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

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

|                          | Case subjects | Control subjects | P       |
|--------------------------|---------------|------------------|---------|
| n                        | 363           | 168              | <0.0001 |
| Age (years)              | 41.4 ± 10.5   | 35.7 ± 7.7       | <0.0001 |
| Diabetes duration (years)| 24.4 ± 9.3    | 15.4 ± 6.7       | 0.04    |
| Males (%)                | 52.3          | 48.8             | 0.45    |
| BMI (kg/m²)              | 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  | 127.0 ± 21.7  | 114.9 ± 13.1     | <0.0001 |
| (mmHg)                   |               |                  |         |
| Diastolic blood pressure | 75.8 ± 11.7   | 73.7 ± 10.6      | 0.04    |
| (mmHg)                   |               |                  |         |
| 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 (ng/ml)              | 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 (5.6–8.1)    | 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 (250.0–1,363.0) | 0.18   |

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

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%) had at least one microvascular complication, apart from 123 (35.3%) had both AER, 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). shHSP27 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- and macroalbuminuria (P = 0.03). Although shHSP27 levels were also slightly higher in case

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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-$\alpha$ (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 ($\leq 1,135$ 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 lower 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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REFERENCES

1. Welsh MJ, Gaestel M: Small heat-shock protein family: function in health and disease. Ann N Y Acad Sci 851:28–35, 1998
2. Arrigo AP: The cellular “networking” of mammalian Hsp27 and its functions in the control of protein folding, redox state and apoptosis. Adv Exp Med Biol 594:14–26, 2007
3. Dunlop ME, Muggli EE: Small heat shock protein alteration provides a mechanism to reduce mesangial cell contractility in diabetes and oxidative stress. Kidney Int 57:464–475, 2000
4. Kamiya H, Zhang W, Sima AA: Apoptotic stress is counterbalanced by survival elements preventing programmed cell death of dorsal root ganglia in subacute type 1 diabetic BB/Wor rats. Diabetes 54:3288–3295, 2005
5. Zochodne DW, Verge VM, Cheng C, Sun H, Johnston J: Does diabetes target ganglion neurones? Progressive sensory nerve involvement in long-term experimental diabetes. Brain 124:2119–2234, 2001
6. Joussen AM, Huang S, Poulaki V, Camphasen K, Beeckman WD, Kirchhof B, Adams AP: In vivo retinal gene expression in early diabetes. Invest Ophthalmol Vis Sci 42:3047–3057, 2001
7. Park HK, Park EC, Bae SW, Park MY, Kim SW, Yoo HS, Tudev M, Ko YH, Choi YH, Kim S, Kim DI, Kim YW, Lee BB, Yoon JB, Park JE: Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. Circulation 114:886–890, 2006
8. De AK, Roach SE: Detection of the soluble heat shock protein 27 (hsp27) in human serum by an ELISA. J Immunol Assay Immunochrom 25:159–170, 2004
9. Martin-Ventura JL, Duran MC, Blanco-Colio LM, Mehlhac O, Leclercq A, Michel JB, Jensen ON, Hernandez-Merida S, Tunon J, Vivanco F, Egidio J: Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. Circulation 110:2216–2219, 2004
10. The EURODIAB IDDM Complications Study Group: Microvascular and acute complications in IDDM patients: the EURODIAB IDDM Complications Study. Diabetologia 3:278–285, 1994
11. Chaturvedi N, Spoele AK, Porta M, Aldington SJ, Fuller JH, Songini M, Kohner EM; EURODIAB Prospective Complications Study: Markers of insulin resistance are strong risk factors for retinopathy incidence in type 1 diabetes. Diabetes Care 24:284–289, 2001
12. Chaturvedi N, Schalkwijk CG, Abrahamian H, Fuller JH, Stehouwer CD; EURODIAB Prospective Complications Study: Circulating and urinary transforming growth factor beta1, Amadori variant, and albumin concentrations in type 1 diabetes: the EURODIAB prospective complications study. Diabetes Care 25:2320–2327, 2002
13. Schram MT, Chaturvedi N, Schalkwijk C, Giorgino F, Ebeling P, Fuller JH, Stehouwer CD; EURODIAB Prospective Complications Study: Vascular risk factors and markers of endothelial function as determinants of inflammatory markers in type 1 diabetes: the EURODIAB Prospective Complications Study. Diabetes Care 26:2165–2173, 2003
14. Schram MT, Schalkwijk CG, Bootma AH, Fuller JH, Chaturvedi N, Stehouwer CD; EURODIAB Prospective Complications Study Group: Advanced glycation end products are associated with pulse pressure in type 1 diabetes: the EURODIAB Prospective Complications Study. Hypertension 46:232–237, 2005
15. Schram MT, Chaturvedi N, Schalkwijk CG, Fuller JH, Stehouwer CD; EURODIAB Prospective Complications Study Group: Markers of inflammation are cross-sectionally associated with microvascular complications and cardiovascular disease in type 1 diabetes: the EURODIAB Prospective Complications Study. Diabetes Care 28:370–375, 2005
16. Giunti S, Bruno G, Lilaz E, Gruden G, Lollì V, Chaturvedi N, Fuller JH, Veglio M, Cavallo-Perin P; EURODIAB IDDM Complications Study Group: Incidence and risk factors of prolonged QTe interval in type 1 diabetes: the EURODIAB Prospective Complications Study. Diabetes Care 30:2057–2062, 2007
17. Aldington SJ, Kohner EM, Meurer S, Klein R, Spoele AK, the EURODIAB IDDM Complications Study Group: Methodology for retinal photography and assessment of diabetic retinopathy: the EURODIAB IDDM Complications Study. Diabetologia 38:437–444, 1995

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18. Shipchandler MT, Moore EG: Rapid, fully automated measurement of plasma homocysteine with the Abbott IMx analyzer. Clin Chem 41:991–994, 1995

19. Rothman KJ, Greenland S: Modern Epidemiology. 2nd ed. Philadelphia, Lippincott Williams & Wilkins, 1998

20. Muchowski PJ, Wacker JL: Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11–22, 2005

21. Evgrafov OV, Mersiyanova I, Irobi J, Van Den Bosch L, Dierick I, Leung CL, Schagina O, Verpoorten N, Van Impe K, Fedotov V, Dadali E, Auer-Grumbach M, Windpassinger C, Wagner K, Mitrovic Z, Hilton-Jones D, Talbot K, Martin JJ, Vasserman N, Tverskaya S, Polyakov A, Liem RK, Gettemans J, Robberecht W, De Jonghe P, Timmerman V: Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. Nat Genet 36:602–606, 2004

22. Dierick I, Irobi J, De Jonghe P, Timmerman V: Small heat shock proteins in inherited peripheral neuropathies. Ann Neurol 37:413–422, 2005

23. Tezel G, Wax MB: The mechanisms of hsp27 antibody-mediated apoptosis in retinal neuronal cells. J Neurosci 20:3552–3562, 2000