusted model, a significant linear trend of ORs across quartiles of HSP27 was evident for DSP ($P = 0.03$), whereas a significant linear trend for micro-/macroalbuminuria and retinopathy was present exclusively in the age- and duration-adjusted model (model 2) (Table 2).

HSP27 values in the upper quartile (>1,135 pg/ml) conferred a 38% increased OR (95% CI 0.77–2.49) of any complications compared with HSP27 values in the lower quartiles (≤1,135 pg/ml). Final models, performed separately for each complication, showed that higher HSP27 values were associated with a more than twofold increased OR of DSP, which was statistically significant (OR 2.45 [95% CI 1.20–5.03]), even after further adjustment for AER values (2.41 [1.11–5.24]). ORs for other complications were increased in the upper versus lowers quartiles, but they did not reach statistical significance (Table 2). Study center did not contribute significantly to the final model and did not modify estimated ORs.

DISCUSSION

In this cross-sectional sample of type 1 diabetic patients from the EURODIAB Prospective Complications Study,

we have provided the first evidence of an independent association between sHSP27 levels and DSP. Mean sHSP27 levels were significantly higher in case subjects with DSP than in control subjects, even after adjustment for age and AER. Furthermore, in logistic regression analysis, higher circulating HSP27 levels conferred a two-fold increased OR of DSP, independently of conventional risk factors, markers of inflammation, and AER. The lack of circulating markers for DSP represents an important limit of clinical research in this field; therefore, our findings may be of potential clinical relevance. Availability of a surrogate marker of DSP, which can be easily and noninvasively obtained, may facilitate diagnosis, measurement of progression, and assessment of therapeutic interventions.

The rise in circulating HSP27 expression in patients with DSP may result from neuronal overexpression. Consistent with this hypothesis, studies in experimental diabetes have shown HSP27 induction in the sensory neurons of the dorsal root ganglia (4,5). Intracellular HSP27, a key survival factor for neurons, plays an important role in axonal regeneration (20), and mutations of the *HSPB1* gene encoding for HSP27 cause inherited distal peripheral neuropathies, such as hereditary distal motor neuropathy and Charcot-Marie-Tooth disease type 2 (21). The mechanism of HSP27 neuroprotection is unclear, but preservation of the cytoskeletal stability and both chaperone-like and anti-apoptotic activities have been implicated (22). In diabetic patients with DSP, overexpression of HSP27 may thus be aimed to counteract the neurological damage caused by the diabetic milieu. On the other hand, HSP27 release can also contribute to the neuronal damage, as anti-HSP27 autoantibodies, which are produced in response to extracellular HSP27 exposure, can induce neuronal apoptosis (23).

There are certain limitations to our study. First, this is a cross-sectional study, hence restricting our ability to assess temporal relationships between sHSP27 levels and microvascular complications and to identify causal biological mechanisms underlying this association. However, no data on HSP27 in large groups of type 1 diabetic patients exist; therefore, this study may serve as a reasonable starting point to explore the role of this molecule in type 1 diabetes. Second, the number of control subjects was lower than the overall number of case subjects, thus reducing the power of analyses; comparisons between control subjects and case subjects with single complications allowed a more favorable case-to-control ratio, but multiple comparisons within the same case-control study base might have caused significant results due to chance. Third, although serum samples were adequately stored, the possibility of protein degradation cannot be excluded; however, random misclassification would have biased our estimates downward, without affecting significant associations. Unlike previous studies, a key strength here is the ability to account for confounding by other risk factors and complications, and the large sample size provides sufficient power for these analyses. In addition, our patients were from a representative sample of people with type 1 diabetes across Europe, and our results are therefore likely to be generalizable.

In conclusion, this is the first study measuring sHSP27 in a large group of subjects, and the results provide evidence that sHSP27 levels are independently associated with DSP in type 1 diabetic patients. Further studies are required to determine causal relationships and elucidate underlying mechanisms.

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